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Soybean and Health

Edited by Hany El-Shemy

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http://dx.doi.org/10.5772/1007
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Notice

First published in Croatia, 2011 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Soybean and Health
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p. cm.

ISBN 978-953-307-535-8

eBook (PDF) ISBN 978-953-51-5171-5

Meet the editor

Professor Hany A. El-Shemy received his two Ph.D. degrees in biochemistry and genetic engineering from the University of Cairo, Egypt and Hiroshima University, Japan. He became an assistant professor with Biochemistry Department of Cairo University, Egypt from Sept, 1996, and advanced to associate professor in Sept, 2002, as well as full professor in March 2007. His research interests are in the fields of plant biotechnology and medicinal plants. He received 2 patents, wrote 2 book chapters, published more than 60 SCI Journal papers, and 25 conference presentation, and served as the technique committee member as well as chair in many international conferences and the editors including PLoS ONE, BMC Genomics, CIMB journals, also reviewer for more than 10 SCI cited journals. He received several awards, including State prize Award from Academy of Science, Egypt (2004), Young Arab Researcher prize Awarded from Shuman Foundation, Jordan (2005).

Contents

Preface

Worldwide, soybean seed proteins represent a major source of amino acids for human and animal nutrition. Soybean seeds are an important and economical source of protein in the diet of many developed and developing countries. Soy is a complete protein, and soyfoods are rich in vitamins and minerals.

Soybean protein provides all the essential amino acids in the amounts needed for human health.

Recent research suggests that soy may also lower risk of prostate, colon and breast cancers as well as osteoporosis and other bone health problems and alleviate hot flashes associated with menopause. This volume is expected to be useful for student, researchers and public who are interested in soybean.

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Soybean Products Consumption in the Prevention of Cardiovascular Diseases

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1. Introduction

Soybean is a leguminous plant native to eastern Asia (Liu et al. 2008). Soybean is cultivated worldwide, with the U.S. being responsible for more than 50% of the world's production of this important food. Soybean grains are rich in protein content (see aminoacids composition, Table 1); therefore constitute a useful source of food. It is consumed as cooked beans, soy sauce, soy milk and tofu (soybean curd). Also, a vegetable oil is obtained from soybeans, rich in polyunsaturated fatty acids. This legume is also a good source of several phytochemicals such as isoflavones and lignans, molecules with antioxidant properties that, among other effects, might help to fight and prevent several pathologies. For these reasons, these compounds have been intensively studied at basic and at the clinical level.
Soybean consumption benefits, especially in the cardiovascular system, have been related to its important protein content, high levels of essential fatty acids, vitamins and minerals. Nevertheless, there has been controversy as to the extent to which soybean is a health-promoting food. We address some aspects of these issues in this chapter.

2. Agronomic characteristics of soybean plants

Soybean (*Glycine max* L. Merr., subfamily *Papilionoidae*) is an annual plant that measures up to 1.5 m tall, with pubescent leaves and pods. The stems are erect and rigid. The root system has as main root which can reach a meter deep, the average being between 40 and 50 centimeters**.** In its primary and secondary roots, are located a variable number of nodes. One of the characteristic of the root system development is its sensitivity to variations in the supply and distribution of inorganic nutrients in the soil (Forde & Lorenzo 2001).
Soy leaves are alternate, compound (trifoliate), with the exception of the basal leaves, which are simple. The leaflets have an oval - lanceolate with a green color turning yellow, at maturity stage**.** Soybean flowers are found in variable number of axillary racemes as

inflorescences with white or purple colors, depending on the variety. Fruits of soy are pods dehiscent on both sutures (Sánchez A et al. 2004). The pods reach two to three inches in length. Each fruit has three to four seeds, yellow, but there are usually black, green and brown seeds, depending on the variety (Nadal S et al. 2004). The phenologic stages of soybean were described by Ferh and colleagues (Fehr et al. 1971). This scale includes the growth stages of soybean (Table 2). The numbering of the vegetative state is determined by the count of existing nodes on the main stem. Above that, usually there are fully developed leaves. A leaf is already fully developed when the edges of the leaflets of the blade located immediately above are not touching. The reproductive stages are based on flowering, growth of pods and seeds and plant maturity.

Amino acid	g/16 g Nitrogen
Isoleucine	4.54
Leucine	7.78
Lysine	6.38
Methionine	1.26
Cysteine	1.33
Phenylalanine	4.94
Tyrosine	3.14
Threonine	3.86
Tryptophan	1.28
Valine	4.80
Arginine	7.23
Histidine	2.53
Alanine	4.26
Aspartic acid	11.70
Glutamic acid	18.70
Glycine	4.18
Proline	5.49
Serine	5.12

Table 1. Amino acid composition of soybeans seeds. Source: Adapted by authors from FAO (1970) and FAO/WHO (1973).

Soybean is a spring-summer crop with a growing period fluctuating between three and seven months. The best zones to cultivate this crop are in temperate regions. This species develops with optimum temperatures between 20 and 30 ºC. The vegetative growth is achieved above 10 ºC, stopping the development at 4 °C. However, it resists frost of -2 to -4 ºC. In the sowing season, it requires optimum temperatures that fluctuate between 15 and 18 ºC and 25 °C at flowering stage. For this reason, soybean is established at warmer seasons. The seed maturity is obtained at early and mid-autumn, when the leaves of the soybean crop fall and turn to yellow, and the seeds begin to loss moisture. It is suggested harvesting the crop when the water content of the seed is 13%, the maximum safe moisture level for long-range storage. If the humidity content at harvest is higher, forced-air drying of the seeds will be required prior to storage. Otherwise, the seeds split at mechanical harvest (Lopez Bellido 1988). Soy is a plant sensitive to day length, so it is regarded as a plant with a short-day photoperiod. In relation to moisture, during the growing period, soybeans

requires at least 300 mm of water, whether provided by irrigation or rain on those wet rainy temperate zones. However, for good yields it is necessary to apply irrigation of 500 to 700 mm, especially during the period of pod and seed development (Lopez Bellido 1988). Soybeans grow well on most types of soil, but the crop is affected when plants are sown in deep sands with scarce water retention; the optimum soil pH fluctuates between 6 and 6.5 (Lopez Bellido 1988). This species is able to fix nitrogen through symbiosis with nodulating bacteria in the soil. Literature indicates that up to 50% of the total nitrogen of the plant may be supplied by the nitrogen fixing mechanism (Berk 1992).

Vegetative stages

Ve: Emergency cotyledons on the ground surface.
Vc: Cotyledon: unifoliates sufficiently developed so that its edges or sides are not touching.
V1: First Node: fully developed leaves at the node of unifoliates
V2: Second Node: two nodes on main stem with fully developed leaves beginning with the knot unifoliates
Vn N º nodes: number of nodes on main stem with fully developed leaves beginning with the knot unifoliates.

Reproductive stages

R 1 Top Flowering: an open flower at any node on main stem.
R 2 Full Bloom: an open flower at one of the upper main stem nodes with a full developed leaf.
R 3 Top Fruit: pod 5 mm long in any of the four upper nodes on main stem with a fully developed leaf
R 4 Full Fruit: pods 2 cm long in any of the four upper nodes on main stem with a fully developed leaf
R 5 Top grain filling: seeds 3 mm long in a pod in some of the top four nodes on main stem with a fully developed leaf.
R 6 Maximum grain size: the green seed pods are completely fills the cavity of the fruit, in some of the top four nodes on main stem with fully developed leaves.
R 7 Beginning of Maturity: one normal pod on main stem has reached mature color characteristic.
R 8 Commercial Maturity: 95% of pods with the typical color of maturity

Table 2. Stage of Development Descriptions for Soybeans.

3. Soybean and cardiovascular diseases

Cardiovascular diseases are the principal cause of death in the Western population. Among other factors, increased plasma cholesterol levels is an important risk factor for developing atherosclerosis and associated coronary and cerebrovascular diseases (Faergeman 2006; Fernandez & Webb 2008; Goldstein & Brown 2009). Control of cholesterol, mainly LDL-cholesterol, using pharmacological agents, such as statins, have significantly reduced the risk of cardiovascular diseases (Farmer 1998; Vaughan et al. 2000). However, some side effects associated with statins (Thompson *et al.* 2003) or others pharmacological therapies,

have driven the search for natural therapies or alternative approaches for managing hypercholesterolemia, especially in patients with borderline plasma cholesterol (Deng 2009). Food supplements and/or nutraceuticals have become attractive alternatives to prevent or treat hypercholesterolemia and reduce the risk for cardiovascular diseases with few side effects. Soy-based foods, mainly soy protein extract, have been widely associated with beneficial effects on lipoprotein profile, being identified as beneficial for cardiovascular disease (Anderson et al. 1995; Sacks et al. 2006).

Today, numerous epidemiological and clinical studies have linked the consumption of soy based foods with lower incidence of a number of chronic diseases, such as cardiovascular diseases (Anderson et al. 1995; Dewell et al. 2006; Sacks et al. 2006), cancer (Messina *et al.* 2006), and osteoporosis (Messina & Messina 2000). However, the mechanisms that explain these effects are not yet fully clarified. Over the past decades, intense research has focused in identifying bioactive components in soy that could be responsible for these effects. Soy protein and isoflavones have been suggested as the major bioactive components in soy and have received considerable attention. In the middle of the 1990s a meta-analysis based in numerous studies in humans showed that soy protein consumption resulted in a significant decrease of 9.3% of total cholesterol, 12.9% of LDL (low-density lipoprotein) cholesterol and 10.5% of triglycerides (Anderson et al. 1995). The effectiveness of the treatment with soy protein was higher in hypercholesterolemic subjects, in other words, it was dependent on the initial plasma cholesterol levels (Anderson et al. 1995; Crouse, III et al. 1999), having only marginal and sometimes null effects in normocholesterolemic subjects. Based mainly in this meta-analysis, the US Food and Drug Administration (FDA) approved the labeling of foods containing soy protein as protective against coronary heart disease. The claim of FDA recommended intake of 25 g of soy protein per day to reduce risk of developing coronary heart disease (Food and Drug Administration, HHS: final rule, 1999). However, in this health claims, the FDA also stated that there was no sufficient background or evidences to relate isoflavones with the reduction of plasma cholesterol levels.

3.1 Soy protein hypothesis and LDL cholesterol

Early evidences linking soy protein consumption and the reduction in cardiovascular risk were obtained in laboratory animals models, where the substitution of animal protein by soy protein extract caused a significantly decrease in plasma cholesterol levels in animals fed with a cholesterol-free diet (Anderson et al. 1995; Carroll 1982; Kritchevsky 1979). The soy protein hypothesis has gained support after the epidemiological observations in Asian countries, where the diet is rich in soy-based foods, with a low incidence of hypercholesterolemia and cardiovascular diseases (Keys 1980). However, the diet is not the only difference between Asian and Western countries. There are important differences in lifestyle that may help to explain the differences in lipoprotein profile and cardiovascular risks. However, the results in human trials have been controversial. The substitution of animal protein with soy protein showed effects only in subjects with increased plasma cholesterol levels, without or only marginal effects in normocholesterolemic or borderline subjects (Crouse, III et al. 1999; Jenkins et al. 2002). Some researchers found that the substitution of almost all animal protein with soy protein resulted in a significantly decrease in plasma cholesterol levels, mainly LDL-cholesterol, but in subjects with severe hypercholesterolemia (Descovich *et al.* 1980). In 1995, a meta-analysis of numerous studies in humans populations published by Anderson JW (Anderson et al. 1995) reinforced the soy protein hypothesis in subjects with hypercholesterolemia, where a significant decrease in

total plasma cholesterol and mainly LDL-cholesterol level were observed after replacing animal protein with soy protein. This meta-analysis was the base for the Food and Drugs Administration (FDA) claims published in 1999 in regard to soy consumption. In 2000, the American Heart Association Nutrition Committee published a scientific advisory about soy protein consumption and cardiovascular diseases. This Committee recommended including soy protein in a diet low in saturated fatty acids and cholesterol to reduce the cardiovascular risk (Erdman, Jr. 2000). This Scientific advisory was reviewed in 2006, after evaluating other parameters of cardiovascular risk, such as lipoprotein and blood pressure, and regarding isoflavones as possible responsible of benefits related with cardiovascular diseases (Sacks et al. 2006).

3.1.1 Isoflavones

After the publication of the meta-analysis by Anderson and colleagues (Anderson et al. 1995), many well-controlled studies have been designed to explain the soy protein hypothesis with higher specificity and with approaches directed to resolve the molecular mechanisms involved in this effect. Soy protein extracts contain variable amounts of isoflavones called phytoestrogens. Their abundance in soy protein preparations is widely dependent on the methods used to obtain the protein extracts (Erdman, Jr. *et al.* 2004). The main isoflavones in soy protein extracts include genistein, daidzein, glycitein, their naturally occurring glycosides (genistin, daidzin and glycitin), and malonyl glycosides. Several studies have been performed to establish whether soy protein and/or isoflavones could be responsible for the hipocholesterolemic effects of soy-based diets and therefore, their benefits by cardiovascular health. Some studies used only soy protein and others used purified isoflavones. The results of these numerous experimental trials were analyzed in a meta-analysis and concluded that isoflavones have no impact on plasma cholesterol levels (Jenkins et al. 2002). It is interesting to comment that despite isoflavones did not change significantly the lipoprotein profile or cholesterol levels, the plasma concentration of isoflavones are increased when these biocomponents are included in the diet. Isoflavones are phytoestrogens and have a weak agonistic activity on the mammalian estrogens receptors (ER), due to the similar spatial structure with 17-β-estradiol. Isoflavones have higher binding affinity for the ERβ receptor than ERα. For this reason, isoflavones are not considered as typical estrogens and have been considered as modulators of ER. In some tissues, isoflavones could be even antagonist o partially agonist of ER. Estrogens and other modulators of these receptors are considered hypolipidemic agents and for this reason, protective for cardiovascular health. However, some modulators are considered inhibitors of the cardioprotective effects of estrogen; in other words, they can increase the cardiovascular risk. Today, still there is not conclusive evidence about isoflavones and their role as modulators of estrogen pathways. Thus, it remains to be determined (Sacks et al. 2006) the results obtained in human:

1) Whether isoflavones are the active ingredients for soy hypocholesterolemic effect; and 2) whether isoflavones act as agonists or antagonists of ER to exert their possible hypocholesterolemic activity.

Recently, a mechanism independent of lipid-lowering effect has been proposed to explain the benefits in reduction of cardiovascular risk (Nagarajan 2010). Atherosclerosis and cardiovascular disease have been widely associated to lipoprotein profile alteration, mainly, cholesterol levels. Recently, pro-inflammatory process has been involved in the etiology of these chronic diseases. Early events that occur in the atherogenesis process involve a complex interaction between activated monocytes and vascular endothelial cells (Palomo et

al. 2008). When activated, monocytes and vascular endothelial cells react and transmigration occurs, starting a pro-inflammatory process. In the intimae layer, monocytes are transformed into macrophages which take up oxidized LDL-cholesterol to become foam cells. A possible anti-inflammatory role is attributed to soy based diets and this role could explain the anti-atherosclerotic and protective cardiovascular effects of soy products consumption.

4. Beneficial effect of soybean on the metabolic syndrome

The metabolic syndrome is a cluster of metabolic alterations associated to insulin resistance (IR) , but conceptual differences exist between the currently available definitions (Reaven 2004). Reaven was one of the first to describe this combination as a syndrome that he called IR syndrome or simply "X Syndrome"; later the World Health Organization (WHO) named it Metabolic Syndrome. Aspects of metabolic syndrome include a clustering of cardiovascular risk factors: abdominal obesity, dyslipidemia, blood pressure, insulin resistance, and a proinflammatory state (Borgman & McErlean 2006).
Current studies estimate that about 50% of the adult population of the United States is obese (Mokdad *et al.* 2003). About half of the obese population also suffers from pre-diabetes or metabolic syndrome. Both insulin resistance and obesity are key features of the metabolic syndrome and type 2 diabetes mellitus (Park *et al.* 2003). One potential mechanism linking obesity and insulin resistance involves the production of hormones or adipokines by adipose tissue. Plasma concentrations of several adipokines, such as leptin, tumor necrosis factor alpha (TNF-α), and the plasminogen activator-inhibitor 1 (PAI-1), have been associated positively with insulin resistance, whereas adiponectin is negatively associated (Kadowaki et al. 2006; Shoelson et al. 2006). Furthermore, lower plasma concentrations of adiponectin are associated with increased incidence of metabolic syndrome, diabetes, and vascular disease (Lihn et al. 2005; Tan et al. 2004).
The metabolic syndrome is associated with a poorly healthy life style and low consumption of fruits and vegetables; among vegetables we found soybean.
The soybean is the most widely grown and utilized legume in the world. The benefits of soybean consumption are related to its high protein content (Table 1), high levels of essential fatty acids, numerous vitamins and minerals, isoflavones and fibre. Soybean is regarded as equal in protein quality as animal foods. Just one cup of soybeans provides 57.2% of the daily value for protein for less than 300 calories and only 2.2 g. of saturated fat. In addition, soy protein tends to lower cholesterol levels, while protein consumption from animal sources tends to raise them, since they also include saturated fat and cholesterol. Besides healthy protein, some of the nutritional high points of soybeans include an important amount of well absorbed iron: 49.1% of the daily value for iron in that same cup of soybeans plus 37.0% of magnesium and 41.2% of the daily value for essential omega-3 fatty acids (Morgado 2007).
In addition, soybean products may have a direct impact on the intermediate metabolism. Interestingly, genistein and daidzein, found in soybeans and processed soy protein, have been shown to bind to the peroxisome proliferator-activated receptors (PPAR), particularly to PPARγ as well as PPAR α and δ (Dang et al. 2003; Mezei et al. 2003), suggesting the potential value of isoflavones as a nutritional approach to modulate the action of insulin. PPARs belong to a family of transcription factors of which include three isoforms, PPARα, PPARδ (β) and PPARγ (Moore-Carrasco et al. 2008). Soy is the most commonly used botanical in the US, and the FDA has approved a health claim for soy protein and soy-based

food products, based largely on the evidence that soy consumption improves plasma lipid and lipoprotein concentrations and might reduce risk of coronary heart diseases, and does not appear to increase cancer risk (Wagner *et al.* 2001).
Wagner and colleagues (Wagner *et al.* 2008) showed that in male monkeys, consumption of soy protein with its isoflavones increased the insulin secretion following a glucose challenge. There are few studies suggesting that the benefits of soy on carbohydrate metabolism are more evident in females than males. In addition to the binding of isoflavones to PPAR, they have estrogenic activity, binding to both ERα an ERβ, but with greater affinity to ERβ (Wagner et al. 2001). Genistein is also a tyrosine kinase inhibitor (Akiyama *et al.* 1987) and high concentrations of genistein may inhibit insulin signalling pathways. However, the potential effects of soy foods in human health remain highly controversial (Erdman, Jr. 2000; Messina et al. 2004; Mezei et al. 2003; Sacks et al. 2006). It has been suggested that many of the health effects of soy may be related to activation of ERα and ERβ, mediated by soy-associated phytoestrogens, especially genistein and daidzein (Badger *et al.* 2002).

4.1 Biochemical features of the metabolic syndrome and the impact of soybean

The progressive increase in obesity, cardiovascular diseases and metabolic syndrome prevalence motivated the National Cholesterol Education Program (NCEP) on its third panel: *Treatment of High Blood Cholesterol in Adults* (ATP III 2004), to propose clinical criteria to define metabolic syndrome by the presence of three or more of these altered factors: high blood pressure (BP), dyslipidemia (hypertriglyceridemia, low HDLc), high plasmatic glucose and abdominal obesity. A number of cardioprotective benefits have been attributed to dietary isoflavones, contained in soy products, including a reduction in LDL cholesterol, an inhibition of pro-inflammatory cytokines, cell adhesion proteins and inducible nitric oxide production, potential reduction in the susceptibility of the LDL particle to oxidation, inhibition of platelet aggregation and an improvement in vascular reactivity (Rimbach *et al.* 2008).
With this in mind, it would be expected that high soy consumption would be associated with a lower risk of metabolic syndrome, but little evidence exists regarding the effects of soy consumption on the metabolic syndrome in humans. We will analyze the effect of soybean consumption on each parameter of the metabolic syndrome separately.

4.1.1 Impact of soy products on blood pressure and endothelial function

The renin-angiotensin system is one of the most important blood pressure control systems in mammals (Paulis & Unger 2010). The effect of commercial purified soybean saponin on renin activity and blood pressure was investigated by Hiwatashi and colleagues (Hiwatashi *et al.* 2010). They found that soybean saponin inhibited renin activity *in vitro* and that oral administration of soybean saponin at 80 mg/kg of body weight per day to spontaneously hypertensive rats for 8 weeks significantly decreased blood pressure. In addition, a study investigated the effects of soy protein and soy isoflavones on blood pressure and cholesterol levels in 61 middle-aged men at high risk of developing coronary heart disease in Scotland (Sagara et al. 2004). For five weeks, half the men consumed diets containing at least 20 g of soy protein and 80 mg of soy isoflavones each day. The effects on blood pressure, cholesterol levels, and urinary excretion of isoflavones were measured, and then compared to those of the other half of the men who were given a placebo diet containing olive oil. Men that consumed soy in their diet presented significant reductions in both diastolic and

systolic blood pressure. Furthermore, Welty and colleagues (Welty *et al.* 2007) found that soy nut supplementation significantly reduced systolic and diastolic pressure in all 12 hypertensive women and in 40 of the 48 normotensive women. The order of diets did not affect the response. Hypertensive women (mean baseline systolic BP of 152 mm Hg and diastolic BP of 88 mm Hg) had a mean 9.9% decrease in systolic BP and a 6.8% decrease in diastolic BP on the soy diet compared with the control diet. Normotensive women (mean systolic pressure of 116 mm Hg) had a 5.2% decrease in systolic pressure and a 2.9% decrease in diastolic pressure on the soy diet compared with the control diet. On the other hand, Teede and colleagues found no effect of soy/isoflavones on blood pressure in a study that enrolled 41 hypertensive subjects (Teede et al. 2006).

Regarding endothelial function, it has been reported that the consumption of soy isolated protein improved the flow-induced dilatation in postmenopausal women (Cuevas et al. 2003; Hall et al. 2008), independently of changes in the lipid profile. This effect has been also observed using soybean isoflavones (Colacurci et al. 2005; Lissin et al. 2004).

The mechanisms of action by which soybean products may improve endothelial function include effects on the production of nitric oxide, a key regulator of the cardiovascular function. Soybean products have been shown to increase the levels of endothelial nitric oxide synthase (eNOS). For instance, genistein increased the levels of NOS in spontaneously hypertensive rats (Si & Liu 2008; Vera et al. 2007) as well as NOS activity (Lin et al. 2011; Si & Liu 2008). Equol, a metabolite of daidzein produced by intestinal bacteria, has also been shown to activate eNOS and produce vasorelaxation in an acute fashion (Joy et al. 2006).

4.1.2 Metabolic syndrome dyslipidemia

In the last decades several publications have shown beneficial effects of soybean products consumption on lipids metabolism.

As mentioned previously, the meta-analysis by Anderson and colleagues (Anderson et al. 1995) indicated that soy protein was effective in lowering plasma cholesterol. Also beneficial results were published in 1998 by Tikkanen and colleagues, showing in six healthy volunteers who consumed soy protein (60 mg isoflavones per day) for 2 weeks, a reduction in LDL oxidation, which is atherogenic (Tikkanen *et al.* 1998). Later, a similar study confirmed the same in 24 subjects, where dietary supplementation with high-isoflavone soy protein for 17 days prolonged the lag time of copper-induced LDL oxidation compared to a low-isoflavone soy diet and also reduced plasma concentrations of F2-isoprostanes (marker of lipid peroxidation) (Wiseman *et al.* 2000). In the same way, Potter in 1998 studied the impact of bioactive components of soy in cardiovascular disease and confirmed that the major components of soybean flour (soy proteins; soy cotyledon fiber and isoflavones) independently decrease serum cholesterol (Potter 1998). A meta-analysis conducted by Ho (Zhan & Ho 2005) showed that soy protein containing isoflavones significantly reduced serum total cholesterol, LDL cholesterol, and triacylglycerol and significantly increased HDL cholesterol, but the changes were related to the level and duration of intake, and gender and initial serum lipid concentrations of the subjects.

4.1.3 Glucose metabolism and abdominal obesity

The effects of soy consumption on glucose metabolism are more controversial than the beneficial effects on lipids, and still remain uncertain since there are many contradictory publications. Clear differences in the carbohydrate profiles were observed following soy intervention, suggesting a soy-induced alteration in energy metabolism (Solanky *et al.* 2005).

These results were later supported by metabonomic analyses of 24 h urine samples from the same individuals and combination of both analyses suggested an inhibition of glycolysis and a general shift in energy metabolism from carbohydrate to lipid metabolism due to isoflavone intervention. In 2007 Ho and colleagues (Ho *et al.* 2007) studied the long-term effects of soy-derived isoflavones on glycemic control, specifically fasting glucose and found that one-year of soy isoflavone supplementation showed a moderate but significant favorable effect in fasting glucose in postmenopausal Chinese women. On the other hand Liu et al (Liu *et al.* 2010) published recently an interesting placebo-controlled trial, where they investigated the effects of soy protein and isoflavones on glycemic control and insulin sensitivity: in a 6-months control-case trial with postmenopausal Chinese women, with prediabetes or untreated early diabetes. They concluded that the results did not support the hypothesis that soy protein had favorable effects on glycemic control and insulin sensitivity. The same year, another publication aimed to determine the effects of soy consumption on the components of the metabolic syndrome (Azadbakht *et al.* 2007) was published. They randomly assigned to 42 postmenopausal women with the metabolic syndrome a control diet (Dietary Approaches to Stop Hypertension, DASH), a soy-protein diet, or a soy-nut diet, each for 8 weeks. Consumption of soy-nut reduced fasting plasma glucose more significantly than the soy-protein or control diet. Soy-nut consumption significantly reduced serum C-peptide concentrations compared with control diet, but consumption of soy-protein did not. The soy-nut regimen also decreased LDL cholesterol more than did the soy-protein period and the control diet.

5. Soybean effects on hemostasis

Epidemiological studies suggest that soy consumption is associated, at least in part, with lower incidence of a number of chronic diseases (Anderson et al. 1995; Boyapati et al. 2005). The lower rates of several chronic diseases in Asia, including cardiovascular diseases and certain types of cancer, have been partly attributed to consumption of large quantities of soy foods (Kang et al. 2010; Messina 1995; Wu et al. 1998).

Cardiovascular diseases are leading causes of death worldwide, especially in developed and developing countries. As mentioned before, several risk factors for cardiovascular diseases, including dyslipidemia, hypertension and diabetes mellitus, in large part are influenced by diet (De et al. 2006; Liu et al. 2000).

Epidemiological evidence suggests that a diet rich in fruits and vegetables promotes health, reducing the risk of developing cardiovascular and other types of chronic diseases (Rimm 2002). The contribution in antioxidants from fruits and vegetables is vastly recognized, however, their antithrombotic effects are less known (Torres-Urrutia et al. 2008; Torres-Urrutia et al. 2011).

In order to contextualize, we refer to hemostasis, a complex process that prevents the spontaneous loss of blood and stops bleeding caused by damage to the vascular system. This process, traditionally, has been separated into: primary hemostasis, secondary hemostasis (clotting) and fibrinolysis.

5.1 Primary hemostasis

This phase includes vasoconstriction and platelet-endothelium interactions. Upon vessel wall injury, rapid and complex interactions between circulating platelets and exposed (sub)endothelial structures occur, resulting in platelet adhesion to the damaged endothelium (Santos et al. 2009). The mechanism by which platelets adhere to the vascular

wall to achieve hemostasis is well understood, with von Willebrand Factor (vWF)-mediated platelet adhesion being the most important. Another adhesive protein crucially involved in platelet-vessel wall interaction is collagen, that binds to platelet glycoproteins receptors Ia/IIa and GPVI (Clemetson & Clemetson 2001; Jung & Moroi 2000).
The roles of platelets include adhesion, secretion (ADP, serotonin and thromboxane A2 (TXA2), and aggregation (interaction between glycoprotein GPIIb-IIIa and fibrinogen). Forming the base of the hemostatic plug, the platelet membrane turns into a phospholipid surface upon activation due to a change in shape. This negatively charged surface is necessary for the assembly of activated coagulation factor complexes which in turn are required for thrombin generation. Platelets form a strong link between the processes of primary and secondary hemostasis.

5.2 Secondary hemostasis

The coagulation system corresponds to a cascade of proteolytic activation of plasma factors, typically in two ways, the intrinsic and extrinsic pathways, whose purpose is the formation of fibrin. The fibrin deposit on and between platelets, stabilizes the platelet plug (Palomo et al. 2009).
The coagulation system has three overlapping phases: initiation, amplification and propagation (Hoffman 2003; Hoffman & Monroe 2007). The coagulation requires the formation of an impermeable platelet and fibrin plug at the site of vessel injury, but it also requires that the powerful procoagulant substances activated in this process remain localized to the site of injury. This control of blood coagulation is accomplished by localizing the procoagulant reactions on specific cell surfaces (Hoffman & Monroe 2007).
Coagulation *in vivo* is initiated by the tissue factor. The initiation step is thus localized to the cells that express tissue factor, which are normally found outside the vasculature. A disruption in the vascular wall exposes collagen that causes accumulation and activation of platelets, while exposed tissue factor initiates the process of generating thrombin (Hoffman & Monroe 2007; Romney & Glick 2009) . The small quantity of thrombin generated during this phase is the key factor for further thrombin and subsequent fibrin production (Furie & Furie 2008).
The amplification step sets the stage for subsequent large-scale generation of thrombin in the propagation phase. The small amount of thrombin generated on the tissue factor-bearing cell has several important functions: among others, activation of platelets, activation of the cofactors V and VIII on the activated platelet surface and activation of factor XI to XIa (Hoffman & Monroe 2007; Oliver et al. 1999). In the propagation phase, the tenase (FVIIIa/FIXa) and prothrombinase (FVa/FXa) complexes are formed on the platelet surface. The tenase complex activates FX, which then binds to its cofactor, FVa. The prothrombinase complex on the surface of the platelet initiates a burst of thrombin that brings about the conversion of fibrinogen to fibrin. Thrombin produced in this phase also activates factor XIII, which stabilizes the fibrin clot by catalyzing covalent crosslinkage (Hoffman & Monroe 2007; Schenone et al. 2004; Zimmerman 2007).

5.3 Fibrinolysis

This process involves the breakdown of fibrin clots and the restoration of blood flow. The fibrinolytic system activates the process by which a fibrin clot is digested by plasmin to release soluble fibrin degradation products. Plasmin is generated from plasminogen by tissue-type and urinary-type plasminogen activators (tPA and uPA), and the process is

regulated by the plasmin inhibitor α-2-antiplasmin, plasminogen activator inhibitor (PAI-1) and the Thrombin- Activable Fibrinolysis Inhibitor (TAFI)(Palomo et al. 2009).

5.4 Antiplatelet activity of soybean products

Flavonoids The effects of soybean products on platelet aggregation were initially described for genistein (McNicol 1993; Nakashima et al. 1991). In these reports, genistein was able to inhibit platelet activation induced by collagen and TXA2 analogs, but not by thrombin. Daizein, another soy flavonoid that lacks tyrosine kinase inhibitory activity also inhibited the response to collagen and the TXA2 analog, suggesting that these flavonoids inhibit platelet aggregation by competition for the TXA2 receptor (TXA2R) rather than through tyrosine kinase inhibition. These observations were confirmed by other groups (Gottstein et al. 2003; Guerrero et al. 2005; Guerrero et al. 2007). Guerrero and colleagues suggested that this competitive binding was due to structural features of these flavonoids such as the presence of a double bond in C2-C3 and a keto group in C4. Interestingly, it has been recently described that equol, a metabolite of daidzein, showed higher affinity in binding the TXA2R (Munoz et al. 2009) (Figure 1).

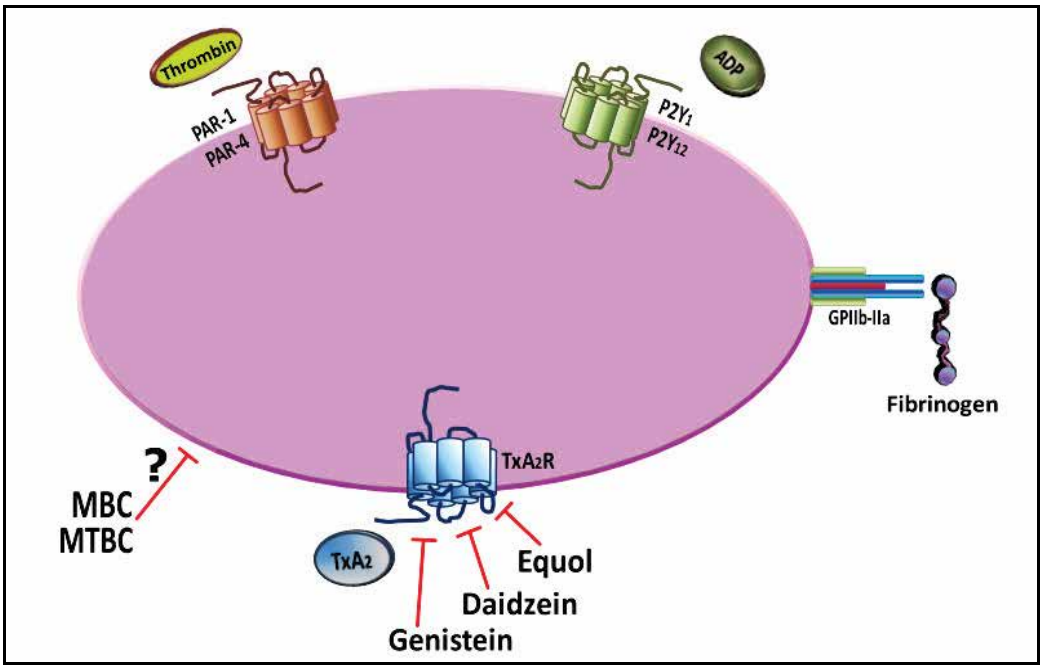

Fig. 1. Antiplatelet effects of soybean products. Equol, daidzein and genistein antagonize the thromboxane A2 receptor. MBC (1-methyl-*β*-carboline) and MTBC (-methyl-1,2,3,4-tetrahydro-*β*-carboline) probably act by affecting the plasma membrane fluidity.

Alkaloids From a extraction of soy sauce, two kinds of components with anti-platelet activity were isolated and structurally identified: 1-methyl-1,2,3,4-tetrahydro-*β*-carboline (MTBC) and 1-methyl-*β*-carboline (MBC). MTBC shows IC_{50s} ranging from 2.3 to 65.8 μg/mL for aggregation response induced by epinephrine, platelet-activating factor (PAF), collagen, ADP and thrombin (Tsuchiya et al. 1999).

Membrane fluidity regulates the platelet function and various membrane-fluidizing agents are known to inhibit platelet aggregation (Kitagawa *et al.* 1993). Certain *β*-carbolines influence the fluidity of model membranes (Peura *et al.* 1982). The alteration of membrane fluidity may be involved in the antiplatelet effects of MTBC and MBC.

5.5 Soybean fibrinolytic activity

Among popular soy foods in Asian countries, natto is a traditional fermented product. Fermented soybeans are prepared using *Bacillus subtilis* natto (*B. natto*). Natto extracts are known to include nattokinase, a potent fibrinolytic enzyme having approximately four-times stronger activity than plasmin in the clot lysis assay (Fujita *et al.* 1993). It is composed of 275 amino acid residues (molecular weight, 28 KDa) and exhibits a high homology with subtilisins (Fujita *et al.* 1995b). Large-molecule proteins usually are inactive because of non-specific lysis in the digestive organs. However, in rats after intraduodenal administration, nattokinase was absorbed and decreased fibrinogen levels in plasma (Fujita et al. 1995a; Suzuki et al. 2003). This effect was also observed in humans that received nattokinase, in which the treatment reduced fibrinogen levels as well as those of factor VII and factor VIII (Hsia et al. 2009).

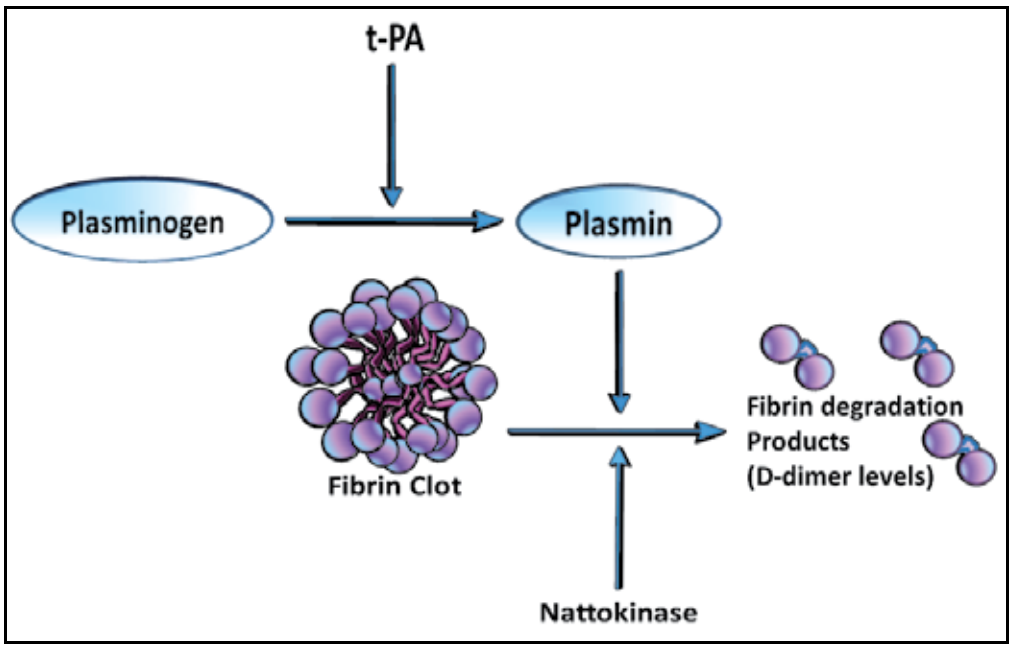

Fig. 2. Fibrinolytic effect of nattokinase, a product of soybean fermentation. tPA, tissue plasminogen activator.

When the proteolytic fragments of fibrin(ogen) were compared with those of plasmin at the same molarity of nattokinase, similar fragments were obtained from the process of cleavage of fibrin(ogen). The cleavage of fibrinogen by nattokinase was three times less efficient than by plasmin as measured from kcat/*K*m. However, the cleavage of cross-linked fibrin by nattokinase was six times more efficient than by plasmin. These results suggest that nattokinase is less sensitive to the cleavage by fibrinogen, but is more sensitive to the cleavage by cross-linked fibrin as compared to plasmin (Fujita et al. 1995b).

Its remarkable thrombolytic efficacy was also shown in a chemically induced thrombosis model, in the common carotid artery of rats in which the endothelial cells of the vessel wall were injured by acetic acid (Fujita et al. 1995a). The thrombolytic activity of nattokinase using this model was compared with fibrino(geno)lytic enzymes, plasmin or elastase. On a molar basis, the recovery of the arterial blood flow with nattokinase, plasmin and elastase were 62.0 +/- 5.3%, 15.8 +/- 0.7% and 0%, respectively. These results indicate that the thrombolytic activity of nattokinase is stronger than that of plasmin or elastase *in vivo* (Fujita et al. 1995a).

6. Conclusion

The experimental and epidemiological evidence of the last decades using soy based foods are promising in relation with cardiovascular protection, mainly due to hypocholesterolemic effects. These evidences were consider by FDA, that published claims that recommended soy protein extract as an alternative to reduce blood cholesterol concentrations and increase cardiovascular protection. However, in the last years, several studies have been performed and have been unable to confirm the beneficial effects of soy in blood LDL-cholesterol concentrations and others cardiovascular risk parameters, such as lipoprotein (a) concentrations or blood pressure. Moreover, a very large amount of soy protein, more than protein diary recommended ingest, may lower LDL-cholesterol but this effect depends on initial blood cholesterol concentrations and is modest. The experimental evidences related soy protein more than soy isoflavones as responsible for the observed effects. At present is not possible to discard other components present in soy as responsible by these effects. Neither has been possible to know the molecular mechanism involved in these effects on lipoprotein profile. Today, research is focused on protective effects not associated with lipoprotein profile,such as anti-inflammatory properties, for example, by isoflavones. However, isoflavones are not recommended because there are not conclusive evidences about their role in some cancer types. On the other hand, soy based diets also contain high levels of polyunsaturated fatty acids, fibre and vitamins which could be related with cardiovascular benefits. In conclusion, the evidences obtained from meta-analysis and well-controlled studies are not conclusive in relation to hypolipidemic effects of soy as mechanism to explain cardioprotective effects. Recently, studies have begun to propose anti-inflammatory properties as possible mechanism to explain protective cardiovascular health. In addition, interesting inhibitory effects of soybean products on platelet activation and aggregation have been shown and suggest that some of the positive effects of soybean products may be mediated by anti-thrombogenic activity.

7. Acknowledgments

This work was supported by the Programa de Investigacion en Factores de Riesgo Cardiovascular (PIFRECV), Universidad de Talca, Talca, Chile. http://pifrecv.utalca.cl
We thank Mrs. Adriana Treuer for the design of the figures.

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2

Fermented Soybean Products and Their Bioactive Compounds

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1. Introduction

Soybean (*Glycine max* MERILL), first grown in Eastern Asia thousands of years ago, have long been important protein sources, complementing grain proteins, in Asian countries. In addition to essential nutrients, soybean products, especially fermented soybean products, contain various functional components including peptides, isoflavonoids and more (Davis et al., 2005). Due to these nutritional and functional facts, soybean products were included in the world's top 5 healthiest foods in magazine 'Health (2006)': Due to dozens of studies showing soy is good for your heart; the FDA even allows certain soy products to have a heart-healthy claim on their labels. A number of epidemiological studies have suggested that consumption of soybeans and soy foods is associated with lowered risks for several cancers including breast, prostate, and colon, and cardiovascular diseases (Anderson et al., 1998; P.C. Butler et al., 2007; Messina, 1995; Peterson & Barnes, 1991, 1993) and improves bone health (Bhathena & Velasquez, 2002). Furthermore, some studies have shown that a diet rich in soy can reduce breast cancer risk (Messina, 1999a, 1999b).

Fermentation is one of the major processes used in the production of food from soybeans. Fermented soybean paste is indigenous to the cuisines of East and Southeast Asia. Bibliographically, Korea developed and used its own traditional fermented foods two thousand years ago (Kwon et al., 2011a). Korean soy foods are increasingly present on the worldwide market, and because kochujang (fermented red pepper paste with soybean flour) and fermented soybean pastes (doenjang and chungkukjang; both chungkukjang and chungkookjang are used in the reports) were registered in CODEX in July, 2009, they are now internationally accepted foods (Kwon et al., 2010). Most fermented soybean pastes are salty and savory and some are spicy. They are often used as condiments to flavor foods such as stir-fries, stews, and soups. This fermentation changes the physico-chemical and organoleptic properties of soy products such as color, flavor and active components.

Differences in their color, flavor and active components are due to different production methods such as the conditions of fermentation; the addition of wheat flour, pulverized meju, rice; and the presence of different microflora such as bacteria or yeasts used in their production, as well as whether the soybeans are roasted (as in chunjang) or aged (as in tauchu) before being ground. In addition to physicochemical properties, the fermentation of these soybean products changes the bioactive components, such as isoflavonoids and peptides, in ways which may alter their physiological properties in terms of healthy functions and efficacies.

Although soybean is well known to be good for preventing obesity, diabetes, heart disease and breast cancer, the scientific evidence for these health effects are not well enough documented to satisfy the inquisitiveness of oriental fermented foods. The need to satisfy this curiosity is increasing, so we have investigated the health effect of Korean traditional fermented foods, identified the active compounds produced during fermentation and the possible mechanisms that support and challenge the hypothesis. Also we designed the new noble compounds based on the structure which was isolated from fermented soybeans products by modeling the active conformation of linear peptides. Metabolic diseases such as obesity, diabetes and cardiovascular diseases that are very close related with dietary foods are the focus of this study.

Here we introduced the fermented soybean foods and discussed their compositional changes that occur during fermentation to obtain scientific evidence for the health effects and possible mechanism of action of these fermented soybean products. We reviewed the epidemiologic studies, cellular/animal experiments or metabolomic approached studies about soybeans and fermented soybeans and the most of the research reviewed here was performed by our research teams.

2. Korean fermented soybean products

We already described the typical fermented soybean foods are consumed in Asian countries such as Korea, China, Japan, Indonesia, and Vietnam (Kwon et al., 2010). The most common Korean fermented soybean foods are chungkukjang, doenjang, kanjang (soy sauce), and kochujang. Originally natto and miso were Japanese versions of chungkukjang and doenjang, respectively. China also has various fermented soybean products such as doubanjiang, douche (sweet noodle sauce), tauchu (yellow soybean paste), and dajiang.

Chungkukjang is a short-term fermented soybean product similar to Japanese natto, whereas doenjang, kochujang, and kanjang (soy sauce) undergo long term fermentation as do Chinese tauchu and Japanese miso (Fig. 1).

Traditionally chungkukjang was made by fermenting the cooked but non-crushed soybeans for two or three days in the living room, usually they prepared chungkukjang in autumn and winter after harvesting soybeans. Doenjang and kanjang were prepared by three step fermentations: firstly they prepared the meju (dried soybean block) from cooked beans in late of October or early of November and fermented it for 1 or 2 months like as solid fermentation under the outdoor roof of the Korea traditional house in winter; secondly they aged meju for another 1 or 2 months in large earthenware jars by adding a salt solution as liquid fermentation, and then they decanted the supernatant liquid to prepare kanjang from liquid and doenjang from the remaining soy paste; finally, both liquid and paste were aged for longer periods. In comparison, Japanese style doenjang (miso) and kanjang were prepared by directly fermenting the cooked soybean without making meju individually.

Kochujang is a unique and representative Korean traditional food for more than a thousand years (Kwon et al., 2011a). Kochujang was usually prepared by mixing powdered red peppers, powdered meju, salt, malt-digested rice syrup, and rice flour, and the mixture fermented for more than 6 months.

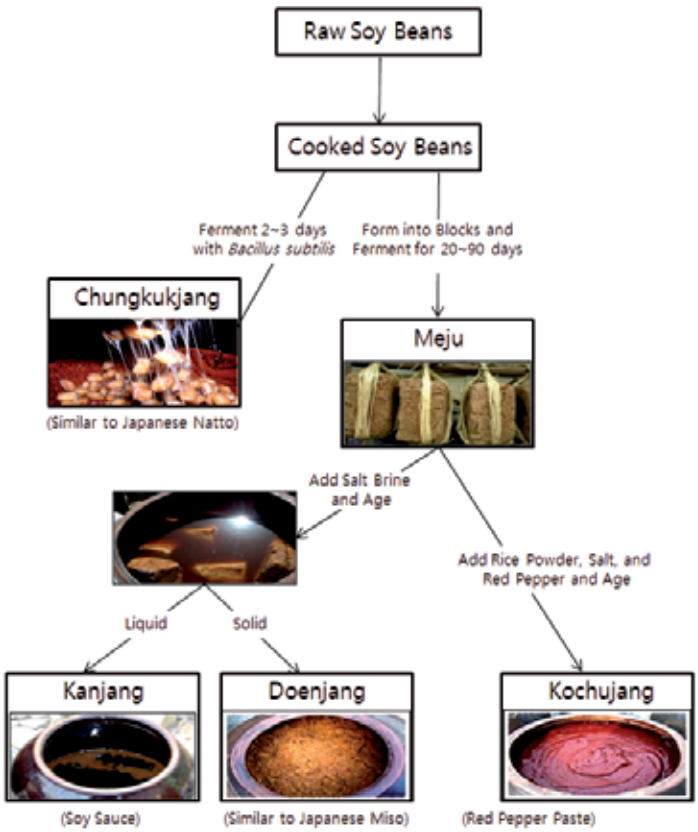


Fig. 1. The preparation of Korean fermented products from soybeans

3. Microorganisms in fermented soybean products

Meju fermentation is the most important step in producing doenjang and kanjang, because many metabolite changes occur during this fermentation, whereas the changes in flavor, color and bioactive components were primarily depends on aging after fermentation. Chungkukjang, doenjang, kanjang and kochujang are fermented with varying microorganisms when traditionally made, because fermentation conditions and ambient microbiota are different among regional environments. To make these products by the traditional method, cooked soybeans are formed into meju and fermented outdoors by micro-organisms naturally present in the environment for 1-2 months. Meju can be fermented by traditional methods, being typically fermented primarily by *Bacillus* species during the early stages of fermentation, followed by *Aspergillus* species, which predominate during the remaining fermentation period. *Aspergillus oryzae* is the major microorganism in the final product of meju when it is made in the traditional method. In traditionally

fermented chungkukjang, many different bacterial strains were identified by rRNA gene sequencing or recA sequence using randomly amplified polymeric DNA-PCR method (G.H. Kwon et al., 2009), *Bacillus subtilis*, *B. amyloliquenfaciens*, *B. licheniformis* and *B. thuringiensis* were representative microorganisms during the chungkukjang fermentation. Among them, *B. subtilis* is the predominant strain in traditional chungkukjang fermentation.

It is well known that poly-γ-glutamate (PGA) and nattokinase (fibrinolytic enzyme) are major components for maintaining a healthy immune system (Messina, 1995) and cardiovascular system (Sumi et al., 1987), respectively. Fibrinolytic microorganisms screened from chungkukjang, doenjang, meju and natto, were identified as *B. subtilis*, *Staphylococcus sciuri*, *Enterococcus faecalis*, and *Citrobacter* or *Enterobac ter* (Yoon et al., 2002). Even in tempeh (Indonesian fermented food), the microorganism producing fibrinolytic enzyme has been screened. We isolated *B. subtilis* from chungkukjang which secreted four different fibrinolytic proteases into the culture medium. Also we cloned the fibrinolytic enzyme gene, aprE2, from this *B. subtilis* into another *B. subtilis* strain to over-express the enzyme successfully using *Esherichia coli-Bacillus* shuttle vector (Jeong et al., 2007). Sometimes chungkukjang became highly viscous due to fibrous PGA polymer accumulation, Ashiuchi et al. (2001) isolated strains of *B. subtilis* from chungkukjang which produced a high contents of PGA.

4. Changes in soybean components by fermentation

4.1 Nutritional and functional compounds in soybeans

Soybean products have been known as healthy foods due to being an excellent source of high quality protein as well as providing various health benefits. The protein content of soybean is 32% to 42%, depending on the variety and growth conditions, of which approximately 80% is composed of 2 storage globulins, 7S globulin (β-conglycinin) and 11S globulin (glycinin), having various functional and physicochemical properties (Garcia et al., 1997; Kwon et al., 2002, 2003). Soybean products are considered as a good substitute for animal protein, and their nutritional value except sulfur amino acids such as methionine and cysteine is almost equivalent to that of animal protein because soy proteins contain most of the essential amino acids for human nutrition.

In addition to high-quality protein, soybeans contain high levels of unsaturated fatty acids, dietary fiber, isoflavones and minerals, which possess numerous health benefits (Young, 1991). In particular, the association of high-quality protein and phytochemicals, especially isoflavones, is unique among plant-based proteins because isoflavones are not widely distributed in plants other than legumes (Velasquez & Bhathena, 2007). Soybeans contain 0.1 to 5 mg total isoflavones per gram, primarily genistein, daidzein, and glycitein. These nonsteroidal compounds, commonly known as soy phytoestrogens, are naturally present as the β-glucosides genistin, daidzin, and glycitin, representing 50% to 55%, 40% to 45%, and 5% to 10% of the total isoflavone content, respectively (Murphy et al., 1999), depending on the soy products (Murphy et al., 1999; Velasquez & Bhathena, 2007; Young, 1991).

4.2 Changes of nutritional compounds and functional components during fermentation

The qualitative and quantitative composition of soybean components is dramatically changed by physical and enzymatic processes during the preparation of soy-based foods (L.M. Baek et al., 2008; Garcia et al., 1997; Jang et al., 2008; Y.W. Lee et al., 2007; Nakajima et

al., 2005; J.S. Park et al., 1994; Yamabe et al., 2007). Fermentation is an excellent processing method for improving nutritional and functional properties of soybeans due to the increased content of small bioactive compounds. The conformation of soy protein (glycinin) is easily changed by heat (steaming) and salt (K.S. Kim et al., 2004). The large protein, lipid, and carbohydrate molecules in raw soybean are broken down by enzymatic hydrolysis during fermentation to small molecules such as peptides, amino acids, fatty acids, and sugars, which are responsible for the unique sensory and functional properties of the final products. Short-term fermented soy foods such as chungkukjang, which are fermented with *B. subtilis*, for less than 72 hr have a much greater concentration of large molecules than do long-term fermented foods including meju and doenjang, which are fermented for more than 6 months with *Bacillus* and *Aspergilus* species from rice straw and koji, respectively (Jang et al., 2008; Y.W. Lee et al., 2007; J.S. Park et al., 1994; Yamabe et al., 2007). Proteomic analysis for soluble proteins from chungkukjang at different fermentation periods suggested that most of the soluble soy proteins were degraded into smaller forms within 20 hr, and many microbial proteins, such as mucilage proteins which, are assimilated into the bacterial biomass, dominated by the soluble protein fraction. The proteomic profile of chungkukjang was very different from that of natto, in terms of the 2-D gel protein profile (Santos et al., 2007) (Fig. 2).

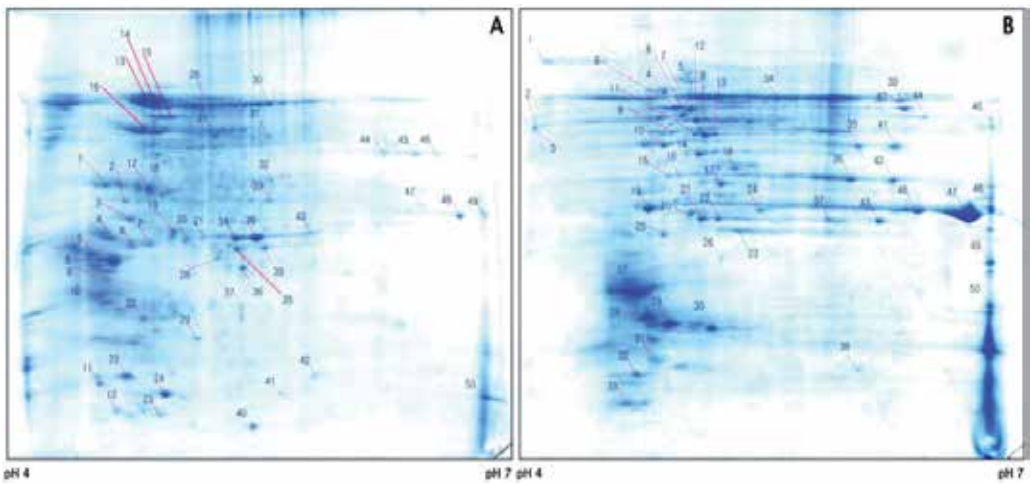


Fig. 2. A comparison of soluble proteins from chungkukjang and natto on a 2-D gel. Phenol extracted soluble proteins from chungkukjang (A) and natto (B) were separated on pH 4-7 IPG strip and 12% SDS-PAGE gel (18×20 cm), and 50 randomly selected protein spots were analyzed by MALDI-TOF MS (Santos et al., 2007).

The degradation of lipids and carbohydrates proceeds especially rapidly during the initial stage of fermentation, since these are used as the major energy sources for the microorganisms (Yamabe et al., 2007). After the initial stage of fermentation, however, soy proteins are rapidly degraded and only a small amount of the crude protein remains at the end stage of long-term fermentation. Soybean isoflavones appear to be the major components responsible for the bioactive functions such as lowering the risks of cancers of breast, prostate, and colon, cardiovascular diseases and osteoporosis.

Isoflavones, which are mostly present as 6-O-malonylglucoside and β-glucoside conjugates and associated with proteins in soybean, are also broken down by heat treatment and fermentation (Barnes et al., 1998; H.K. Choi et al., 2007). During preparation of fermented soy foods, 6-O-malonylglucosides, the most prevalent soybean isoflavones, are converted to 6-O-acetylglucosides or β-glucosides by heating, and β-glucosides are de-conjugated by the action of β-glucosidases secreted by fermentation microorganisms (Murphy et al., 1999). Most isoflavones are not enzymatically hydrolyzed during short-term fermentation, in contrast to long-term fermentation in which 6-o-malonylglucoside content declines with increasing fermentation time with concomitant increases in unconjugated aglycones (genistein and daizein). Total isoflavone content in raw soybeans was about 3,000mg/kg and this decreased by about 50% during cooking prior to chungkukjang preparation. Total isoflavone content of chungkukjang changed slightly during 45 hr fermentation. However, the content of isoflavone glycosides, consisting mainly of daizein, glycitin and genistin, decreased by about 40% during 45 hr fermentation, while the contents of free isoflavones including, daidzein, glycitein and genistein showed a dramatic increase during chungkukjang fermentation, with 2.9-, 54.0-, and 20.6 fold increases in concentration, respectively, by end of fermentation (45 hr) (Jang et al., 2006) (Table 1).

Isoflavone	Fermentation time (hr)										
	raw	0	5	10	15	20	25	30	35	40	45
Daidzin	99±31[1)]	454±17	425±24	401±25	327±28	280±5	245±22	191±5	192±7	212±7	204±10
Glycitin	45±15	101±5	101±5	87±6	77±6	60±6	65±26	69±4	34±5	41±4	35±4
Genistin	184±50	548±22	568±19	513±30	476±32	401±14	333±16	295±12	289±17	281±14	259±22
M-Daidzin	1009±29	129±41	127±29	82±7	97±12	75±9	120±54	96±30	113±22	134±10	120±27
M-Glycitin	157±2	10±5	3±0	12±5	63±5	144±3	217±1	220±0	225±7	244±5	231±1
M-Genistin	1111±8	tr[2)]	tr	tr	tr	tr	tr	tr	tr	tr	tr
A-Daidzin	85±47	75±3	64±8	54±2	56±5	83±2	93±2	91±3	94±2	99±5	97±6
A-Glycitin	101±174	10±2	7±1	5±4	11±5	29±12	44±7	50±10	46±9	38±12	33±12
A-Genistin	tr	43±3	47±3	37±3	35±3	45±0	38±5	41±6	39±5	31±6	27±5
Total glycoside	2818	1370	1341	1191	1142	1117	1156	1020	1033	1079	1006
Daidzein	tr	33±6	50±8	36±2	69±6	79±2	82±2	107±3	112±5	102±6	114±7
Glycitein	tr	4±3	3±4	5±3	43±6	100±0	141±4	152±2	156±2	171±1	165±2
Genistein	7±2	5±4	16±3	9±1	32±9	45±1	39±1	71±4	72±5	52±3	64±4
Total aglycone	7	43	69	51	143	223	262	330	341	325	342
Total isoflavones	2825	1413	1410	1242	1285	1340	1418	1350	1370	1404	1348

[1)]Values are mean ± SD (n-3). [2)]tr : trace.

Table 1. Isoflavone contents (μg/g dry water) of chungkukjang collected at various fermentation times (Jang et al., 2006).

We also investigated changes in isoflavone contents during the fermentation of meju and doenjang (Jang et al., 2008) (Table 2-3). Most of isoflavones in cooked and unfermented soybean existed as glycosides (Table 2), accounting for total isoflavones. While isoflavone

contents were not changed significantly during the first 10 days of meju fermentation, they were changed between the 10 and 90 day from the initiation of fermentation, suggesting that there was the rapid growth of microorganisms and they produced isoflavone-degrading enzymes during those periods.

Isoflavone	Raw	Steamed	Fermented period (day)			
			10	20	40	90
Daidzin	98±18	454±3	367±22	355±19	334±8	110±28
Glycitin	94±51	104±16	93±18	77±37	67±23	13±20
Genistin	148±21	913±11	637±62	623±21	488±9	229±33
M-Daidzin	1,080±44	61±3	280±31	165±14	196±6	92±41
M-Glycitin	233±24	1±1	5±9	12±1	3±5	10±9
M-Genistin	1,244±39	74±17	264±10	Tr[1)]	Tr	Tr
A-Daidzin	23±16	55±5	50±8	47±0	46±8	14±1
A-Glycitin	134±19	26±19	82±8	13±2	11±8	6±2
A-Genistin	57±10	139±3	53±22	45±19	35±17	15±24
Total glycosides	3,111±177	1,827±20	1,830±125	1,337±52	1,181±71	487±220
Daidzein	Tr	Tr	Tr	74±3	68±9	152±19
Glycitein	13±7	6±1	Tr	Tr	4±5	7±2
Genistein	12±2	16±1	6±3	35±3	25±3	170±20
Total aglycones	25±6	22±2	6±4	108±6	97±9	329±56
Total isoflavones	3,137±174	1,849±23	1,836±123	1,445±47	1,278±65	816±276

[1)]Trace

Table 2. Change in isoflavone contents (μg/g) during meju fermentation (Jang et al., 2008)

Isoflavone	Aging period (day)							
	0	20	40	60	80	120	140	160
Daidzin	Tr[1)]	Tr	Tr	Tr	Tr	Tr	Tr	Tr
Glycitin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
Genistin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
M-Daidzin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
M-Glycitin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
M-Genistin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
A-Daidzin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
A-Glycitin	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
A-Genistin	27±5	36±7	32±5	42±7	41±8	42±17	29±8	16±2
Total glycosides	27±5	36±8	32±6	42±8	41±9	42±18	29±9	16±3
Daidzein	222±34	246±67	226±23	282±23	271±50	275±47	325±55	273±24
Glycitein	72±14	78±21	72±4	82±5	82±17	71±16	89±22	79±23
Genistein	298±27	366±81	349±42	411±25	361±40	374±36	334±1	292±55
Total aglycones	592±70	689±167	646±64	775±50	714±106	719±75	749±110	644±14
Total isoflavones	618±75	724±175	678±69	817±56	754±114	761±92	778±111	660±12

[1)]Trace

Table 3. Change in isoflavone contents (μg/g) during aging of doenjang (Jang et al., 2008)

The concentration of aglycones in meju was increased to reach to approximately 40% of total isoflavones in 90 day meju fermentation. However, during the aging step of doenjang production, no significant changes in isoflavone content were observed probably due to inhibition of microorganism growth under high salt condition (Table 3).

In addition to the natural isoflavones found in soybeans, novel isoflavonoids such as equol (7-hydroxyisoflavan), dihydrodaidzein, and O-desmethylangolensin (Joannou et al., 1995; Lampe, 2009), known to be produced by intestinal microflora from daidzein, were found in some fermented soy products, which were metabolized by microorganisms (Fig. 3). Equol exhibits a more powerful estrogenic activity than daidzein and has been reported to exert beneficial health effects on various types of diseases such as osteoporosis, cardiovascular disease, hormone-dependent cancer, and post-menopausal syndrome. Therefore, equol production is probably one factor responsible for the decreased risk of certain cancers and other diseases in humans consuming fermented soybean foods. In general, the chemical profiles of various minor components related to health benefits and nutritional quality of products are also affected by fermentation (Izumi et al., 2000; D.J. Kim et al., 2008)

Among the various small metabolites derived from macromolecules, the changes in amino acid and peptide concentrations were especially prominent, although qualitative and quantitative changes in individual peptides were not studied. Some amino acids increased and others remained almost constant with increased fermentation time, but glutamate, the richest amino acid in soybean, was obviously decreased by fermentation, suggesting that microorganisms might use it as a preferred nitrogen source (Kada et al., 2008). Novel bioactive oligopeptides from soybean protein which are produced by fermentative microorganism is an emerging area of research with great promise. It is well known that various tastes and flavors such as umami, bitter taste and savory flavor originate from soybean peptides.

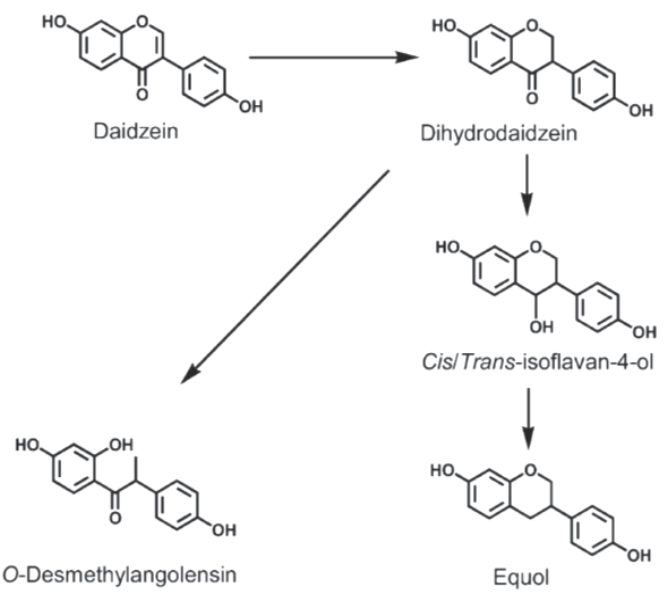


Fig. 3. Metabolism of the soy isoflavone daidzein to O-desmethylangolensin and equol. Adapted from Heinonen et al. (1999).

S.H. Kim & Lee (2003) evaluated the flavor compounds in water-soluble extract from doenjang by fractionating the amino acids and peptides by gel filtration chromatography (Table 4). Umami taste in doenjang and the savory flavor of fermented and non-fermented soy foods, such as soy sauce and hydrolyzed vegetable protein, are the result of the release of small peptides and amino acids from the fermentative digestion or acidic hydrolysis of soy proteins (Aaslyng et al., 1998; Rhyu & Kim, 2011).

In addition to the umami taste, soy protein is reported to have numerous beneficial effects in humans, including improvements in body composition, anti-hypertension, insulin secretion etc. (S.J. Kim et al., 1999; Sites et al., 2007). Many peptides having anti-hypertension and hypocholesterolemic from doenjang and kanjang have been isolated. Detailed structure of bioactive peptides preventing hypertension and hypercholesterolemia will be described in section 7.1.

Amino acids	Fractions						Threshold	Tastes of
(mg/l) (Vt, ml)[a]	2	3	4	5	6	7	value (mg/l)[b]	free amino acid in water[b]
Asp (Na)	0	0	24	36	4	2	1000	Umami
Ser	1	2	2	61	13	2	1500	Sweet
Glu (Na)	1	0	150	65	10	2	300	Umami
Gly	1	0	5	16	4	1	1300	Sweet
His	1	0	2	27	3	1	200	Bitter
Thr	1	1	4	67	12	0	2600	Sweet
Arg	1	1	2	19	14	0	500	Bitter
Ala	1	1	4	120	13	0	600	Sweet
Pro	0	2	9	98	8	2	3000	Sweet, Bitter
Cys	0	18	150	18	0	5	nd[c]	Bitter
Tyr	0	3	11	2	2	50	nd	Bitter
Val	0	1	6	82	9	0	400	Bitter
Met	1	3	4	0	6	0	300	Sweet
Lys	1	3	82	27	6	2	500	Bitter
Ile	1	2	9	70	11	1	900	Bitter
Leu	1	1	3	120	18	2	1900	Bitter
Phe	0	1	1	1	22	25	900	Bitter
Total	11	39	468	829	155	95		

[a]Vt = Total volume of the fractions obtained after chromatography of 10 ml water-soluble extract.
[b]defined as in Kato et al. (1989).
[c] not detected.

Table 4. Concentration of free amino acids identified in each tasted Sephadex G-25 gel filtrate obtained from the water-extract of doenjang (S.H. Kim & Lee, 2003).

5. Metabolite profiling of fermented soybean foods

Thousands of metabolites are among the fermented products produced by microorganism during fermentation. Most of metabolites such as fatty acids, organic acids, amino acids, peptides or isoflavone derivatives as described above are produced from the soybean by degradation, however, some metabolites do not originate from soybean. This means that fermenting microorganisms, especially *Aspergillus* species, might produce new metabolites by themselves, using soybean as a substrate source in the long fermentation period.

Chungkukjang has a characteristic flavor which is generally acceptable to Koreans but hardly tolerable to some foreign people. Volatile organic acids, such as acetic acid, propionic acid, 2-methylpropanoic acid, butanoic acid and 3-methylbutanoic acid, have been identified as the characteristic flavor in chungkukjang by gas chromatography-mass spectrometry (GC-MS) (M.K. Park et al., 2007). The contents of volatile organic acids in chungkukjang were highly dependent on the fermentation period; increasing during fermentation. Moreover, the branched-chain organic acids (2-methypropionic acid and 3-methylbutanoic acid) were formed earlier and were present in much higher concentration than the corresponding straight-chain organic acids during chungkukjang fermentation. We investigated which metabolites were made from different chungkukjang in terms of fermentation time and inoculated strains, *B. subtilis*, *B. amyloliquefaeciens* and *B. licheniformis* by GC-MS: the metabolite profiling of chungkukjang by PCA (principal component analysis) and PLS-DA (partial least square discriminant analysis) showed that some sugars and organic acids including sucrose, fructose, glucose, mannose, succinic acid, and malonic acid, as well as most amino acids, contributed mainly to differentiation of the different chungkukjangs according to fermentation time. The levels of most amino acids decreased in the early stage of fermentation and in increased in the late stage of chungkukjang fermentation with *B. subtilis* (M.K. Park et al., 2010). The levels of fatty acids generally increased throughout the fermentation process and levels of most organic acids, except for tartaric acids, decreased. Tryptophan, citric acid, β-alanine, itaconic acid, 2-hydroxyglutaric acid, γ-aminobutyric acid, leucine, malic acid, and tartaric acid were the major components that discriminated various chungkukjangs according to fermentation period. On the other hand, mannose, xylose, glutamic acid and proline were mainly responsible for differentiating the chungkukjang according to the various inoculated strains (Baek et al., 2010).

In addition to metabolic profiling by GC-MC analysis, metabolomic profiling of chungkukjang during fermentation was also carried by ^{1}H NMR spectrometry and PCA. The major peaks in the ^{1}H NMR spectra of the 50% methanol fraction of chungkukjang corresponded with isoleucine/leucine, lactate, alanine, acetic acid, citric acid, choline, fructose, sucrose, tyrosine, phenylalanine and formic acid (H.K. Choi et al., 2007) (Fig. 4).

The first two principle components (PC1 and PC2) of the ^{1}H NMR spectra of the aqueous fraction (Fig. 5) allowing discrimination of chungkukjang extracts obtained after different periods of fermentation showed that samples obtained after 0 hr and 5 hr of fermentation were separated but relatively close proximity to each other. Similarly, score plots of the samples obtained after 10, 20, and 40 hr of fermentation were separated clearly from each other. The spectra for samples obtained during the later stages of fermentation (20-40 hr) were less pronounced than those that occurred in samples obtained earlier. Therefore, fermentation appeared to occur primarily between 5 and 20 hr after the start of the fermentation process. The major compounds that contributed to discrimination were isoleucine/leucine, lactate,

acetic acid, citric acid, choline, fructose, tyrosine and phenylalanine. Among them, lactate, acetic acid, citric acid, fructose and sucrose contributed mainly to discrimination by PC1, while lactate, acetic acid, citric acid, choline, sucrose, tyrosine and phenylalanine contributed mainly to discrimination by PC2. The GC-MC and NMR results revealed similar metabolic profiling of chungkukjang according to fermentation periods.

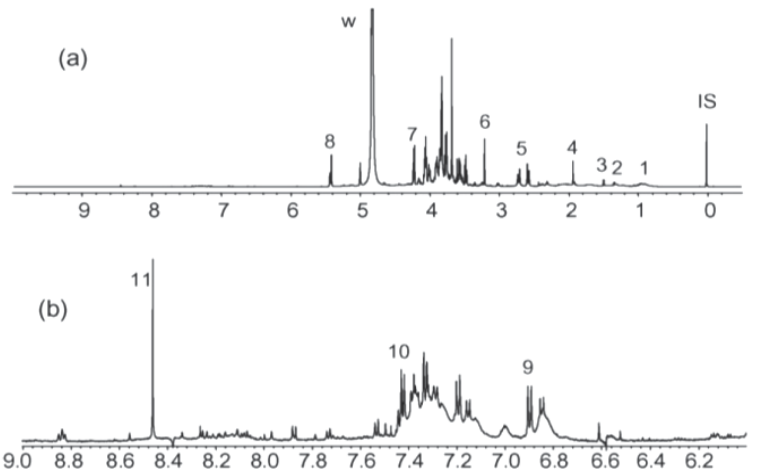


Fig. 4. Representative ^{1}H nuclear magnetic resonance (NMR) spectra of the total (a) and aromatic (b) region of the aqueous fraction of chungkukjang extracts from samples obtained at the start of fermentation. IS, internal standard; 1, isoleucine/leucine; 2, lactate; 3, alanine; 4, acetic acid; 5, citric acid; 6, choline; 7, fructose; 8, sucrose; 9, tyrosine; 10, phenylalanine; 11, formic acid; w, water. Values on the X-axis are the chemical shift in ppm relative to TSP (H.K. Choi et al., 2007).

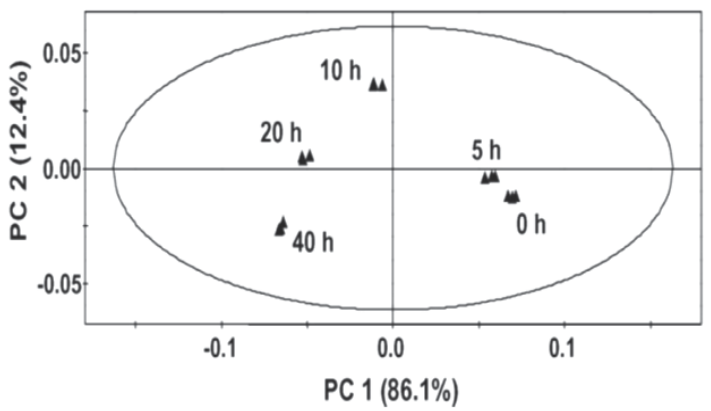


Fig. 5. Principal component analysis (PCA)-derived score plot of the first two principal components (PC1 and PC2) of the aqueous fraction of chungkukjang extracts. (H.K. Choi et al., 2007).

Metabolomic analysis of meju during fermentation by ultra performance liquid chromatography-quadrupole-time of flight mass spectrometry (UPLC-Q-TOF MS) revealed

that various metabolites, including amino acids, small peptides, nucleosides, urea cycle intermediates, and organic acids, responsible for the unique taste and nutritional and functional quality of fermented soy foods, were clearly altered by increasing the fermentation period (Kang et al., 2011). Changes in these metabolites allowed discrimination among meju samples with different fermentation periods (0, 10, 20, 40, and 60 day) on a PLS-DA score plot, and the fermentation was mainly processed between 10 and 40 day of fermentation. Twenty-two metabolites (phenylalanine, glutamic acid, leucine, adenine, citrulline, arginine, glutamine, γ-aminobutyric acid, proline, acetylornithine, valine, pipecolic acid, methionine, citric acid, xanthine, tyrosine, isoleucine, Glu-Tyr, Ser-Pro, tryptophan, Glu-Phe, and Leu-Val-Pro-Pro) with high PLS-DA values over 1.00 were determined to be the major compounds contributing to the discrimination of meju samples. These metabolites, which were positively related to the sensory quality of meju, can be used as fermentation biomarkers for the production of meju and construction of the meju fermentation metabolic pathway. Based on the metabolites found, we constructed a meju fermentation metabolic pathway that shows the overproduction and underproduction of metabolites during fermentation and the several related cycles, including the urea cycle, TCA cycle, glutamine cycle, and methionine cycle (Kang et al., 2011) (Fig. 6).

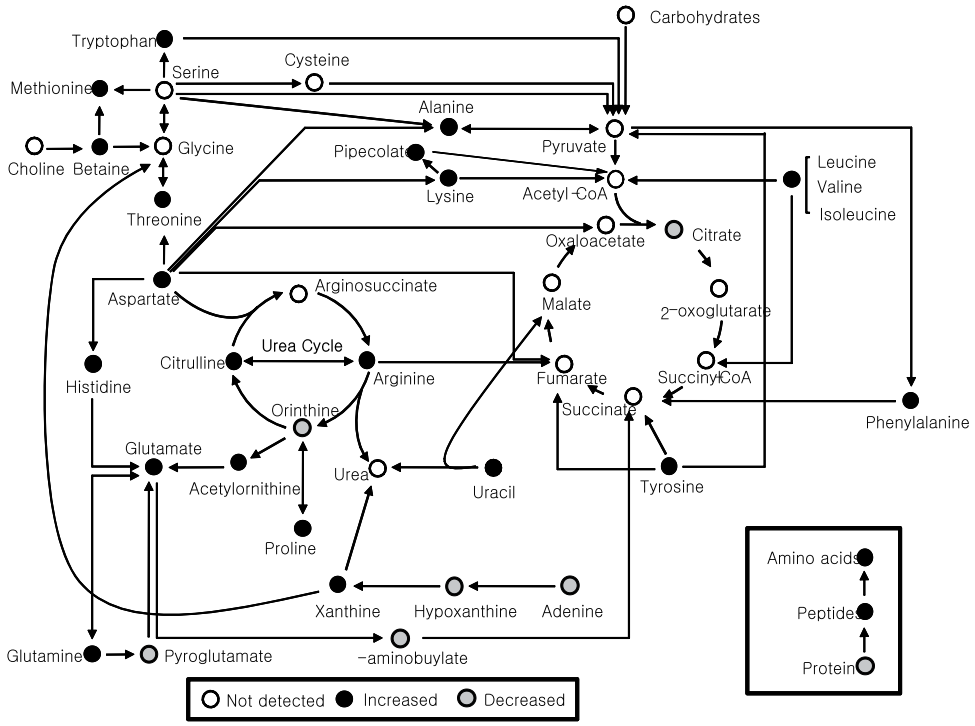


Fig. 6. Schematic representation of metabolites produced during meju fermentation. Metabolites identified by UPLS-Q-TOF are marked with black and grey. Black, grey, and open circles represent increased, decreased, and undetected metabolites, respectively. Most metabolites were physically and enzymatically produced from soybean biomass during fermentation, but some metabolites might originate from the biomass of microorganisms (Kang et al., 2011).

Some microbial metabolites being independent of proteins and other large molecules from soybean were produced by the proposed pathway during meju processing. Some metabolites, such as citrulline, for example, were likely produced via some part(s) of the pathways.

The metabolomic profiling of doenjang by NMR-PCA and GC-MS (Namgung et al., 2010; S.O. Yang et al., 2009) demonstrated that the predominantly produced amino acids included alanine, valine, leucine, isoleucine, proline, glutamine, phenylalanine and lysine, showing remarkable increases in amounts during the later stages of fermentation. Carbonic acid, citric acid, lactic acid and pyrogultamic acid were identified as the major organic acids. Significant amounts of erythrose, xylitol, inositol and mannitol were also detected during fermentation. Among fatty acids, relatively higher amounts of palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid were found in the doenjang at each fermentation time point. Our results indicate that monitoring the changes in metabolites during meju fermentation might be important for producing meju-related foods (doenjang and kanjang) with good nutritional and sensory quality.

The study of metabolomics plays a crucial role in determining the changes in metabolites in fermented foods but also detecting bioavailability of metabolites after the consumption of foods. The function of soybeans and fermented soybeans in humans are somewhat different possibly due to their different metabolite in the blood. Thus, it is necessary to study bioavailability of metabolites in the blood using metabolomics methods in humans after consuming soybean products.

6. Functionality of soybean products

6.1 Soybeans

Soybeans are well known for their health-promoting benefits which include antioxidation, anti-obesity, anti-diabetes, properties and prevention of osteoporosis and cancers such as breast and prostate cancer (Anderson et al., 2008; Anderson & Pasupuleti, 2008; Messina et al., 1999). Many studies, but insufficient and inconclusive, suggest that these kinds of health function are primarily due to esterogenic properties of isoflavones but the results are still controversial (Kwon et al., 2010). There are hundreds of *in vitro* studies showing that genistein has anti-oxidant properties, inhibits the growth of a wide range of both hormone-dependent and hormone independent cancer cells, including breast (Pagliacci et al., 1994; Peterson et al., 1996; Zava & Duwe, 1997), prostate (Kyle et al., 1997; Zhou et al., 1999), colon (Kuo, 1996; Kuo et al., 1997), and skin (Rauth et al., 1997) cells (Adlercreutz & Mazur, 1997; Akiyama & Ogawara, 1991; Constantinou & Huberman, 1995) and that genistein also inhibits the metastatic activity of both breast (Peterson & Barnes, 1996; Scholar & Toewa, 1994) and prostate (Santibáñez et al., 1997) cancer cells independent of the effects on cell growth. Daidzein, one of the 2 primary isoflavones in soybeans, exhibits anticancer effects (Jing et al., 1993). Recently, genistein was also demonstrates to have anti-inflammatory properties (Hernandez-Montes et al., 2006; Verdrengh et al., 2003), and we found that genistein decreased cisplatin induced apoptosis by regulation p53 induction in kidney, and reactive oxygen species production in cisplatin-treated normal kidney HK-2 cells (Sung et al., 2008). Our previous review (Kwon et al., 2010) described in detail the anti-obesity effects of soybean for type 2 diabetes. Some studies of the effects of soybeans, including isoflavonoids and soy proteins, on glucose metabolism are inconsistent, and the mechanisms have not been extensively studied. However, other studies showed positive

effects on hypocholesterolemia, for example, 46 postmenopausal women taking isolated isoflavone extracts had significantly increased high-density lipoprotein cholesterol and a decrease in apolipoprotein B, the primary apolipoprotein in low-density lipoprotein particles (Clifton-Bligh et al., 2001; Goodman-Gruen & Kritz-Silverstein, 2001).
In soy protein nutrition, the sulfur amino acids, methionine and cysteine, are the limiting amino acids. However, the relatively low sulfur amino acid content of soybeans may actually provide an advantage in terms of calcium retention ironically. The reported hypercalciuric effect of protein is likely to be at least partially due to the metabolism of sulfur amino acids. The skeletal system serves as one of the main buffering systems in the body; as a result, the hydrogen ions produced from the metabolism of sulfur amino acids cause demineralization of bone and excretion of calcium in the urine (Remer & Manz, 2001). Thus, bean protein may improve calcium retention relative to animal and grain proteins.
Soy protein also has a cholesterol lowering effect in monkey (Terpstra et al., 1984) and in men (Wong et al., 1998), and protein hydrolyzate or hydrolyzed peptides of soybeans decrease blood cholesterol and glucose levels (Kwon et al., 2002; Yoshikawa et al., 1999). Lunasin, a 43-amino-acid peptide from soy, has been shown to have numerous biologic properties including anticancer and anti-inflammatory activities (Galvez et al., 2001; Mejia & Dia, 2009; K.Y. Park et al., 2001). In fact, we identified hypocholesterolemic peptides from fermented soy products, including doenjang (see following section), and soy protein isolates are known to activate peroxisome-proliferator–activated receptors (PPARs) and liver X receptor signaling and to inhibit sterol regulatory element-binding protein-1c signaling, contributing to insulin sensitization (Jhala et al., 2003; Ronis et al., 2009)]. In addition, soy-fed CD-1 mice exhibited enhanced insulin sensitivity, especially in white adipose tissue, due to the potentiation of phosphorylation of AMP-activated protein kinase and acetyl-CoA carboxylase and up-regulation of the expression of genes involved in peroxisomal fatty acid oxidation and mitochondrial biogenesis and in skeletal muscles by increasing glucose uptake (Cederroth et al., 2008).
It is also well-known that substituting vegetable oils, such as soybean oil, for animal fat reduces the risk of high blood pressure, thrombosis, platelet generation, and cholesterol accumulation (Meydani et al., 1991). Soybean oils contain health promoting linoleic, linolenic acids, and phytochemicals.

6.2 Chungkukjang

Recently, there has been growing interest in chungkukjang due to its effects on health. Previous reports indicate that the consumption of chungkukjang decreases blood pressure and serum lipids (J.L. Yang et al., 2003). In addition, dietary supplementation with chungkukjang exerted not only hypoglycemic effects but also antioxidant effects in diabetic rats (Kwon et al., 2007a). After fermentation, isoflavonoid glycones are changed into isoflavonoid aglycones, which seem to have greater anti-diabetic activity than do isoflavonoid glycones (Table 1, 2, 3) (Jang et al., 2006). Kawakami et al. (2005) demonstrated that an isoflavone aglycone–rich diet reduced liver and serum total cholesterol levels, and liver triglyceride levels in rats fed cholesterol. Thus, fermented soybean products may be more effective for ameliorating metabolic disorders due to increased isoflavonoid aglycone content.
We demonstrated that the ethanol extract of chungkukjang inhibits the generation of 1,1-diphenyl-2-picryl hydrazine (DPPH) radicals and prevents LDL oxidation. Chungkukjang

and its constituents (genistein and daidzein) also inhibited H_2O_2-induced DNA damage from NIH/3T3 fibroblasts and exhibited cytoprotective effects against H_2O_2-induced cell death. An *in vivo* study also demonstrated that an oral administration of chungkukjang water extract potently inhibited the formation of malondialdehyde, DNA damage and the formation of micronucleated reticulocytes in $KBrO_3$-treated mice (N.Y. Kim et al., 2008); ethanol extract of chungkukjang showed anti-inflammation activity by inhibiting 5-lipoxygenase from A23187-treated RBL-1 cells, and reducing leukoriene production (Y.H. Choi et al., 2008). Chungkukjang significantly reduced passive cutaneous anaphylaxis in rats at 400 mg/kg/day and also showed *in vivo* anti-inflammatory activity against arachidonic acid-induced mouse ear edema. We found that the methanol extract of 40 hr fermented chungkukjang exhibited the highest degrees of free-radical-scavenging and tyrosinase-inhibition activities, which have been indicated to prevent hyperpigmentation. These results suggest that chungkukjang extracts possess antioxidative anti-inflammatory and tyrosinase-inhibition activities, which are attributable to phenolics other than flavonoids (H.K. Choi et al., 2008).

Anti-diabetic effect was intensively studied with colleagues (D.J. Kim, 2008; Kwon et al., 2006, 2007a, 2007b); several animal studies and a few human studies have evaluated the effects of fermented soybeans on glucose metabolism as reviewed previously (Kwon et al., 2010). We found that chungkukjang improves glucose homeostasis by enhancing hepatic insulin sensitivity and insulinotropic actions in 90% pancreatectomized rats, a type 2 diabetic animal model (Kwon et al., 2007a, 2007b). In addition, chungkukjang enhanced glucose-stimulated insulin secretion in a hyperglycemic clamp study in diabetic rats and increased pancreatic β-cell mass via increased proliferation and decreased apoptosis. D.J. Kim et al. (2008) also showed similar results in C57BL/KsJ-db/db mice. Chungkukjang supplementation induced a significant decrease in blood glucose and glycosylated hemoglobin levels and improved insulin tolerance compared to the diabetic control group via increasing serum insulin and pancreatic insulin contents. Therefore, chungkukjang delayed diabetic symptoms in type 2 diabetic rats more than non-fermented soybeans, and this was related to increased isoflavonoid aglycones such as daidzein and genistein and small peptides.

In addition to anti-diabetic effects of chungkukjang, there is epidemiological evidence that chungkukjang has anti-obesity effects, but insufficient data exist. It is known that soybean and soy components have an anti-obesity and anti-diabetes (Anderson & Pasupuleti, 2008), however, the active compounds from fermented soybeans for obesity and diabetes are not fully identified. The hydrolyzated/fermented peptides or other phytochemicals produced during fermentation will be potential candidates for these actions. Although certain types of peptides isolated from the breakdown of soybeans or fermented by microbes have shown antihypertensive and anti-inflammatory properties (Inoue et al., 2009; Kato et al., 1989; S.H. Kim et al., 1999; Mejia & Dia, 2009), no specific peptides have revealed anti-obesity and anti-diabetic actions due to the difficulties of isolation and identification of bioactive compounds such as peptides.

We tried to identify the anti-obesity effects of chungkukjang by analyzing the hepatic mRNA expressions of enzymes related to fatty oxidation by RT-PCR analysis in high fat-fed mice (Soh et al., 2008). The expression of hepatic ACS (acyl-CoA synthase), CTP-1 (carnitine palmitoyltransferase-1), ACO (acyl-CoA oxidase), and UCP2(uncoupling protein-2) were increased by chungkukjang supplementation. The data demonstrated that chungkukjang supplementation leads to increased mRNA expressions of enzymes and protein involved in

fatty acid oxidation in liver, reduced accumulation of body fat and improvement of serum lipids in high fat diet fed mice (Kwon et al., 2007a; Soh et al., 2008). In addition, the hepatic transcriptional profiles using cDNA microarray showed that several genes involved in fatty acid catabolism (Acaa2, Mgll, Phyh, Slc27a2, Slc27a5), which were the main genes that had altered expressions by high fat diet, were normalized by chungkukjang consumption (Table 5) (Soh et al., 2011). Gene expression profiles obtained by both microarray and RT-PCR analysis were very similar with regard to the direction (up- or down-regulation) and degree of differences in expression. The result means that chungkukjang consumption improves serum lipid profiles and body fat accumulation, probably by modulating transcriptional levels of enzymes for utilization of fatty acids.

Genbank No	Gene	cDNA microarray		Real time RT-PCR	
		HDcon	HDC	HDcon	HDC
BG085346	Acetyl-Coenzyme A acyltransferase 2 (Acaa2)	-4.18	↔[1)]	0.28[2)]	0.94
A277495	Carnitine palmitoyltransferase 1a, liver (Cpt1a)	ND[3)]	ND	1.73	3.41
BF457090	24-dehydrocholesterol reductase (Dhcr24)	2.1	↔	1.45	0.78
BG063933	Dolichyl-phosphate (UDP-N-acetylglucosamine) acetylglucosaminephosphotransferase 1 (Dpagt1)	2.3	↔	3.02	1.91
AA023077	Hydroxysteroid 11-beta dehydrogenase 1 (Hsd11b1)	-8.95	↔	0.23	1.14
AI835105	Monoglyceride lipase (Mgll)	-8.99	↔	0.29	0.62
W82212	Phytanoyl-CoA hydroxylase (Phyh)	-5.78	↔	0.37	1.21
AI893897	Paraoxonase 1 (Pon1)	2.0	↔	1.68	0.69
AA259329	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit (Prkag1)	2.2	↔	2.20	1.41
AA108401	Solute carrier family 27 (fatty acid transporter), member 2 (Slc27a2)	-4.06	↔	0.25	0.86
AA254935	Solute carrier family 27 (fatty acid transporter), member 5 (Slc27a5)	-11.73	↔	0.25	0.83

[1)]The double headed arrow symbol (↔) denotes that there is no significant difference when gene expression in HDcon (high fat diet control) or HDC (high fat diet with chungkukjang) groups were compared with the NDcon (normal diet control) group. [2)]Data are expressed as fold changes, normalized to β-actin mRNA expression, where the values for the NDcon mice were set at 1.00. The analyses were performed in duplicate. [3)]ND, not detected. (Soh et al., 2011)

Table 5. A comparison between cDNA microarray analysis and real time RT-PCR (3-12).

6.3 Meju and doenjang

It is known from *in vitro* studies that doenjang has various beneficial properties, such as anticancer, antimetastatic, antihypertensive and antimutagenic activities (Jung et al., 2006; K.Y. Park et al., 2003; S.O. Yang et al., 2009) reported doenjang showed higher antimutagenic activity than raw soybeans, cooked soybeans, and other fermented soybeans in the Ames test (A.E. Burtler et al., 2003). The active compounds that were identified included genistein, linoleic acid, β-sitosterol glucoside, soya saponin, etc. Anticancer and antimetastatic properties of doenjang were enhanced as aging time progressed (see Fig. 1) (Jung et al., 2006). The 24 month fermentation was the most effective in preventing cancer by decreasing tumor formation and increasing natural killer cell activity in spleens and glutathione S-transferase activity in livers of mice. Unlike chungkookjang, doenjang has not been demonstrated to affect glucose homeostasis because sample preparation is very difficult for studying anti-diabetic effects due to the high content of salt. However, some compounds from doenjang have been shown to have greater activity for reducing blood pressure in terms of angiotensin converting enzyme (ACE) inhibition (Hwang, 1997; S.H. Kim et al., 1999).

The health effects of meju have not attracted much research interest because meju is not the food that is eaten directly. Recently, however, interest in its functional properties has increased gradually because of its impact on functionality when making doenjang and kanjang. Like as doenjang, ACE inhibitory factors associated with its peptides were found in solid meju extract which was fermented with *B. subtilis* (Hwang, 1997). In addition, Min6 insulinoma cells treated with genistein, chungkukjang or meju extract (60 day fermentation) had greater glucose-stimulated insulin secretion capacity and greater β-cell viability than those treated with unfermented soybeans (Kwon et al., 2011b). This improvement was associated with the activation of insulin/insulin-like growth factor-1 signaling; the tyrosine phosphorylation of insulin receptor substrate-2 and serine phosphorylation of Akt was potentiated, and this in turn increased the expression of pancreatic and duodenal homeobox-1 involved in β-cell proliferation. Furthermore, genistein, daidzein, and meju extract stimulated glucagon-like peptide-1 secretion in enteroendocrine NCI-H716 cells, which generated insulinotropic actions, meaning that meju has a better anti-diabetic action than soybeans soybeans (Kwon et al., 2011b).

6.4 Kochujang

Kochujang containing meju and red pepper may affect energy, lipid, and glucose metabolism. Current investigations are concentrating their efforts on investigating the biological and physiological functions of kochujang because red pepper, a major component of kochujang, and its active principle capsaicin are known to enhance energy and lipid metabolism, possibly by increasing catecholamine secretion from the adrenal medulla through the activation of the sympathetic nervous system (Diepvens et al., 2007; Karlsson, 1994). In fact, several studies have indicated that traditional kochujang exhibits anti-mutagenic activity (S.J. Kim et al., 1999), and antitumor effects (K.Y. Park et al., 2001) are reported. Anti-obesity effects were reported in mice and rats fed with high fat diets (Ashiuchi et al., 2001; Choo, 2000; Koo et al., 2008); fermented kochujang supplementation of a high fat diet prevented obesity in mice by reducing fat deposition and decreased circulating levels of cholesterol, triglycerides, and blood glucose, mediated by down-regulating expression of lipogenic enzymes and up-regulating the lipolytic enzymes and the thermogenesis gene UCP-1 (Koo et al., 2008).

The decreased numbers of adipocytes by lipolysis may improve glucose tolerance by the enhancing insulin sensitivity. Although capsaicin content in red pepper was reduced by approximately 50% during the fermentation process, kochujang has been shown to reduce body weight, visceral fat, and serum leptin levels without modulating energy intake in diabetic rats (Kwon et al., 2009). It also improves glucose tolerance by enhancing insulin sensitivity. The improvement in hepatic insulin sensitivity lowered hepatic glucose output and triglyceride accumulation and increased glycogen storage. The possible mechanism is the potentiated phosphorylation of signal transducer and activator of transcription-3 → AMP-activated protein kinase → acetyl CoA carboxylase and the reduced the expression of phosphoenolpyruvate carboxykinase, a regulatory enzyme for gluconeogenesis, in the liver (Choi & Suh, 2004).

7. Bioactive soypeptides and design of new peptides

Many oligopeptides produced from soy protein by digestive endogenous or microbial proteinase during fermentation, demonstrated a range of biological activities – angiotensin converting enzyme (ACE) inhibition, anti-thrombotic, surface tension and antioxidant properties (Gibbs et al., 2004). The biologically active peptides were mostly derived from glycinin, a highly expressed soy protein, therefore some researchers investigated the novel bioactive peptides isolated from soyprotein by treatment with endoproteinase (pronase, trypsin, pepsin, Glu C protease, plasma proteases and kidney membrane proteases). Bioactive peptides were isolated from the fermented soybean products by *Bacillus* species or *Aspergillus* species and identified the structures. We also isolated ACE inhibitory peptides, antithrombotic and hypocholesterolemic peptides and identified the structures from doenjang (S.H. Kim et al., 1999; Kwon et al., 2002).

7.1 Bioactive peptides

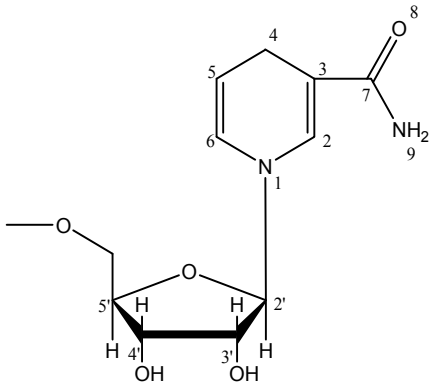
A Chinese group tried to isolate the ACE inhibitory peptides from soy protein by treating it with protease and testing the fraction for hypotensive effects in SHR (spontaneous hypertensive rats) (Wu & Ding, 2001; Gouda et al. 2006) also isolated active fraction from soyprotein peptides hydrolyzed by protease P and its sequential structure was identified as Val-Leu-Ile-Val-Pro (VLIVP). The sequence of VLIVP corresponded to Val_{397}-Pro_{401} of the glycinin subunit G2 of soybean. From kidney bean protein hydrolyzate, ACE inhibitory peptide was isolated and identified as Val-Ile-Pro-Ala-Ala-Tyr (VIPAAY) (J.R. Lee et al., 1999). Fermented soy peptides from doenjang (S.H. Kim et al., 1999) and miso (Inoue et al., 2009) have also been shown to possess ACE inhibitory activity *in vitro* and a dipeptide (Ala-Pro, AP) and tripeptides (Val-Pro-Pro, VPP and Ile-Pro-Pro, IPP) from doenjang and miso are reported to act as antihypertensive agents in SHR. The primary structural and configuration properties of ACE inhibitory peptides from soybean were similar; it starts with an alipathic nonpolar amino acid group such as Val, Ala, and Ile at the N-terminal and ends with Pro at the C-terminus (S.H. Kim et al., 1999). A certain in ACE inhibitory peptide, His-His-Leu (HHL) isolated from doenjang (Shin et al., 1995), exerts a significant decrease of ACE activity in the aorta and leads to lowered systolic blood pressure (SBP) in SHR (Shin et al., 2001). After determining the sequence of these peptides, efficacy studies were done by synthesizing the authentic and analogue peptides and the mechanism was also elucidated (Gouda et al., 2006; Shin et al., 2001). It was not determined which of the peptides in kanjang had ACE inhibitory, anti-thrombotic or antioxidant properties, however, a couple of papers

on the ACE inhibition activity of peptides and nicotinamide in Japanese soy sauce or soy seasoning were reported (Kinoshita et al., 1993; Makahara et al., 2010; Zhu et al., 2008).
Based on the studies that observed hypocholesterolemic effects of soybean protein in humans and monkeys (Terpstra et al., 1984; Wong et al., 1998), hypocholesterolemic peptides were isolated from glycinin hydrolyzate by trypsin and pepsin digestion. The identified primary structure was Leu-Pro-Tyr-Pro (LPYP) (Kwon et al., 2002) and Ile-Ala-Val-Pro-Gly-Glu-Val-Ala (IAVPGEVA) and Ile-Ala-Val-Pro-Thr-Gly-Val-Ala (IAVPTGVA) (Pak et al., 2004, 2005c) from glycinin hydrolyzed by trypsin and pepsin, respectively. Hypocholesterolemic activity was determined by assaying the inhibition of 3-hydroxcy-3-methylglutaryl CoA reductase (HMG-CoA reductase) *in vitro* and by determining the serum cholesterol levels in mice. The sequence (LPYP) was very similar to Yoshigawa's peptide (LPYPR) (Yoshikawa et al., 1999) also isolated from glycinin hydrolyzate, and the sequences including SPYPR correspond to the $A_5A_4B_3$ and A_3B_4 regions of glycinin (Fukazawa et al., 1985; Momma et al., 1985). LPYPR was a little better HMG CoA reductase inhibitor than LPYP *in vitro*, but both reduced serum cholesterol levels by the same amount (30%) (Kwon et al., 2002). Our results indicate a positive correlation between the binding of cholic and deoxycholic acids and the hypocholesterolemic effects of the pepsin hydrolyzate peptide (IAVPGEVA), while peptide hydrolyzates from trypsin digestion (LPYP and LPYPR) had hypocholesterolemic effects and gave a negative correlation for binding of bile acids. Therefore, the degree of interaction of bile acids is not ssential for identifying hypocholesterolemic activity (Pak et al., 2005a, 2005b).

7.1.1 Mode of actions of isolated peptides

Design of more potent compounds based on known bioactive compounds in terms of structure and mode of action is one of the best way to reduce the risks of failure and to save time and expense in the development of bioactive compounds. Using the peptides previously identified, therefore, we tried to design more powerful HMG CoA reductase inhibitors from soy peptide and compared them to statins and statin derivatives. Statins are known inhibitors that effectively lower plasma cholesterol levels. All statins have a chemical structure similar to HMG in their molecules (Istvan, 2003; Istvan & Deisenhofer, 2000a, 2000b; Wilding et al., 2000).
In general, understanding the relationship between structure and activity (mode of action) for two peptide structures, namely LPYP and IAVP are essential for a new peptide design. In cholesterol synthesis, isoprenoids comprise a large group of natural compounds that are formed in living cells from mevalonic acid and are involved in various cellular functions such as growth regulation of higher plants, fungi, and mammals, including sterol synthesis (Bloch, 2009). Investigations of the mechanism of mevalonic acid synthesis using enzymes of HMG-CoA reductase (HMGR) (Frimpon & Rodwell, 1994), which are key enzymes in sterol biosynthesis, established that the conversion of 3-hydroxy-3-metylglutaryl-coenzyme-A (HMGR-CoA) (main substrate) into mevalonate involves two molecules of nicotinamidedinucleotidephosphate (NADPH) (complementary substrate) and occurs through two sequential hydryl shifts. Although isoprenoids are necessary for normal cell function, an excess of certain products synthesized from mevalonate, e.g., cholesterol, can lead to progressive atherosclerosis and associated cardiovascular diseases.
We examined the capability of synthetic LPYP, LPYPR, SPYPR, IAVP and their derivatives to occupy the binding site for NADPH which is one of the substrates of HMGR. We started with the knowledge that these peptides contain a proline unit [2] in the fourth residue from

the N-terminus (this is the same in IAVP and IAVPTGVA or IAVPGEVA). Such a sequence may play an important role in HMGR inhibition (Pak et al, 2005a, 2005b). On the other hand, proline is the only unit with an aliphatic ring containing both the main and side chains. Its presence in the amino-acid sequence makes the conformation of the peptide relatively rigid. Thus, we assumed that proline in the above sequence can form a structure similar to the nicotinamide part [1] of NADPH and, therefore, has similar interactions with the binding site for nicotinamide in HMGR. We compared the peptide structures with those of the nicotinamide part of NADPH using data from an x-ray structure analysis of the enzyme active center with $NADP^+$ (Istvan & Deisenhofer, 2000a, 2000b; Istvan et al., 2000) and selected four atoms (C4, O8, N9, O6′) with the shortest H-bond distances. The peptide structures and their conformations calculated using AM1, PM3 and MNDO (Pak et al., 2005a, 2005b).



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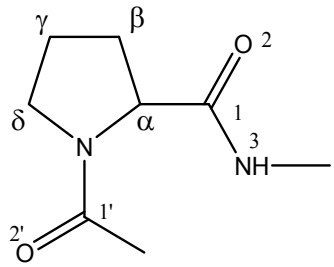

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The steric similarity of these structures were determined by calculating the percent of the projections of the selected bonds of the peptides to the corresponding bonds in the nicotinamide part of NADPH in two mutually perpendicular planes. The first plane passed through the pyridine ring of nicotinamide; the second, through C2′ of the ribose ring. Thus, the results indicate that these peptides have structural features similar to the nicotinamide part in the studied positions and correlate with the inhibitory ability of these peptides. Therefore, they may bind to the active center of HMGR and interact similarly with it as NADPH. Also, LPYP, LPYPR, IAVP, IAVPTGVA and IAVPGEVA may occupy the part of the binding site for NADPH in the active center of the enzyme. According to the mathematical model of the structure - activity relationship for these peptides, a hydrophobic part of these peptides is a required structural element for their biological activity, and the proline acts as a key component for these compounds to be recognized as residues for the nicotinamide part of the NADPH binding site (Pak et al., 2005b, 2006). Kinetic experiments support that these peptides inhibit HMGR competitively with respect to HMG-CoA and NADPH and interact with this enzyme like a bisubstrate (Pak et al., 2005d).

7.2 Peptide modeling and designing

7.2.1 Peptide design for competing with HMG-CoA

Hypocholesteroemic peptides with HMGR (HMG–CoA reductase) inhibiting activity were isolated from soybean, and found to act as inhibitors by competing with NADPH binding at

the active site of HMGR due to the structural and conformation similarity between peptides and NADPH. Based on the structures and reaction mechanisms of isolated peptides and the bioactive conformation of statins (commercial simvastatin and rosuvastatin) that also act as competitive inhibitors of HMG-CoA for HMGR (Istvan & Deisenhofer, 2000a, 2000b), design of another type of peptide sequence was proposed competing with HMG-CoA rather than NADPH. The active space in peptide design was defined by using bioactive conformations of simvastatin and rosuvastatin extracted from the crystal structure of HMGR–statin complex as shown in Fig. 7 (Pak et al, 2006).

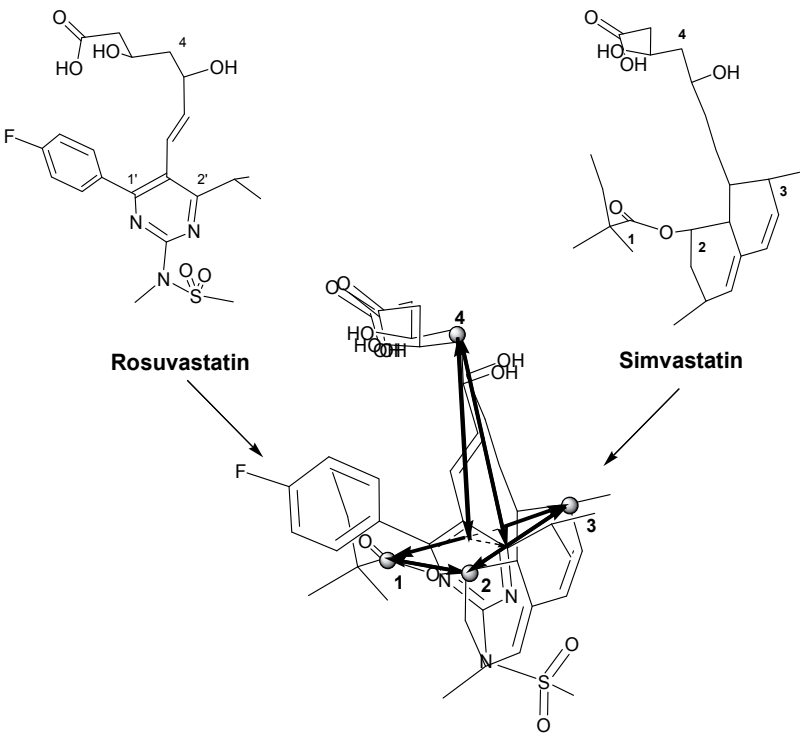

Fig. 7. Modeling of an ''active space'' in the binding site of HMGR using the bioactive conformations of simvastatin and rosuvastatin extracted from the crystal structure of HMGR–statin complexes (PDB codes: 1HW9 (simvastatin), 1DQA (rosuvastatin)). The tetrahedron 1-2-3-4 was used as a model of the ''active space'' in the present study (Pak et al., 2006).

A conformational aspect relating to an analysis of the flexibility of the peptide molecules and their occupied volumes was applied to the peptide design by extrapolation from the bioactive conformation of statin molecules. The design criterion was formulated in terms of a proximity parameter (Pr), reflecting the probability of an active peptide conformation to approximate the statin (Pak et al., 2006). It led to the proposed modeling of the peptide sequence as a tetrapeptide with an E-residue in C-terminus and L-, I-, or Y-residues at the N-terminus (Pak et al., 2005b, Istvan et al., 2000). A- and V-residues have at least a steric effect due to their aliphatic side chains and were selected for introduction into positions 2 and 3. Based on these considerations, nine peptides IAAE, LAAE, YAAE, IAVE, LAVE,

YAVE, IVAE, LVAE, and YVAE were chosen as candidate peptides for the peptide library. To elucidate a role of the E-residue in biological activities, the IAVA peptide was additionally selected. IAVP, having the highest inhibitory activity against HMGR among previously synthesized peptides (Pak et al., 2004), was chosen as a control compound to compare the biological activities of the newly designed peptides. Comparing the calculated Pr, four peptides (IAVE, YAVE, IVAE, and YVAE) from the library were selected and synthesized. This finding proposes that the obtained configurations of the peptide backbones can be seen as a basis to extend the peptide library in search of more active competitive inhibitors of HMG–CoA. Among all peptides, YVAE showed the greatest ability to inhibit HMGR. A kinetic analysis (Pak et al., 2006) revealed that this peptide is a competitive inhibitor of HMG–CoA with an equilibrium constant of inhibitor binding (K_i) of 15.2±1.4 μM. The HMGR inhibitory activity of YVAE was about 10 times greater than LPYP: IC_{50} of YVAE for HMGR is 41.8 μM, whereas the IC_{50} for the original peptide LPYP is 484 μM; a kinetic study showed that YVAE is a competitive inhibitor of HMGR. The calculated coefficient correlation (R) between log (IC_{50}) and the inverse value of proximity parameter (1/Pr) was found to be 0.99, indicating a high degree of correlation and efficacy of the given approach in the peptide sequence design.

7.2.2 Design of peptides conserving recognized residue

Previously, the structure-functional analysis in IAVPTGVA and IAVPGEVA peptides by making mutant peptides by substituting some amino acids for alanine (A-substitution) revealed that the activities suggest that the P-residue (A-substitution with P) is a recognized residue for the nicotinamide part of the NADPH binding site, and that the T- and E-residues can be seen as mimics of an HMG-moiety for the HMG-CoA binding site (Pak et al., 2005b, 2006). A conformational analysis revealed the existence of a 'β-turn' structure in these peptide sequences (Pak et al., 2004). Thus, VPTG and VPGE fragments in IAVPTGVA and IAVPGEVA peptides isolated from soy protein by pepsin treatment were shown to play an important role as a recognition site for peptide activity. Based on the presuppositions, the maintained conformation close to the bioactive conformations of VPTG and VPGE fragments was the focus for developing lead peptide candidates, which include constrained structures (maintaining VPTG and VPGE fragments) in a recognized sequence through a number of peptides in the design of a competitive inhibitor.

Location of the side chain of peptides was compared to that of iso-butyl (simvastatin) and the benzene ring of the 4-fluorophenyl radical of statins (rosuvastatin). The design criterion was formulated in terms of a 'V' parameter, reflecting an occupied volume in conformation space by an individual peptide adduced to the conformation space occupied by all peptide candidates from a library (Pak et al., 2007). Twelve peptide cycles were selected for the peptide library. Comparing the calculated 'V' parameters, two cyclic peptides (GLPTGG and GFPTGG) were selected as lead cycles from the library (Table 6). The constrained GLPTGG and GFPTGG peptides were designed according to obtain the most rigid peptide backbone (Pak et al., 2007). Based on sequences GLPTGG and GFPTGG, six linear peptides obtained by breaking the cycle at different positions were selected as lead peptide candidates. It is proposed that activities of the linear peptides based on an identical amino acid sequence, which are obtained from a less flexible peptide cycle, would be relatively higher than those obtained from more flexible cyclic peptides. The linear GFPTGG peptide, showing the highest inhibitory activity against HMGR, increases the inhibitory potency nearly tenfold

(Table 6). Kinetic analysis reveals that the GFPTGG peptide is a competitive inhibitor of HMG-CoA with an equilibrium constant of inhibitor binding (K_i) of 6.4±0.3 μM and IC_{50} of GFPTGG for HMGR is 16.9 μM. CD spectra GFPTGG peptide in the TFE/water mixture, type II of the β-turn was considered as a major structural element in these peptides (Pak et al., 2007).

Peptide		Position										IC_{50} [μM]
	Set	5	4	3	2	1	1′	2′	3′	4′	5′	
Cyclic				G	L	P	T	G	G			46
				G	F	P	T	G	G			47
				G	W	P	T	G	G			82
Linear					L	P	T	G	G	G		84
	1				F	P	T	G	G	G		60
					W	P	T	G	G	G		357
				G	L	P	T	G	G			19
	2			G	F	P	T	G	G			17
				G	W	P	T	G	G			137
			G	G	L	P	T	G				54
	3		G	G	F	P	T	G				48
			G	G	W	P	T	G				297
			G	G	L	P	T	G	G	G		328
	4		G	G	F	P	T	G	G	G		299
		G	G	G	L	P	T	G	G	G	G	801
		G	G	G	F	P	T	G	G	G	G	698
Control[a]			I	A	V	P	T	G	V	A		152
			I	A	V	P	G	E	V	A		201
Negative control			I	A	V	T	P	G	V	A		Inactive

[a]The previously isolated peptides (Pak et al., 2005c).

Table 6. Sequence structures and inhibitory activities (IC_{50}) of the synthesized peptides used in this study

7.2.3 Modeling of an active backbone of peptide

A structure-function analysis of synthesized peptides proposes that a competitive inhibitory peptide can be designed by maintaining bioactive conformation in a recognized sequence as an aspect of the structure-based approach. A two-stage approach was applied in the peptide library design: The first was the modeling of the peptide backbone of a competitive inhibitory peptide in the active site of HMGR using previously designed peptides; The second stage was the design of new peptide libraries in order to evaluate the effects of the functional residue on peptide affinity. These applications utilized two different approaches in the design of constrained and unconstrained peptides in the investigation of the peptide binding effect for HMGR.

Based on the modeling of an active peptide backbone in the active site of HMGR, two peptide libraries for constrained and unconstrained peptides were designed using different amino acids varying in hydrophobicity and electronic properties. IAVP and the peptides IAVE, YAVE, IVAE, and YVAE as competitive inhibitors of NADPH and HMG-CoA,

respectively, were selected by the design approach for unconstrained peptides (Pak et al., 2006). GLPTGG and GFPTGG, acting as a bisubstrate-mimic, were chosen among designed peptides, having the most constrained structures (Fig. 8) (Pak et al., 2008a). Active peptides were selected by the design parameter 'V' or 'Pr', which reflects the probability of active peptide conformations for constrained and unconstrained peptides, respectively. (Table 7) (Pak et al., 2006, 2007). According to the 'Pr' value, FVAE and F(4-Fluoro)VAE have relatively higher values compared with WVAE and halogen-containing peptides,

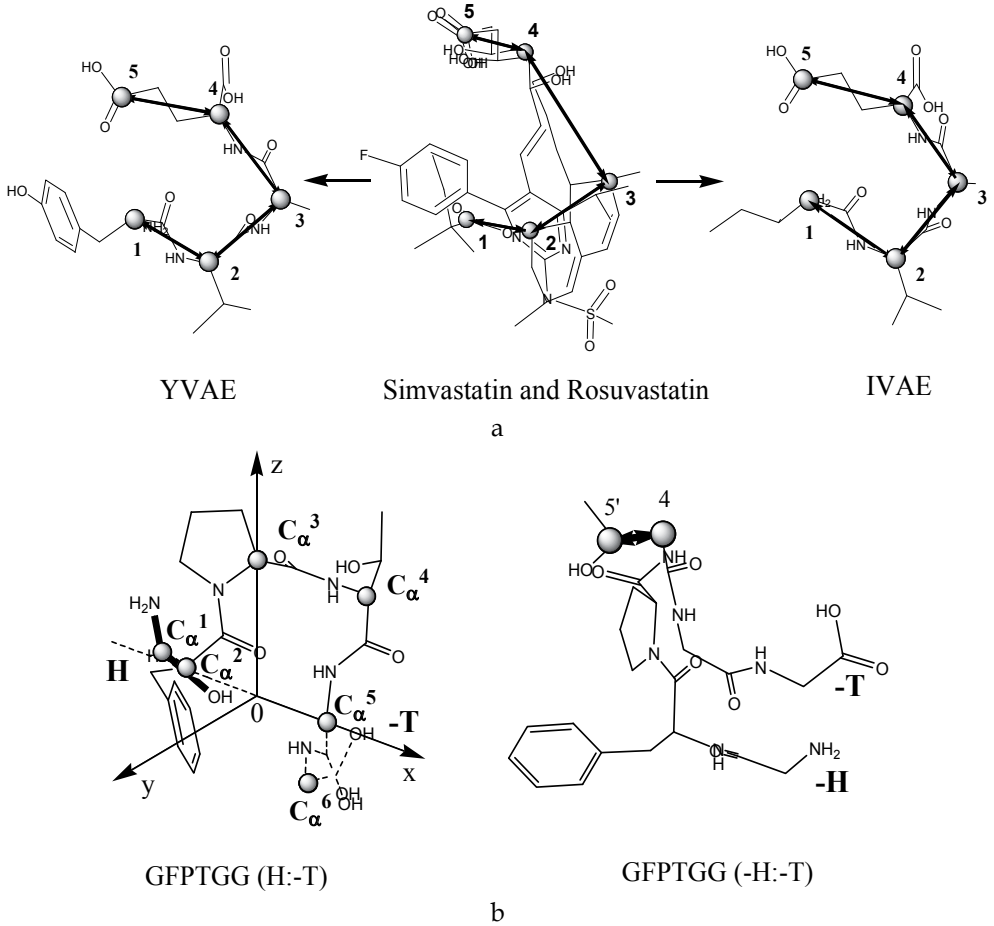

Fig. 8. Models of HMG-CoA competitive inhibitory peptides (a) and peptide acting as a bisubstrate (b). (a) Model of an 'active space' (tetrahedron 1-2-3-4) based on a superposition of the bioactive conformations of simvastatin and rosuvastatin extracted from the crystal structure of HMGR–statin complex (PDB codes: 1HW9 (simvastatin), 1DQA (rosuvastatin)), and the average structures determined for IVAE and YVAE peptides. (b) Model of the type II β-turn in peptide conformer (-H:-T) with an arrangement of peptide head (H-bold) and tail (T-short-dashed) relative to the plane passing through the three α-carbon of 2, 3, and 5 atoms of the peptide backbone, and the peptide conformation (-H:-T) used in this study. The 4–5 and 4–50 fragments in the peptide models were fixed as an HMG-mimic during modeling (Pak et al., 2008a).

respectively (Pak et al., 2008a). By considering the 'V' parameter, the GFPDGG and GFPEGG sequences are the most rigid structures compared to the other peptides, respectively. By considering the 'V' parameter, the GFPDGG and GFPEGG sequences are the most rigid structures compared to the other peptides (Pak et al., 2008a). Each of the synthesized peptides showed an ability to inhibit HMGR, with the exception of the GVAE and GGPTGG peptides (Table 7). The activities of the GVAE and GGPTGG peptides were not detectable, even at high concentrations. This indicates that the location of the I-, L-, F- and Y-side chains plays an important role in the peptide–protein interaction in the active site of HMGR. The GFPDGG peptide (IC_{50} = 1.5 μM), designed on the basis of the rigid peptide backbone, increases the inhibitory potency more than 300 times compared to the first isolated LPYP peptide (IC_{50} = 484 μM) from soybean. The obtained data suggest the possibility of designing a highly potent inhibitory peptide for HMGR and confirms that changes in the secondary structure of the enzyme play an important role in the mechanism of HMGR inhibition.

Peptide sequence	Peptide description	IC_{50} (μM)
LPYP	Peptide isolated from soybean by trypsin	484.7
IAVPGECA	Peptide isolated from soybean by pepsin	152.1
Unconstrained peptide		
IAVP	NADPH competitive inhibitor	97.1
IAVE	HMG-CoA competitive inhibitor	75.2
YAVE	HMG-CoA competitive inhibitor	52.6
IVAE	HMG-CoA competitive inhibitor	44.1
YVAE	HMG-CoA competitive inhibitor	41.8
FVAE	HMG-CoA competitive inhibitor	43.8
F(4-Fluore)VAE	HMG-CoA competitive inhibitor	3.8
GVAE	Negative control (I- and Y- side chains)	Inactive
Constrained peptide		
GLPTGG	NADPH and HMG-CoA competitive inhibitor	19.4
GLPDGG	NADPH and HMG-CoA competitive inhibitor	22.3
GLPEGG	NADPH and HMG-CoA competitive inhibitor	27.2
GFPTGG	NADPH and HMG-CoA competitive inhibitor	16.9
GFPDGG	NADPH and HMG-CoA competitive inhibitor	1.5
GFPEGG	NADPH and HMG-CoA competitive inhibitor	1.7
GGPTGG	Negative control (I- and F- side chains)	Inactive

Table 7. Summary of the sequence structures and inhibitory activities (IC_{50}) of the isolated and designed peptides used in this study (Pak et al., 2008a).

7.2.4 Design of an active peptide using a model with fixing recognized residues

In the proposed design, two binding site points were used: the first was derived from a relative assessment of the ''region bioactivity,'' in HMGR, through an analysis of the active structures of statins; the second from binding of the designed peptides inside the ''region bioactivity'' through a common point/site of the bioactive compounds and peptide structures. Under these points, the peptide conformations fixed by active/recognized residues can be seen as an approach to model the restricted flexibility of the peptides in accordance with the conformational requirements imposed by the ''region bioactivity' (Pak et al., 2008b).

In order to design a linear peptide with an unconstrained structure in a recognition sequence, two subsequent stages were applied based on the YVAE peptide as a basis of the recognition sequence: the first stage was to extend the peptide length for YVAE sequence in accordance with statin structures. For this purpose, structures of all statin molecules extracted from the crystal structures of HMGR–statin complexes were analyzed while focusing on the structural diversity of the rigid hydrophobic groups. The second stage was to investigate the conformational behavior of the peptide models. A principle component analysis (PCA), which projects multidimensional data on low-dimensional subspace, was used to evaluate a head-to-tail peptide cycle as a model of linear analog in order to select a lead peptide candidate. Using the space obtained (V parameter) by an analysis of the bioactive conformations of statins, eight cyclic peptides were selected for a peptide library based on the YVAE sequence as a recognized motif (Fig. 8). For each cycle, the four models were assessed according to the design criterion applied for constrained peptides. Three cyclic peptides (FGYVAE, FPYVAE, and FFYVAE) were selected as lead cycles from the library. The linear FGYVAE peptide (IC_{50} = 0.4μM) showed a 1,200-fold increase in inhibitory activity compared to the first isolated LPYP peptide (IC_{50} = 484μM) from soybean (Table 8). Experimental analysis of the modeled peptide structures confirms the appropriateness of the proposed approach for the modeling of active conformations of peptides (Pak et al., 2008b).

Peptides	Peptide sequence	Fixed residues in set				IC_{50} (μM)
		1	2	3	4	
Design	GGYVAE	E	EA	EAV	EAVY	7.4
	FFYVAE	E	EA	EAV	EAVY	2.5
	FPYVAE	E	EA	EAV	EAVY	1.4
	FGYVAE	E	EA	EAV	EAVY	0.4
	FWYVAE	E	EA	EAV	EAVY	29.5
	GGGGYVAE	E	EA	EAV	EAVY	760.7
Control	IAVPTGVA	T	TG	TGV	TGVA	152.1
	IAVPGEVA	E	EV	EVA	EVAI	201.3
	LPYP[a]					484.7
	YVAE[b]					41.9
Negative control	FGYVAA					Inactive

[a]The LPYP peptide was found by analysis of a digested soy glycinin using trypsin (Kwon et al., 2002).
[b]The YVAE peptide was used as a basis of the recognized sequence, which presents an unconstrained peptide structure (Pak et al., 2006).

Table 8. Peptide sequences, peptide models and inhibitory activities (IC_{50}) of the synthesized peptides used in this study

7.2.5 Peptide fragmentation for design of peptides

In a previous design, while searching for lead peptide candidates, the efficacy of a design approach based on the use of a cyclic peptide as a model of linear analog was demonstrated (Valero, et al., 2000). The conformational behavior of the cyclic peptides showed that the 6-membered cyclic peptide was relatively stable compared to the 8-, and 10-membered cyclic peptides (Pak et al., 2010). Analysis of the conformational behavior of the peptide models showed that an analogical approach could be applied in order to assess the conformational

Peptide sequence	Peptide fragment					IC_{50} (μM)
	1	2	3	4	5	
Control[a]						
LPYP	LPYP					484.72
IAVPGEVA	IAVP	AVPG	VPGE	PGEV	GEVA	201.12
IAVPTGVA	IAVP	AVPT	VPTG	PTGV	TGVA	152.19
Set 1[b]						
GLPTGG	GLPT	LPTG	PTGG			19.43
GLPDGG	GLPD	LPDG	PDGG			22.31
GLPEGG	GLPE	LPEG	PEGG			27.28
GFPTGG	GFPT	FPTG	PTGG			16.94
GFPDGG	GFPD	FPDG	PDGG			1.52
GFPEGG	GFPE	FPEG	PEGG			1.78
GXPTGG[d]	GXPT[d]	XPTG[d]	PTGG			4.82
GXPDGG[d]	GXPD[d]	XPDG[d]	PDGG			6.95
GXPEGG[d]	GXPE[d]	XPEG[d]	PEGG			0.75
GXPGGG[d]	Negative control					Inactive
Set 2[c]						
IAVE			IAVE			75.23
IVAE			IVAE			52.67
YAVE			YAVE			44.81
YVAE			YVAE			41.21
FFYVAE	FFYV	FYVA	YVAE			2.56
FPYVAE	FPYV	PYVA	YVAE			1.47
FGYVAE	FGYV	GYVA	YVAE			0.41
XVAE[d]				XVAE[d]		3.84
FGXVAE[d]		FGXV[d]	GXVA[d]	XVAE[d]		8.52
GFGYVAE	GFGY	FGYV	GYVA	YVAE		0.27
TFGYVAE	TFGY	FGYV	GYVA	YVAE		0.26
DFGYVAE	DFGY	FGYV	GYVA	YVAE		0.16
EFGYVAE	EFGY	FGYV	GYVA	YVAE		0.24
DFGYVAG	Negative control					Inactive

[a] 'Control' contains the peptides isolated from soy protein (Kwon et al, 2002; Pak et al., 2005b, c).
[b] 'Set 1' contains the peptides, which includes the recognized T, D or E residue as a corner residue of the β-turn structure (Pak et al., 2007, 2008a).
[c] 'Set 2' contains the peptides, where the E residue is a recognized residue for the HMG-binding pocket, and a β-turn structure is located in the N-terminus of the hexapeptides (Pak et al., 2008b).
[d]The substituted (4-fluoro)phenylalanine residue is indicated as 'X'.

Table 9. Peptide sequence, peptide fragment, and inhibitory activities (IC_{50}) of the synthesized peptides (Pak et al., 2010).

space that was occupied by a peptide by using peptide fragments. In order to assess the proposed method, a competitive inhibitor for HMGR was designed by using two starting points: (1) determined recognition residues and (2) the structural preference of a peptide, such as a β-turn conformation in the present design. For testing the proposed design, 13

peptides that were designed in previous studies were used (Pak et al., 2007, 2008a, 2008b). These peptides were divided into two sets in accordance with the location of a 'turn' structure relative to the recognized residue that is essential for binding. Set 1 comprised six peptides: GLPTGG, GLPDGG, GLPEGG, GFPTGG, GFPDGG, and GFPEGG (Table 9).
A common structural element of these peptides was a turn conformation, which included T, D, or E as a corner residue of the β-turn structure and was recognized by a HMG-binding pocket. All peptides inhibited HMGR in a competitive manner. Set 2 included seven peptides, in which E residue was also a recognition residue for the HMG-binding pocket. A conformational analysis of the IAVE, IVAE, YAVE, and YVAE peptides revealed no observable patterns that were related to a well-populated secondary structure conformation (Pak et al., 2006). For the FFYVAE, FPYVAE, and FGYVAE peptides, a turn conformation was determined at the N-terminus of these peptides. All of these peptides presented as HMG-CoA competitive inhibitors (Pak et al., 2008b) (Table 9).

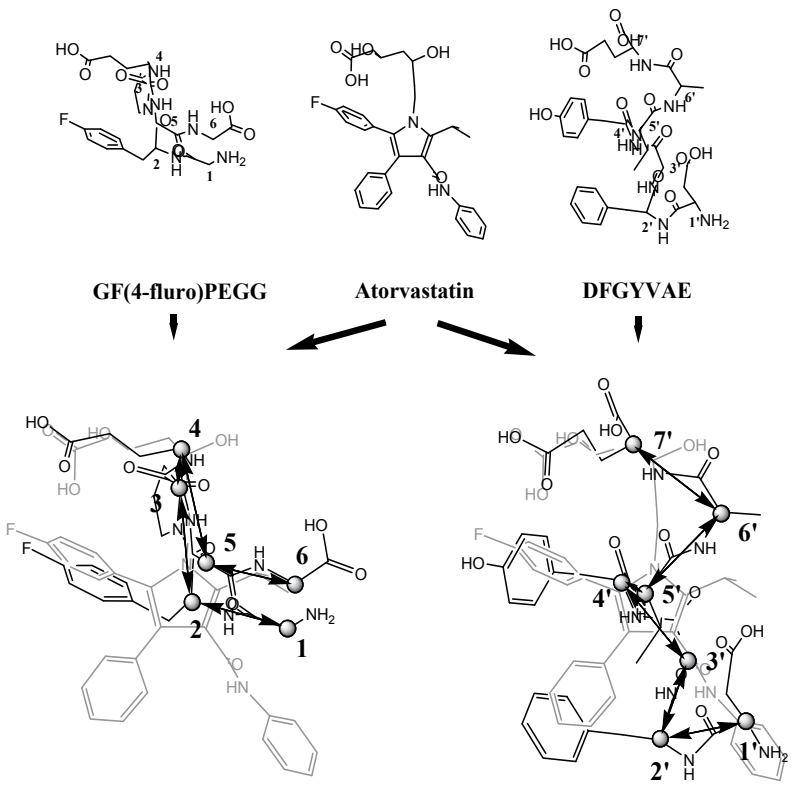

Fig. 9. Spatial compatibility of the linear GF(4-fluoro)PEGG and DFGYVAE peptides with atorvastatin. The alpha-carbon atoms are indicated by marks from 1 to 6 for the GF(4-fluoro)PEGG peptide and from 10 to 70 for the DFGYVAE peptide. The model of GF(4-fluoro)PEGG was built as type II of the β-turn on the basis of the fixed backbone dihedral angles adopted by the two corner proline and glutamic acid residues (φ_{i+1} = -60°, ψ_{i+1} = 120°, φ_{i+2} = 80°, and ψ_{i+2} = 0°). For the DFGYVAE peptide, the model was constructed as type I of the β-turn by using the fixed backbone dihedral angles adopted by the two corner glycine and tyrosine residues (φ_{i+1} = -60°, ψ_{i+1} = -30°, φ_{i+2} = -90°, and ψ_{i+2} = 0°). The orientations of the side-chains and other residues were determined by an optimization procedure.

Two sets of peptides were developed based on the different locations of a β-turn structure relative to a recognition residue (Table 9). Set 1 contains peptides in which a recognition residue is included in turn conformation. In Set 2, the β-turn structure is located distantly from the recognition residues. The design parameter 'V' that was applied in previous studies was slightly modified for the purpose of the current research. The 17 previously and 8 newly designed peptides were estimated by this parameter. In each set, one sequence was selected as a lead peptide candidate for each set: GF(4-fluoro)PEGG for Set 1 and DFGYVAE for Set 2. The inhibitory activities improved in each set, the proposed contribution of the fluorine atom of GF(4-fluoro)PEGG peptide during binding led to an increase in the inhibitory activity for this peptide compared to the other members of Set 1 (Fig. 9). This was explained by a similar location of the 4-fluorophenyl group of atorvastatin and GF(4-fluoro)PEGG peptide. Probably, the increase of inhibitory activity of the DFGYVAE peptide can be interpreted in terms of the contribution of the oxygen atom of the carbonyl group of the D side chain, while the increase of inhibitory activity of the GFGYVAE peptide by the contribution of the oxygen atom from the amide bond between N-terminus' G and F residues in binding. The IC_{50} for the GF(4-fluoro) PEGG peptide was found to be 0.75μM, while the linear DFGYVAE peptide (IC_{50} = 0.16μM) showed a 3,000-fold increase in inhibitory activity compared to the first isolated LPYP peptide (IC_{50} = 484μM) from soybeans. The comparison of the structure–activity relationship (SAR) data between Set 1 and 2 provided an opportunity to design the peptides in terms of peptide selectivity.
In conclusion, the present study not only shows the design of a more potent inhibitor for HMGR, but also defines a design tool to model active conformations for linear peptides.

8. Conclusions

One possible reason for the lower incidence of some diseases such as obesity, diabetes, or even breast cancers among Asians is that they consume fermented soybean products, which are unique to traditional Asian diets. Asians have prepared and eaten their own soy products such as tofu and fermented soy products for thousands of years. Soybean fermented foods such as doenjang (long-term fermented soy products), chungkukjang (short-term fermented soy products), kanjang (fermented soy sauce) are highlighted due to their healthy functionalities. Both short- and long-term fermented soybeans contain more beneficial components to ameliorate metabolic disorders than unfermented soybeans. The changes in nutritive and non-nutritive biofactors during fermentation and their capacity for ameliorating metabolic disorders were reported in Asian countries, especially Korea. The changes in metabolomic profiling were analyzed using GC, LC, and NMR with mass spectrometry for the metabolites produced during the fermentation by *Bacillus* for a short-term fermentation and *Aspergillus* for a long-term fermentation. Fermentation of soybeans increased isoflavonoid aglycones, modified isoflavonoids such as equol and small peptides and these changes enhanced the prevention of metabolic disorders. Chungkukjang and meju improved anti-diabetic action by improving β-cell function and mass via potentiating insulin/IGF-1 signaling in the islets. Thus, daily consumption of fermented soybean products can prevent and/or delay metabolic disorders in humans. From fermented soybeans we found some effective peptides for reducing the hypertension and hypercholesterolemia by analyzing ACE (angiotensin converting enzyme) inhibition and HMG(3-hydroxy-3-methylglutaryl)-CoA reductase inhibition, respectively. Active hypotensive and hypocholesterolemic peptides were isolated and structures identified as HHL for hypotensive, and LPYP and IAVPGEVA for hypocholesterolemic peptide, respectively. Based on the conformation of these peptide structures and mechanisms of the

reactions, new peptides were designed using peptide modeling to strengthen their activity as candidates for a new drug and nutraceuticals. Novel peptide (DFGYVAE) was designed to increase the hypocholesterolemic effectiveness to three thousand times higher than origin peptide (LPYP). Currently the peptides from soy or other beans can be the subject of investigation for new drugs and functional food ingredients for gut health and modulating the intestinal absorption of nutrients. Research into bioactive soy peptides is still in its infancy, but holds great promise.

9. Acknowledgment

This work was supported by a grant from the National Research Foundation in Korea for Biofoods (M10510120001-05N1012-00111). The authors have no conflict of interest for the information presented in this review.

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3

Effects of Soya Oil upon the Metabolic Syndrome of ω3-Depleted Rats

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1. Introduction

The bolus intravenous injection of a novel medium-chain triglyceride:fish oil emulsion was recently proposed as a suitable tool to increase within 60 min the long-chain polyunsaturated ω3 fatty acid content of cell phospholipids (Carpentier et al., 2010). The optimal procedure for the dietary correction of the metabolic and hormonal perturbations found in situations of ω3-depletion, in terms of the total lipid content and source of ω3 fatty acids in the diet, as well as the time course for the enrichment of tissue lipids in these ω3 fatty acids, merits, however, further attention (Malaisse et al., 2011). In such a perspective, the present report deals mainly with the possible suitability of a diet containing 5% (w/w) soya oil, as assessed in rats first exposed for 3 months to an ω3-deficient sunflower oil (also 5%) diet.

2. Materials and methods

Six groups of 4 fed female rats each were examined in the present study. Two groups consisted of control animals exposed from the age of 8 weeks after birth for 3 months to a diet containing 5% (w/w) soya oil and examined either at the end of this 3 months initial period or 8 weeks later whilst being maintained on the soya oil diet. The other 4 groups of rats were first exposed during the initial 3 months period to a diet containing 5% (w/w) sunflower oil (ω3D rats) and examined either at the end of this 3 months initial period or after further exposure for one, two or eight weeks to the soya oil diet. Six further ω3D rats were maintained after the end of the initial 3 months period and for the ensuing 8 weeks to the sunflower oil diet, but were only examined for their food intake and body weight. The rats were eventually sacrificed by CO_2 inhalation. The methods used for collection of intestinal mucosa at the jejunal and caecal level (Hacquebard et al., 2009), liver (Malaisse et al., 2009), red blood cell (Carpentier et al., 2011a), brain (Portois et al., 2009) and both visceral and parametrial adipose tissue (Portois et al., 2007) sampling, lipid extraction (Folch et al., 1957), separation by thin-layer chromatography (Dahlan et al., 1992) and determination of lipid fatty acid pattern by gas-liquid chromatography (Lepage & Roy, 1986), food intake measurements (Zhang et al., 2010) and plasma D-glucose (Lowry & Passonneau, 1972) and insulin (Leclercq-Meyer et al., 1985) determinations were previously described in the cited references.

All results are presented as mean values (± SEM) together with the number of individual observations (n) or degree of freedom (df). Except if otherwise mentioned, all tabulated values refer to four individual determinations. In the case of RBC, however, the measurements listed for ω3D rats examined 1 or 2 weeks after the switch in diet refer to single measurements made in pooled material from 4 rats. In the Tables, the indication N.T. refers to non-tabulated data. The statistical significance of differences between mean values was assessed by use of Student's *t*-test.

3. Results

3.1 Fatty acid pattern of intestinal phospholipids

3.1.1 Long-chain polyunsaturated ω3 fatty acids

At the end of the 3 months initial period, the relative weight content of all ω3 fatty acids (C18:3ω3, C20:5ω3, C22:5ω3 and C22:6ω3) was significantly lower ($p < 0.005$ or less), whether in the jejunum or caecum, in the ω3D rats than in the control animals (Table 1). When the former rats were then exposed for one week to the soya oil diet, a significant increase ($p < 0.02$ or less) in the C18:3ω3, C22:5ω3 and C22:6ω3 relative content of jejunal phospholipids and in the C22:5ω3 and C22:6ω3 content of caecal phospholipids was observed, whilst such was not the case for either the C20:5ω3 content of jejunal phospholipids or C18:3ω3 and C20:5ω3 content of caecal phospholipids. Nevertheless, the mean values reached in the ω3D rats exposed for one week to the soya oil remained, as a rule, lower than those found in the control animals at the end of the 3 months initial period. Even, in the two out of eight instances in which such was not the case, i.e. for the C18:3ω3 content of jejunal phospholipids and C22:5ω3 content of caecal phospholipids, no significant difference ($p > 0.3$ or more) was found between the two groups of rats under consideration.

Rats		C18:3ω3	C20:5ω3	C22:5ω3	C22:6ω3
			Jejunum		
Control	before	7.10 ± 1.46	0.00 ± 0.00	4.18 ± 0.39	39.51 ± 2.00
ω3D	before	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	14.86 ± 1.62
	after (1 wk)	8.30 ± 1.35	0.42 ± 0.42	2.89 ± 0.32	25.88 ± 2.18
			Caecum		
Control	before	1.46 ± 1.46	2.76 ± 1.60	2.74 ± 2.74	49.94 ± 6.83
ω3D	before	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	5.49 ± 5.49
	after (1 wk)	0.00 ± 0.00	0.00 ± 0.00	5.60 ± 0.65	23.80 ± 1.57

Table 1. Relative weight content (‰) of long-chain polyunsaturated ω3 fatty acids in jejunum and caecum phospholipids

3.1.2 Long-chain polyunsaturated ω6 fatty acids

As shown in Table 2, no significant difference between the three groups of rats was observed in terms of the relative weight content of C18:2ω6, C20:3ω6 and C20:4ω6 in the jejunal phospholipids. However, the content of C20:2ω6, C22:4ω6 and C22:5ω6, expressed relative to their respective overall mean values averaged 38.2 ± 12.0% (n = 12) in the control animals examined at the end of the 3 months initial period, as distinct ($p < 0.001$) from 166.2

± 17.6% (n = 12) in the ω3D rats examined before the switch in diet. After one week exposure to the soya oil diet, the latter percentage was decreased ($p < 0.005$) to 95.6 ± 8.4% (n = 12), the latter value remaining higher ($p < 0.001$) than that recorded in the control animals.
The relative weight content of ω6 fatty acids differed, on occasion, in the jejunal *versus* caecal phospholipids. For instance, the weight content of C18:2ω6 averaged 320.88 ± 9.60‰ (n = 12) at the jejunal level, as distinct ($p < 0.001$) from only 187.42 ± 5.53‰ (n = 12) at the caecal level. Moreover, as a rule, no significant difference between the mean values recorded in the three groups of rats was observed for the relative weight content of ω6 fatty acids in the caecal phospholipids. Only in the case of C22:5ω6, a significant increase ($p < 0.001$) in the content of this fatty acid in caecal phospholipids was found when comparing control animals and ω3D rats both examined at the end of the 3 months initial period, with an in-between value ($p < 0.01$ or less) in the ω3D rats examined one week after the switch in diet.

Rats		C18:2ω6	C20:2ω6	C20:3ω6	C20:4ω6	C22:4ω6	C22:5ω6
				Jejunum			
Control	before	314.40 ± 19.28	1.16 ± 0.68	5.71 ± 0.45	175.68 ± 21.41	4.24 ± 0.60	0.00 ± 0.00
ω3D	before	316.85 ± 16.43	4.61 ± 0.71	6.31 ± 0.75	173.77 ± 8.19	7.94 ± 1.66	16.80 ± 3.09
	after (1 wk)	331.38 ± 17.79	2.87 ± 0.41	4.94 ± 0.58	167.09 ± 11.11	4.94 ± 0.39	8.48 ± 1.84
				Caecum			
Control	before	192.02 ± 12.80	3.40 ± 1.96	16.50 ± 0.36	164.85 ± 12.04	16.69 ± 5.70	2.13 ± 1.32
ω3D	before	189.76 ± 8.28	0.00 ± 0.00	7.42 ± 4.34	200.00 ± 13.13	29.56 ± 3.28	26.47 ± 1.37
	after (1 wk)	180.50 ± 8.92	3.66 ± 2.12	16.59 ± 0.62	214.29 ± 10.21	24.39 ± 1.05	16.12 ± 2.25

Table 2. Relative weight content (‰) of long-chain polyunsaturated ω6 fatty acids in jejunum and caecum phospholipids

3.1.3 Saturated fatty acids

The relative weight content of saturated fatty acids often differed significantly in jejunal *versus* caecal phospholipids (Table 3). For instance, the C14:0 content was close to 15-fold higher ($p < 0.001$) in the caecal phospholipids (9.44 ± 1.13‰; n = 12) than in the jejunal phospholipids (0.64 ± 0.23‰; n = 12). Whether at the jejunal or caecal level, no significant difference was observed between the relative weight content of a given saturated fatty acid in the three groups of rats under consideration.

Rats		C14:0	C16:0	C18:0	C20:0	C22:0	C24:0
				Jejunum			
Control	before	0.00 ± 0.00	167.22 ± 3.42	198.25 ± 9.42	7.00 ± 0.79	7.58 ± 0.53	6.25 ± 0.51
ω3D	before	0.78 ± 0.45	164.31 ± 10.29	204.77 ± 5.02	9.32 ± 3.02	10.83 ± 2.73	9.24 ± 2.38
	after (1 wk)	1.15 ± 0.39	160.78 ± 4.72	198.89 ± 6.32	7.30 ± 0.60	8.33 ± 0.69	8.43 ± 0.99
				Caecum			
Control	before	7.79 ± 3.03	247.74 ± 15.67	123.27 ± 10.61	15.05 ± 3.28	18.09 ± 5.06	18.20 ± 7.70
ω3D	before	9.01 ± 0.69	242.60 ± 10.81	129.05 ± 4.07	11.83 ± 2.12	12.85 ± 2.12	9.61 ± 1.39
	after (1 wk)	11.51 ± 1.38	233.53 ± 12.15	120.62 ± 4.78	10.55 ± 1.18	10.11 ± 1.24	7.98 ± 1.50

Table 3. Relative weight content (‰) of saturated fatty acids in jejunum and caecum phospholipids

3.1.4 Monodesaturated fatty acids

The C16:1ω7 and C18:1ω9 relative weight content of jejunal phospholipids averaged, respectively, 2.97 ± 0.39‰ and 60.66 ± 2.82‰ (n = 12 in both cases), as distinct ($p < 0.001$)

from 10.01 ± 1.56‰ and 117.51 ± 2.01‰ (n = 12 in both cases) in caecal phospholipids. Such contents failed to differ significantly from one another, whether in the jejunum or caecum, in the three groups of rats under consideration (Table 4). A sizeable amount of C20:1ω9 in intestinal phospholipids was only detected in one out of 4 rats in the ω3D rats examined before or after the switch in diet. No C22:1ω9 could be detected in any of the 24 rats examined for such a purpose.

Rats		C16:1ω7	C18:1ω9	C20:1ω9	C22:1ω9
			Jejunum		
Control	before	3.70 ± 0.70	58.03 ± 2.03	0.00 ± 0.00	0.00 ± 0.00
ω3D	before	2.50 ± 0.92	64.77 ± 2.11	0.43 ± 0.43	0.00 ± 0.00
	after (1 wk)	2.71 ± 0.20	59.18 ± 3.94	0.05 ± 0.05	0.00 ± 0.00
			Caecum		
Control	before	5.54 ± 3.34	120.28 ± 1.42	0.00 ± 0.00	0.00 ± 0.00
ω3D	before	12.18 ± 2.09	118.44 ± 4.76	1.01 ± 1.01	0.00 ± 0.00
	after (1 wk)	12.30 ± 1.17	113.82 ± 3.49	0.91 ± 0.91	0.00 ± 0.00

Table 4. Relative weight content (‰) of monodesaturated fatty acids in jejunum and caecum phospholipids

3.2 Relation between intestinal and hepatic phospholipids

In the case of long-chain polyunsaturated ω3 fatty acids, highly significant positive correlations prevailed between the relative content of C18:3ω3 in jejunal and hepatic phospholipids ($r = + 0.8368$; $n = 12$; $p < 0.001$), that of C22:5ω3 also in jejunal and hepatic phospholipids ($r = + 0.9153$; $n = 12$; $p < 0.001$), and that of C22:6ω3 either in jejunal and hepatic phospholipids ($r = + 0.9716$; $n = 12$; $p < 0.001$) or in caecal and hepatic phospholipids ($r = + 0.8679$; $n = 12$; $p < 0.001$). The paired ratio between the C22:6ω3 relative weight content in hepatic/jejunal phospholipids did not differ significantly ($p > 0.14$ or more) in the three groups of rats under consideration, i.e. control and ω3D rats examined at the end of the 3 months initial period and ω3D rats exposed for one week to the soya oil diet, with an overall mean value of 4.13 ± 0.16 (n = 12).

In the case of long-chain polyunsaturated ω6 fatty acids, a significant positive correlation ($r = + 0.5868$; $n = 12$; $p < 0.05$) was found between the C22:4ω6 relative weight content of jejunal and hepatic phospholipids in the same 12 rats. Such was not the case ($r = + 0.4924$; $n = 12$; $p > 0.1$) for the C20:2ω6 content of the same phospholipids. The tightest correlations concerned the C22:5ω6 or liver phospholipids and that of either jejunal phospholipids ($r = + 0.9776$; $n = 12$; $p < 0.001$) or caecal phospholipids ($r = + 0.9242$; $n = 12$; $p < 0.001$), with, in the latter two series of comparisons, either only one or no negative xy product among the 12 sets of data.

3.3 Fatty acid pattern of liver phospholipids

3.3.1 Long-chain polyunsaturated ω3 fatty acids

In fair agreement with the jejunal data, the relative weight content of C18:3ω3 in liver phospholipids averaged at the end of the initial 3 months period 0.35 ± 0.35‰ (n = 4) in the

ω3D rats, as distinct ($p < 0.005$) from 2.30 ± 0.19‰ ($n = 4$) in the control animals, increasing ($p < 0.005$) in the former ω3D rats to, respectively, 1.91 ± 0.09 and 2.09 ± 0.17‰ ($n = 4$ in both cases) one and two weeks after the switch to the soya oil diet.

The relative weight content of the three other long-chain polyunsaturated ω3 fatty acids in liver phospholipids yielded comparable information (Table 5). First, the mean values recorded in the control animals before the switch in diet and 8 weeks thereafter never differed significantly ($p > 0.6$ or more) from one another, with overall mean values of 4.99 ± 0.77‰ ($n = 8$) for C20:5ω3, 9.29 ± 0.58‰ ($n = 8$) for C22:5ω3 and 170.43 ± 2.92‰ ($n = 8$) for C22:6ω3. Second, these mean values were all significantly higher ($p < 0.002$ or less) than those recorded in the ω3D rats at the end of the 3 months initial period. Third, in the ω3D rats, higher values ($p < 0.025$ or less) were found after the switch in diet then before such a switch, with mean values over the 8 weeks exposure to the soya oil diet averaging 2.47 ± 0.55‰ ($n = 12$) in the case of C20:5ω3, 7.15 ± 0.67‰ ($n = 12$) in the case of C22:5ω3 and 130.02 ± 6.80‰ ($n = 12$) in the case of C22:6ω3. Last, the latter three percentages all remained significantly lower ($p < 0.02$ or less) than the corresponding mean values recorded in the control animals over the same period of 8 weeks. In the ω3D rats, a progressive time-related increase in the relative weight content of a long-chain polyunsaturated ω3 fatty acid in the liver phospholipids between the first and last week of exposure to the soya oil diet was only observed in the case of C22:6ω3 ($r = + 0.6374$; $n = 12$; $p < 0.04$).

These findings indicate that, except as far as C18:3ω3 is concerned, a period of 8 weeks exposure of the ω3D rats to the soya oil diet was not quite sufficient to restore the liver phospholipid content of ω3 fatty acids to the same level as that otherwise found in control animals exposed to the same diet from the 8th week after birth.

Rats		C20:5ω3	C22:5ω3	C22:6ω3
Control	before	5.05 ± 1.12	9.00 ± 0.41	169.11 ± 4.49
	after (8 wk)	4.93 ± 1.22	9.58 ± 1.17	171.81 ± 4.37
ω3D	before	0.00 ± 0.00	1.56 ± 0.80	54.52 ± 5.32
	after (1 wk)	2.80 ± 1.40	6.81 ± 1.23	112.62 ± 8.54
	after (2 wk)	1.90 ± 0.73	5.96 ± 0.33	128.16 ± 4.91
	after (8 wk)	2.71 ± 0.85	8.68 ± 1.40	149.37 ± 13.55

Table 5. Relative weight content (‰) of long-chain polyunsaturated ω3 fatty acids in liver phospholipids

3.3.2 Long-chain polyunsaturated ω6 fatty acids

Except in the case of C22:4ω6 and C22:5ω6, the mean values recorded in the control animals were not significantly different at the end of the 3 months initial period and 8 weeks thereafter (Table 6). In the former two cases, however, the mean values found at the end of the experiments were higher ($p < 0.02$ or less) than those recorded at the end of the 3 months initial period.

No significant difference between the 6 groups of rats listed in Table 6 was observed for the C18:2ω6 relative weight content of liver phospholipids. Likewise, as far as C20:3ω6 is concerned, the mean value found in the control animals (7.28 ± 0.74‰; $n = 8$) did not differ significantly ($p > 0.5$) from that recorded in the ω3D rats (6.65 ± 0.64‰; $n = 16$)

In the case of the other 5 long-chain polyunsaturated ω6 fatty acids (C20:2ω6, C18:3ω3, C20:4ω6, C22:4ω6 and C22:5ω6), the mean value found in the ω3D rats examined before the switch in diet always exceeded both that recorded in the 8 control animals and those

measured in each of the three groups of ω3D rats examined after the switch in diet. The relative magnitude of the difference between the values recorded in the ω3D rats before the switch from the sunflower oil diet to the soya oil diet and the overall mean value found in the control animals at the end of the initial 3 months period and 8 weeks thereafter was quite variable, ranging from as little as 12.0 ± 2.4% (df = 10; $p < 0.001$) in the case of C20:4ω6 to as much as a tenfold increase ($p < 0.001$) in the case of C22:5ω6.

Rats		C18:2ω6	C20:2ω6	C18:3ω6	C20:3ω6	C20:4ω6	C22:4ω6	C22:5ω6
Control	before	82.51 ± 5.16	1.59 ± 0.18	1.72 ± 0.25	6.05 ± 0.63	303.65 ± 5.77	2.87 ± 0.16	5.27 ± 0.48
	after (8 wk)	78.23 ± 4.70	1.57 ± 0.12	1.64 ± 0.06	8.52 ± 1.06	308.73 ± 8.17	3.99 ± 0.13	9.27 ± 1.07
ω3D	before	77.83 ± 2.73	2.78 ± 0.80	2.03 ± 0.20	5.44 ± 0.44	342.83 ± 4.45	8.54 ± 0.31	72.67 ± 8.82
	after (1wk)	74.16 ± 5.52	1.64 ± 0.17	1.85 ± 0.29	6.73 ± 1.51	330.74 ± 6.44	5.38 ± 0.04	37.21 ± 5.54
	after (2 wk)	77.23 ± 2.55	1.94 ± 0.15	1.61 ± 0.13	6.19 ± 0.97	330.51 ± 2.11	4.63 ± 0.23	23.63 ± 2.55
	after (8 wk)	76.92 ± 6.60	1.44 ± 0.05	1.74 ± 0.19	8.22 ± 1.84	324.40 ± 3.48	4.45 ± 0.25	14.71 ± 4.07

Table 6. Relative weight content (‰) of long-chain polyunsaturated ω6 fatty acids in liver phospholipids

However, when the values recorded in the ω3D rats 1 week, 2 weeks and 8 weeks after the switch in diet, in excess of the reference value found in control animals, were expressed relative to the difference between the measurements made in the ω3D rats before the switch in diet and the same reference value found in control animals, a time-related obliteration of the latter difference was often observed. As illustrated in Fig. 1, such a progressive decrease displayed an exponential pattern, with a half-life somewhat below one week.

3.3.3 Saturated fatty acids

Except in the case of C16:0 and C24:0 ($p < 0.05$ or less), no significant change was observed in the control animals between the liver phospholipid relative weight content of the six saturated fatty acids listed in Table 7, as measured at the end of the 3 months initial period and 8 weeks thereafter.

Rats		C14:0	C16:0	C18:0	C20:0	C22:0	C24:0
Control	before	1.56 ± 0.15	133.92 ± 2.71	237.04 ± 4.67	0.70 ± 0.01	2.23 ± 0.69	6.84 ± 0.34
	after (8 wk)	1.52 ± 0.11	120.29 ± 4.39	241.12 ± 5.76	0.75 ± 0.04	3.62 ± 0.13	8.79 ± 0.05
ω3D	before	1.86 ± 0.22	144.02 ± 5.75	273.82 ± 1.42	0.20 ± 0.20	4.42 ± 0.20	9.63 ± 0.24
	after (1 wk)	1.74 ± 0.14	133.30 ± 3.77	261.74 ± 7.20	0.35 ± 0.20	2.78 ± 0.93	8.01 ± 0.16
	after (2 wk)	1.81 ± 0.10	130.91 ± 1.67	252.75 ± 1.55	0.20 ± 0.20	3.42 ± 0.40	7.86 ± 0.05
	after (8 wk)	1.51 ± 0.15	116.45 ± 4.95	251.72 ± 9.17	0.00 ± 0.00	3.34 ± 0.19	8.42 ± 0.56

Table 7. Relative weight content (‰) of saturated fatty acids in liver phospholipids

The C20:0 relative weight content of liver phospholipids was lower ($p < 0.001$) in the ω3D rats (0.19 ± 0.08‰; n = 16) than in the control animals (0.72 ± 0.02‰; n = 8). The opposite situation prevailed for the other five saturated fatty acids (C14:0, C16:0, C18:0, C22:0 and C24:0). As documented in Fig. 2, using the same analytical procedure as that applied to the data relative to content of long-chain polyunsaturated ω6 fatty acids (Fig. 1), the switch from the sunflower oil diet to the soya oil diet provoked a time-related normalization of the liver

phospholipid content of the just mentioned five saturated fatty acids, with a half-life close to one week. Such a progressive decrease represented a mirror image of the progressive increase in the C22:6ω3 relative weight content of liver phospholipids in the ω3D rats examined after the switch in diet, with again a half-life close to one week.

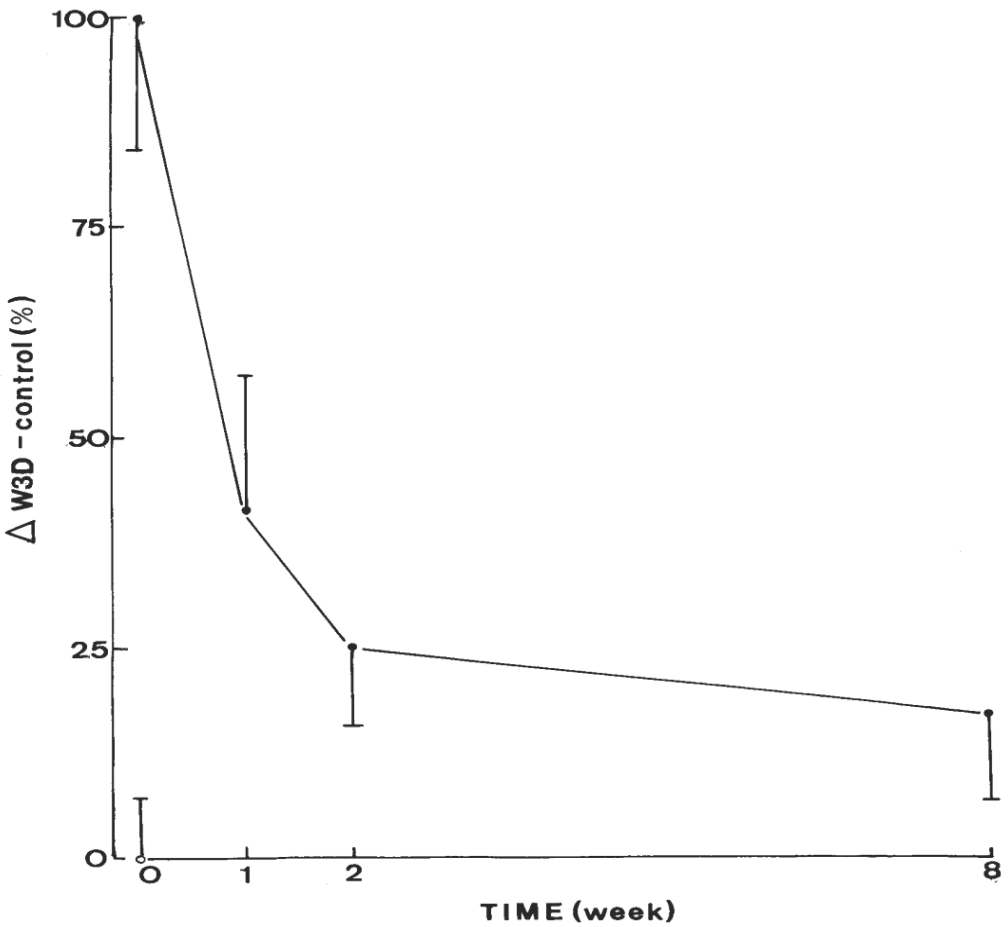

Fig. 1. Time course for the changes in the relative weight content of C20:2ω6, C18:3ω6, C20:4ω6, C22:4ω6 and C22:5ω6 in the liver phospholipids of ω3D rats after the switch from the sunflower oil to soya oil diet. For each of these five fatty acids, all individual results are expressed relative to the difference between the mean value recorded in the ω3D rats before the switch in diet and the overall mean value found in the control animals examined at the end of the 3 months initial period and 8 weeks thereafter. Mean values (± SEM) refer to 20 individual measurements in the ω3D rats (closed circles) and 40 individual measurements in the control animals (open circle)

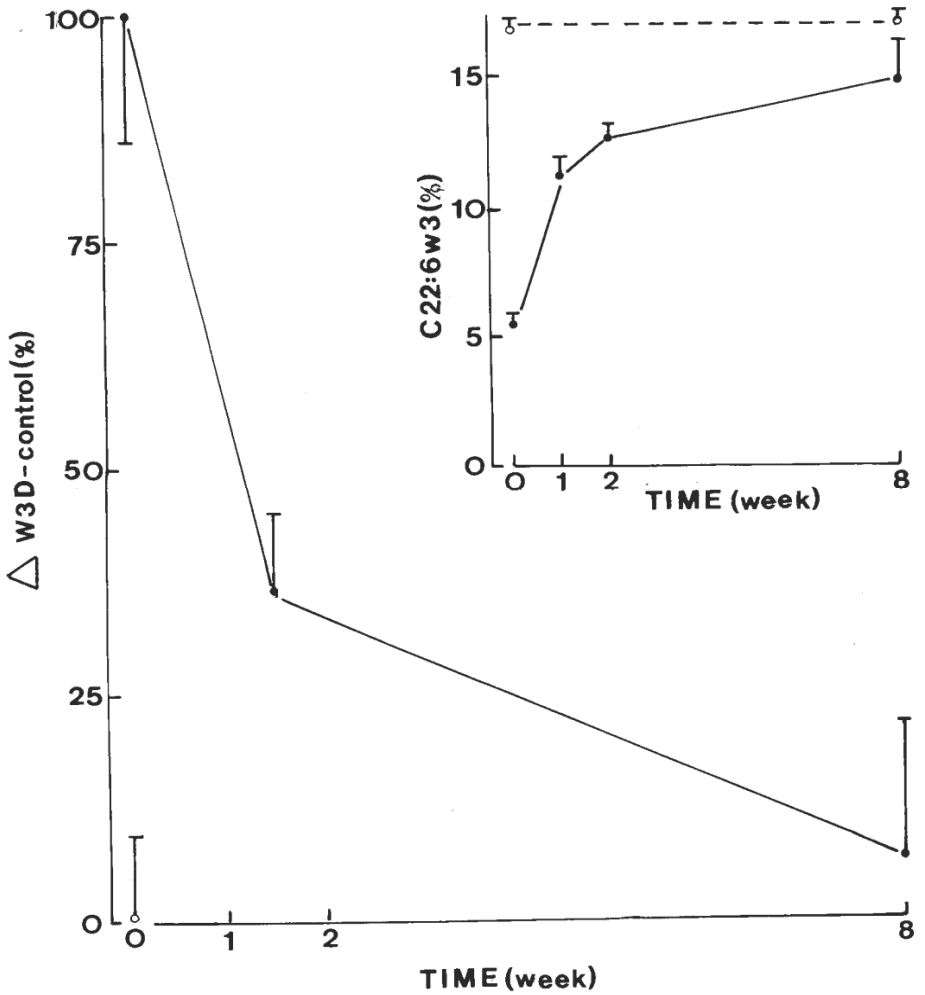

Fig. 2. Time course for the changes in the relative weight content of C14:0, C16:0, C18:0, C22:0 and C24:0 in the liver phospholipids of ω3D rats after the switch from the sunflower oil to soya oil diet. Same presentation as in Fig. 1. The inset illustrates the time course for the changes in the relative weight content (%) of C22:6ω3 in the liver phospholipids of control animals (open circles and dashed line) and ω3D rats (closed circles and solid line); mean values (± SEM) refer to 4 individual determinations in each case.

3.3.4 Monodesaturated fatty acids

No significant difference in the relative weight content of monodesaturated fatty acids (C16:1ω7, C18:1ω9, C20:1ω9 and C22:1ω9) in liver phospholipids was detected when comparing control animals and ω3D rats, whether examined at the end of the 3 months initial period or thereafter (Table 8). For instance, the C22:1ω9 content averaged 2.08 ± 0.20‰ (n = 8) in the control animals, and respectively 2.92 ± 0.63‰ (n = 4) and 2.74 ± 0.40‰ (n = 12) in the ω3D rats examined before and after the switch in diet. None of the latter three percentages differed significantly from one another ($p > 0.2$ or more).

Rats		C16:1ω7	C18:1ω9	C20:1ω9	C22:1ω9
Control	before	5.16 ± 1.06	23.91 ± 2.57	0.33 ± 0.19	2.04 ± 0.24
	after (1 wk)	6.14 ± 0.44	23.04 ± 0.80	0.00 ± 0.00	2.11 ± 0.37
ω3D	before	5.83 ± 0.96	27.04 ± 2.78	0.00 ± 0.00	2.92 ± 0.63
	after (1 wk)	5.20 ± 1.02	23.79 ± 2.17	0.16 ± 0.16	2.05 ± 0.49
	after (2 wk)	5.57 ± 0.74	24.90 ± 1.35	0.00 ± 0.00	2.12 ± 0.54
	after (8 wk)	5.75 ± 0.78	24.58 ± 1.46	0.00 ± 0.00	4.06 ± 0.57

Table 8. Relative weight content (‰) of monodesaturated fatty acids in liver phospholipids

3.4 Fatty acid pattern of RBC phospholipids

3.4.1 Long-chain polyunsaturated ω3 fatty acids

The mean values for the relative weight content of long-chain polyunsaturated ω3 fatty acids in RBC phospholipids are listed in Table 9.

In the ω3D rats, the C18:3ω3 relative weight content of RBC phospholipids increased ($p < 0.005$) from a mean value of 0.62 ± 0.36‰ (n = 4) before the switch in diet to an overall mean value of 1.79 ± 0.07‰ (n = 6) thereafter. The latter mean value remained somewhat lower ($p < 0.06$) than that recorded in the control animals at the end of the 3 months initial period (2.11 ± 0.14‰; n = 4). A minor amount of C18:4ω3 (1.60 ± 0.21‰; n = 4) was only detected in the ω3D rats examined before the switch in diet.

Rats		C18:3ω3	C20:5ω3	C22:5ω3	C22:6ω3
Control	before	2.11 ± 0.14	4.33 ± 0.57	19.11 ± 0.64	68.11 ± 1.39
	after (8 wk)	N.T.	3.01 ± 0.37	21.37 ± 2.25	64.84 ± 2.84
ω3D	before	0.62 ± 0.36	0.00 ± 0.00	1.82 ± 0.14	19.76 ± 1.12
	after (1 wk)	1.88 (1)	1.06 (1)	4.53 (1)	27.75 (1)
	after (2 wk)	1.69 (1)	1.36 (1)	5.80 (1)	36.31 (1)
	after (8 wk)	1.79 ± 0.10	2.86 ± 0.35	14.02 ± 1.10	49.64 ± 3.84

Table 9. Relative weight content (‰) of long-chain polyunsaturated ω3 fatty acids in RBC phospholipids

In the ω3D rats, the C20:5ω3 content of RBC phospholipids also progressively increased from 0.00 ± 0.00‰ (n = 4) before the switch in diet to 1.21 ± 0.15‰ (n = 2) one to two weeks thereafter and 2.86 ± 0.35‰ (n = 4) 8 weeks thereafter. Once again, the latter mean value remained lower ($p < 0.05$) than that recorded in the control animals over the same period of 8 weeks (4.12 ± 0.33‰; n = 8). The time-related increase in the C20:5ω3 content of RBC phospholipids was less rapid than that found, in the same ω3D rats, for the C20:5ω3 content of liver phospholipids. Indeed the paired RBC/liver ratio for such contents increased in the ω3D rats from 0.0 ± 0.0% (n = 4) before the switch in diet to respectively 37.9% (n = 1), 71.6% (n = 1) and 120.9 ± 17.2% (n = 4) one, two and 8 weeks thereafter. The latter mean value was no more significantly different ($p > 0.25$) from the RBC/liver ratio found in the control animals examined either before (98.6 ± 19.6%; n = 4) or after (90.6 ± 16.7%; n = 4) the last 8 weeks of the present experiments.

A delayed alignment of the C22:5ω3 content of RBC phospholipids on that of liver phospholipids in the ω3D rats eventually exposed to the soya oil diet was also observed, as illustrated in Fig. 3. Thus, the liver/RBC ratio for this variable transiently increased in the

ω3D rats switched to the soya oil diet before resuming a mean value (60.9 ± 5.4%; n = 4) still somewhat higher ($p < 0.02$) than that recorded in the control animals over the same period of 8 weeks (46.2 ± 2.5%; n = 8).

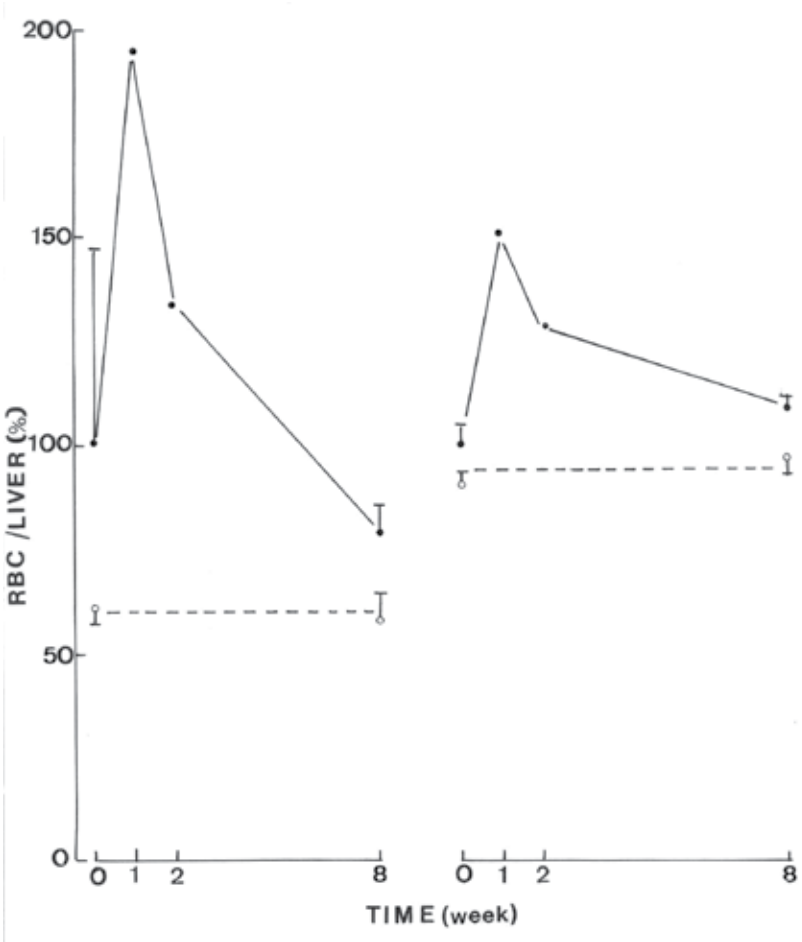

Fig. 3. Time course for the changes in the RBC/liver ratio for the C22:5ω3 (left) and C22:6ω3 (right) relative weight content of phospholipids in control animals (open circles and dashed line) and ω3D rats (closed circles and solid line). Single measurements made in pooled samples from 4 rats (week 1 and 2) or mean values (± SEM; n = 4) are expressed relative to the mean value found in the ω3D rats before the switch in diet.

Likewise, the liver/RBC ratio for the C22:6ω3 content of phospholipids transiently increased in the ω3D rats exposed to the soya oil diet before resuming a mean value of 300.0 ± 6.2% (n = 4), still somewhat higher ($p < 0.003$) than that recorded in the control animals over the same period of 8 weeks (257.9 ± 7.9%; n = 8). Incidentally, Fig. 3 also documents that, in the control animals, the liver/RBC ratio for either the C22:5ω3 or C22:6ω3 content of phospholipids was not significantly different ($p > 0.27$ or more) at the onset and end of the last 8 weeks period in the present experiments, as also observed ($p > 0.7$) for the RBC/liver ratio for the C20:5ω3 content of phospholipids in the control animals (see above).

3.4.2 Long-chain polyunsaturated ω6 fatty acids

A mirror image of that recorded for C22:5ω3 or C22:6ω3 was observed concerning the liver/RBC ratio for the relative weight content of C22:4ω6. Thus, such a ratio, which was significantly higher ($p < 0.001$) in the ω3D rats (33.9 ± 1.1%; n = 4) than in the control rats (21.7 ± 1.3%; n = 4) both examined at the end of the 3 months initial period, first decreased ($p < 0.025$) in the ω3D rats to 22.0 ± 0.1% (n = 2) one to two weeks after the switch in diet and then increased again ($p < 0.02$) to 27.0 ± 1.4% (n = 4) 8 weeks after the switch in diet, reaching a value virtually identical ($p > 0.49$) to that recorded at the same time in the control animals (28.5 ± 1.5%; n = 4). In the case of C22:5ω6, the liver/RBC ratio was again significantly higher ($p < 0.001$) in the ω3D rats (230.2 ± 9.9%; n = 4) than in the control animals (95.5 ± 4.4%; n = 4) both examined at the end of the 3 months initial period. It decreased ($p < 0.005$) in the ω3D rats to 124.5 ± 9.3% (n = 2) one to two weeks after the switch in diet, such a value being comparable to that reached 8 weeks after the switch in diet (125.6 ± 14.3%; n = 4) in the ω3D rats. The latter two mean values were no more significantly different ($p > 0.25$) than that recorded in the control animals at the end of the present experiments (149.2 ± 13.3%; n = 4) and virtually identical to that prevailing in the same animals over the last 8 weeks of such experiments (122.4 ± 12.0%; n = 8).

Rats		C18:2ω6	C18:3ω6	C20:2ω6	C20:3ω6	C20:4ω6	C22:4ω6	C22:5ω6
Control	before	80.09 ± 4.08	0.17 ± 0.17	0.00 ± 0.00	4.44 ± 0.20	330.00 ± 6.00	13.35 ± 0.79	5.48 ± 0.31
	after (8 wk)	72.40 ± 4.76	0.79 ± 0.28	0.38 ± 0.38	5.78 ± 0.55	329.36 ± 3.91	14.06 ± 0.44	6.23 ± 0.44
ω3D	before	78.13 ± 0.51	0.22 ± 0.22	0.00 ± 0.00	3.69 ± 0.22	341.09 ± 10.40	25.21 ± 0.43	31.37 ± 3.06
	after (1 wk)	69.30 (1)	0.00 (1)	0.00 (1)	4.45 (1)	347.89 (1)	24.37 (1)	27.82 (1)
	after (2 wk)	78.11 (1)	0.00 (1)	1.86 (1)	4.23 (1)	350.33 (1)	21.10 (1)	20.50 (1)
	after (8 wk)	75.82 ± 4.75	0.00 ± 0.00	0.96 ± 0.55	5.42 ± 0.48	335.95 ± 2.91	16.57 ± 0.14	11.21 ± 1.73

Table 10. Relative weight content (‰) of long-chain polyunsaturated ω6 fatty acids in RBC phospholipids

3.4.3 Saturated fatty acids

Significant differences were also on occasion observed when comparing the relative weight content of selected saturated fatty acids in the RBC phospholipids of control and ω3D rats (Table 11). For instance, the content of C18:0 averaged 178.46 ± 2.09‰ (n = 8) in control animals, as distinct ($p < 0.003$) from 188.11 ± 1.64‰ (n = 10) in ω3D rats. Inversely, the C24:0 content of RBC phospholipids was higher ($p < 0.025$) in the control animals (17.05 ± 0.87‰; n = 8) than in the ω3D rats examined before the switch in diet (13.46 ± 0.52‰; n = 4), reascending ($p < 0.001$) to 18.50 ± 0.68‰ (n = 6) when the latter ω3D rats were switched to the soya oil diet.

Rats		C14:0	C16:0	C18:0	C20:0	C22:0	C24:0
Control	before	1.36 ± 0.04	212.94 ± 3.33	179.33 ± 2.25	1.15 ± 0.04	6.38 ± 0.26	15.03 ± 0.77
	after (8 wk)	1.51 ± 0.07	217.35 ± 2.26	177.60 ± 3.84	1.00 ± 0.34	7.50 ± 0.32	19.08 ± 0.46
ω3D	before	1.50 ± 0.06	219.93 ± 8.24	189.75 ± 3.12	1.23 ± 0.08	6.52 ± 0.33	13.46 ± 0.52
	after (1 wk)	1.44 (1)	218.9 (1)	187.6 (1)	1.42 (1)	7.52 (1)	16.10 (1)
	after (2 wk)	1.44 (1)	205.6 (1)	184.9 (1)	1.33 (1)	7.77 (1)	17.84 (1)
	after (8 wk)	1.46 ± 0.08	210.37 ± 3.67	187.4 ± 2.93	1.31 ± 0.10	8.16 ± 0.54	19.27 ± 0.66

Table 11. Relative weight content (‰) of saturated fatty acids in RBC phospholipids

3.4.4 Monodesaturated fatty acids

The C16:1ω7 relative weight content of RBC phospholipids averaged 2.90 ± 0.15‰ (n = 8) in the control animals, and 2.58 ± 0.41‰ (n = 4) and 2.96 ± 0.21‰ (n = 6) in the ω3D rats examined, respectively, before and after the switch in diet. The corresponding values for the C18:1ω9 content of RBC phospholipids averaged 53.08 ± 1.46‰ (n = 8), 52.91 ± 2.47‰ (n = 4) and 54.47 ± 1.56‰ (n = 6). No C20:1ω9 was detected in any of the 18 samples concerned by this study. Sizeable amounts of C22:1ω9 (8.14 ± 0.20‰; n = 4) were only detected in the samples prepared from the ω3D rats examined before the switch in diet.

3.5 Fatty acid pattern of brain phospholipids

3.5.1 Long-chain polyunsaturated ω3D fatty acids

No C18:3ω3, C18:4ω3 or C20:5ω3 was detected in any of the 20 samples examined for the fatty acid pattern of brain phospholipids. The C22:5ω3 and C22:6ω3 relative weight content of brain phospholipids were significantly lower in ω3D rats than in control animals both examined at the end of the 3 months initial period and, in the former ω3D rats, slowly returned towards control values after the switch in diet (Table 12).

Rats		C22:5ω3	C22:6ω3
Control	before	2.29 ± 0.04	235.93 ± 6.60
	after (8 wk)	2.30 ± 0.20	213.34 ± 7.43
ω3D	before	0.00 ± 0.00	186.65 ± 5.01
	after (1 wk)	0.27 ± 0.27	193.19 ± 3.72
	after (8 wk)	1.95 ± 0.14	209.04 ± 4.89

Table 12. Relative weight content (‰) of long-chain polyunsaturated ω3 fatty acids in brain phospholipids

3.5.2 Long-chain polyunsaturated ω6 fatty acids

The C18:2ω6 relative weight content of brain phospholipids was higher ($p < 0.02$) in the control animals (7.30 ± 0.24%; n = 8) than in the ω3D rats examined before and one week after the switch in diet (6.27 ± 0.29‰; n = 8), resuming in the ω3D rats exposed for 8 weeks to the soya oil diet a mean value (7.55 ± 0.14‰; n = 4) no more significantly different ($p > 0.5$) from that recorded in the control animals (Table 13). A comparable situation prevailed

Rats		C18:2ω6	C20:2ω6	C20:3ω6	C20:4ω6	C22:4ω6	C22:5ω6
Control	before	7.16 ± 0.39	1.31 ± 0.06	2.48 ± 0.09	131.66 ± 6.04	26.85 ± 1.70	3.12 ± 0.23
	after (8 wk)	7.45 ± 0.31	1.56 ± 0.13	2.99 ± 0.17	116.71 ± 8.21	27.23 ± 0.93	3.08 ± 0.37
ω3D	before	6.27 ± 0.58	1.34 ± 0.14	2.07 ± 0.21	137.94 ± 7.23	30.21 ± 1.63	22.94 ± 1.46
	after (1 wk)	6.27 ± 0.20	1.48 ± 0.07	2.30 ± 0.19	125.04 ± 3.90	28.88 ± 1.40	19.00 ± 1.41
	after (8 wk)	7.55 ± 0.14	1.48/1.58	2.55 ± 0.15	122.74 ± 3.38	26.49 ± 2.79	8.30 ± 0.54

Table 13. Relative weight content (‰) of long-chain polyunsaturated ω6 fatty acids in brain phospholipids

in the case of C20:3ω6, with a lower value ($p < 0.025$) in ω3D rats (2.31 ± 0.11‰; n = 12) than in control animals (2.74 ± 0.13‰; n = 18). Except in one control animal with a C18:3ω6 relative content of 0.79‰ in brain phospholipids, no sizeable amount of this fatty acid could be detected in any of the other 19 samples. In the case of both C22:4ω6 and C22:5ω6, the mean value recorded at the end of the initial 3 months period was higher in ω3D rats than in

control animals, a progressive decrease being recorded in the former ω3D rats after the switch in diet. Such changes were most obvious in the case of C22:5ω6.

3.5.3 Saturated fatty acids

As a rule, the relative weight content of saturated fatty acids in the brain phospholipids of ω3D rats were comparable to the overall mean values found in control animals (Table 14). In the case of C14:0 and C16:0, however, the individual values collected in ω3D rats differed significantly ($p < 0.004$) from those recorded in control animals, averaging 114.0 ± 3.1% (n = 24) of the mean corresponding measurements made in the latter control animals (100.0 ± 2.5%; n = 16).

Rats		C14:0	C16:0	C18:0	C20:0	C22:0	C24:0
Control	before	1.03 ± 0.06	228.96 ± 6.45	184.97 ± 2.01	3.06 ± 0.14	2.90 ± 0.18	6.90 ± 0.58
	after (8 wk)	0.92 ± 0.04	194.74 ± 5.20	193.39 ± 2.13	5.31 ± 0.91	5.50 ± 0.79	12.95 ± 1.42
ω3D	before	1.11 ± 0.07	232.34 ± 6.19	188.85 ± 2.19	3.44 ± 0.38	3.54 ± 0.80	8.65 ± 1.62
	after (1 wk)	1.15 ± 0.03	231.48 ± 8.04	182.90 ± 2.40	4.32 ± 0.44	4.41 ± 0.46	10.13 ± 0.98
	after (8 wk)	1.21 ± 0.13	231.30 ± 22.37	186.66 ± 6.29	3.89 ± 0.40	4.09 ± 0.76	9.08 ± 1.84

Table 14. Relative weight content (‰) of saturated fatty acids in brain phospholipids

3.5.4 Monodesaturated fatty acids

In the same manner as noticed in the case of most saturated fatty acids, the relative weight content of monodesaturated fatty acids in brain phospholipids displayed in the ω3D rats values not vastly different from the overall mean values recorded in control animals (Table 15). Thus, relative to the latter overall mean values for each monodesatuarted fatty acids found in control animals (100.0 ± 12.9%; n = 32), those recorded in the ω3D rats averaged 100.0 ± 7.1%; n = 48).

Rats		C16:1ω7	C18:1ω9	C20:1ω9	C22:1ω9
Control	before	3.20 ± 0.16	149.78 ± 3.34	8.39 ± 1.01	0.00 ± 0.00
	after (9 wk)	3.36 ± 0.13	187.77 ± 13.66	16.71 ± 2.21	2.73 ± 1.04
ω3D	before	3.05 ± 0.24	161.33 ± 13.22	9.70 ± 2.38	0.58 ± 0.58
	after (1 wk)	3.12 ± 0.08	171.48 ± 6.09	12.37 ± 2.34	2.19 ± 0.32
	after (8 wk)	3.34 ± 0.15	168.53 ± 7.92	10.40 ± 1.32	2.06 ± 0.71

Table 15. Relative weight content (‰) of monodesaturated fatty acids in brain phospholipids

3.5.5 Comparison of brain, RBC and liver data

As already observed, in the case of RBC phospholipids, the changes in the relative weight content of selected fatty acids in brain phospholipids, as recorded in the ω3D rats after the switch from the sunflower oil diet to the soya oil diet, occurred less rapidly than those found in liver phospholipids. In such a respect, there was no significant difference ($p > 0.6$ or more) when comparing the data collected in either the RBC or brain phospholipids. For instance, in the case of C22:5ω3 and C22:6ω3, the increment found in the ω3D rats examined one week after the switch in diet, above the mean value recorded before such a switch, only represented, in brain and RBC respectively, 21.6 ± 10.5% (n = 8) and 26.0 ± 3.8% (n = 2) of the corresponding mean increment found 8 weeks after the switch in diet. The latter

percentages yielded an overall mean value of 22.5 ± 8.3% (n = 10), significantly lower ($p < 0.004$) than that recorded under the same experimental conditions in liver, i.e. 67.5 ± 9.3% (n = 8). Likewise, in the case of C22:4ω6 and C22:5ω6, the decrement recorded in the ω3D rats one week after the switch in diet, below the mean corresponding reference values found before such a switch, yielded in the brain and RBC, when expressed relative to the corresponding mean decrement observed 8 weeks after the switch in diet, an overall mean value of 27.8 ± 14.4% (n = 10), significantly lower ($p < 0.03$) than that recorded under the same experimental conditions in liver, i.e. 69.2 ± 5.4% (n = 8). The latter two mean values relative to two long-chain polyunsaturated ω6 fatty acids were virtually identical ($p > 0.75$ or more) to the corresponding mean values found in the case of the two long-chain polyunsaturated ω3 fatty acids under consideration (see above), yielding overall mean percentages of 25.1 ± 8.1% (n = 20) in brain and RBC, as compared ($p < 0.001$) to 68.4 ± 5.2% (n = 16) in liver.

3.6 Fatty acid pattern in liver triglycerides

The total fatty acid content of liver triglycerides did not differ significantly ($p > 0.64$) in the control animals and ω3D rats, with an overall mean values of 18.3 ± 2.5 mg/g wet wt. (n = 24).

3.6.1 Long-chain polyunsaturated ω3 fatty acids

The relative weight content of C18:3ω3, C20:5ω3, C22:5ω3 and C22:6ω3 in liver triglycerides was significantly lower ($p < 0.02$ or less) in the ω3D rats than in the control animals, both examined at the end of the 3 months initial period (Table 16). In the control animals, no significant difference was observed between the former results and those recorded 8 weeks later. In the ω3D rats examined after the switch in diet, C20:5ω3 remained below the limit of detection in all, except one, rats. The C18:3ω3, C22:5ω3 and C22:6ω3 contents increased, however, in the ω3D rats within one week after the switch in diet, remaining then at a plateau level somewhat lower than the mean value otherwise found in control rats. Thus, relative to such a reference value (100.0 ± 7.9%; n = 24), the measurements made in the ω3D rats examined after the switch in diet averaged 66.1 ± 5.3% (n = 36; $p < 0.001$).

Rats		C18:3ω3	C20:5ω3	C22:5ω3	C22:6ω3
Control	before	12.82 ± 2.96	2.01 ± 0.27	3.41 ± 0.56	7.04 ± 1.08
	after (8 wk)	11.94 ± 3.03	1.61 ± 0.51	3.06 ± 0.90	6.45 ± 1.20
ω3D	before	1.81 ± 0.69	0.00 ± 0.00	0.00 ± 0.00	0.48 ± 0.48
	after (1 wk)	8.86 ± 2.26	0.00 ± 0.00	1.81 ± 0.83	3.66 ± 1.48
	after (2 wk)	10.23 ± 0.56	0.00 ± 0.00	2.07 ± 0.19	4.55 ± 0.47
	after (8 wk)	8.84 ± 1.99	0.12 ± 0.12	1.81 ± 0.55	4.80 ± 1.70

Table 16. Relative weight content (‰) in long-chain polyunsaturated ω3 fatty acids in liver triglycerides

3.6.2 Long-chain polyunsaturated ω6 fatty acids

The C18:2ω6 relative weight content of liver triglycerides was virtually identical in control animals and ω3D rats, whether at the onset or end of the last 8 weeks period of the present experiments (Table 17). Likewise, no significant difference was found between the mean values found in the six groups of rats when considering the relative weight content of C18:3ω6, C20:2ω6 and C20:3ω6 in the liver triglycerides. In the case of C20:4ω6, C22:4ω6 and C22:5ω6,

no significant difference ($p > 0.3$ or more) was observed between the control animals examined either at the end of the initial period or 8 weeks thereafter. In the ω3D rats, the C20:4ω6 content progressively decreased as a function of the length of exposure to the soya oil diet ($r = - 0.5059$; $n = 16$; $p < 0.05$), eventually reaching a mean value comparable ($p > 0.9$) to that recorded at the same time in the control animals. A comparable situation prevailed in the case of C22:4ω6 and C22:5ω6. Thus, for the last three fatty acids (C20:4ω6, C22:4ω6 and C22:4ω6), the increment in the relative weight content found in ω3D rats above the mean corresponding value ($n = 8$ in each case) recorded in the control animals, when expressed relative to the initial increment found before the switch in diet, decreased ($p < 0.02$) from 100.0 ± 17.5% ($n = 12$) to 49.6 ± 11.3% ($n = 24$) one to two weeks after the switch in diet and further decreased ($p < 0.025$) to 3.6 ± 15.6% ($n = 12$) eight weeks after the switch in diet.

Rats		C18:2ω6	C18:3ω6	C20:2ω6	C20:3ω6	C20:4ω6	C22:4ω6	C22:5ω6
Control	before	235.21 ± 34.54	5.35 ± 0.38	1.78 ± 0.09	1.65 ± 0.22	20.14 ± 2.57	2.61 ± 0.63	0.73 ± 0.45
	after (8 wk)	174.06 ± 38.86	4.44 ± 0.88	1.80 ± 0.17	1.78 ± 0.49	15.22 ± 4.02	2.92 ± 0.59	0.72 ± 0.27
ω3D	before	237.23 ± 42.90	6.17 ± 0.88	1.75 ± 0.65	1.92 ± 0.83	33.92 ± 6.30	6.15 ± 1.30	4.62 ± 0.78
	after (1 wk)	194.60 ± 39.29	5.01 ± 0.45	1.94 ± 0.34	1.65 ± 0.22	23.07 ± 6.69	4.49 ± 1.47	3.17 ± 1.08
	after (2 wk)	218.50 ± 30.62	4.73 ± 0.30	1.73 ± 0.60	2.24 ± 0.50	24.75 ± 3.55	4.93 ± 0.86	2.42 ± 0.55
	after (8 wk)	173.01 ± 45.75	4.25 ± 0.66	1.76 ± 0.27	1.79 ± 0.63	16.08 ± 5.93	3.29 ± 1.08	0.93 ± 0.55

Tale 17. Relative weight content (‰) of long-chain polyunsaturated ω6 fatty acids in liver triglycerides

3.6.3 Saturated fatty acids

The C12:0 relative weight content of liver triglycerides averaged 0.72 ± 0.16‰ ($n = 8$) and 0.35 ± 0.12‰ ($n = 16$) in the control animals and ω3D rats, respectively, these two mean values failing to differ significantly ($p < 0.09$) from one another. Likewise, the C14:0, C16:0 and C18:0 contents of liver triglycerides were essentially comparable in the six groups of rats considered in Table 18. A significant amount of C20:0 (0.37 ± 0.14‰; $n = 8$; $p < 0.05$) was detected in the control animals, whilst such was not the case (0.02 ± 0.02‰; $n = 16$; $p > 0.3$) in the ω3D rats. Inversely, a significant amount of C22:0 (0.52 ± 0.17‰; $n = 12$; $p < 0.02$) was detected in the ω3D rats examined before the switch in diet and one to two weeks thereafter, whilst such was no more the case 8 weeks after the switch in diet, null values being also recorded in the 8 control animals. No C24:0 was detected in any of the 24 samples examined in this study.

Rats		C12:0	C14:0	C16:0	C18:0	C20:0	C22:0	C24:0
Control	before	0.77 ± 0.26	11.54 ± 1.47	291.91 ± 14.36	21.83 ± 2.40	0.41 ± 0.23	0.00 ± 0.00	0.00 ± 0.00
	after (8 wk)	0.70 ± 0.24	14.22 ± 2.13	345.81 ± 27.78	25.09 ± 3.20	0.33 ± 0.21	0.00 ± 0.00	0.00 ± 0.00
ω3D	before	0.21 ± 0.21	11.92 ± 1.75	307.56 ± 20.99	29.56 ± 3.48	0.00 ± 0.00	0.66 ± 0.38	0.00 ± 0.00
	after (1 wk)	0.73 ± 0.24	13.54 ± 1.17	349.41 ± 34.67	27.83 ± 2.86	0.00 ± 0.00	0.34 ± 0.07	0.00 ± 0.00
	after (2 wk)	0.00 ± 0.00	13.54 ± 1.59	304.80 ± 16.14	24.35 ± 1.64	0.00 ± 0.00	0.56 ± 0.41	0.00 ± 0.00
	after (8 wk)	0.45 ± 0.27	15.13 ± 1.74	344.48 ± 35.98	28.62 ± 2.99	0.08 ± 0.08	0.00 ± 0.00	0.00 ± 0.00

Table 18. Relative weight content (‰) of saturated fatty acids in liver triglycerides

3.6.4 Monodesaturated fatty acids

No significant difference in the relative weight content of either C16:1ω7 or C18:1ω9 in liver triglycerides was observed between the six groups of rats listed in Table 19. Except in one

instance, such was also the case for the C20:1ω9 content of liver triglycerides. No C22:1ω9 was detected in any of the 24 samples under consideration. The sole statistically significant finding consisted in the positive correlation ($r = + 0.9958$; $n = 4$; $p < 0.01$) prevailing between the mean values for the C16:1ω7 and C18:1ω9 relative content recorded in the ω3D rats (all expressed relative to the mean corresponding value found in the eight control animals) and the length of exposure to the soya oil diet (semi-logarithmic analysis). Indeed, relative to such a reference value (100.0 ± 4.9%; n = 16), the measurements made in the ω3D rats averaged 85.7 ± 6.9% (n = 8) before the switch in diet and 91.9 ± 5.7% (n = 8), 93.9 ± 6.6% (n = 8) and 101.5 ± 6.7% (n = 8), respectively, one, two and eight weeks thereafter.

Rats		C16:1ω7	C18:1ω9	C20:1ω9	C22:1ω9
Control	before	54.15 ± 9.90	325.12 ± 16.65	1.30 ± 0.13	0.00 ± 0.00
	after (8 wk)	65.55 ± 5.93	322.72 ± 13.35	1.28 ± 0.15	0.00 ± 0.00
ω3D	before	45.40 ± 6.55	309.22 ± 20.00	1.27 ± 0.49	0.00 ± 0.00
	after (1 wk)	53.92 ± 6.96	303.93 ± 11.47	1.37 ± 0.49	0.00 ± 0.00
	after (2 wk)	51.78 ± 7.12	327.78 ± 16.36	1.42 ± 0.49	0.00 ± 0.00
	after (8 wk)	59.68 ± 8.20	334.84 ± 14.10	0.41 ± 0.25	0.00 ± 0.00

Table 19. Relative weight content (‰) of monodesaturated fatty acids in liver triglycerides

3.7 Fatty acid profile of visceral adipose tissue lipids

As indicated in Table 20, no significant difference was observed between control animals and ω3D rats in terms of body weight and visceral or parametrial adipose tissue weight, whether at the end of the 3 months initial period or 8 weeks thereafter.

Rats		Body wt (g)	Visceral adipose tissue (g)	Parametrial adipose tissue (g)
Control	before	360.5 ± 17.6	9.5 ± 1.4	16.0 ± 3.4
	after (8 wk)	463.0 ± 18.8	16.5 ± 2.1	23.0 ± 3.2
ω3D	before	380.5 ± 21.8	13.5 ± 1.6	14.1 ± 4.0
	after (8 wk)	445.8 ± 49.1	12.3 ± 1.8	19.1 ± 1.1

Table 20. Body weight and visceral or parametrial adipose tissue weight

The total fatty acid content of visceral adipose tissue lipids was not significantly different ($p > 0.5$) in control animals (1.19 ± 0.03 mg/mg wet wt.; n = 8) and ω3D rats (1.21 ± 0.03 mg/mg wet wt.; n = 8).

3.7.1 Long-chain polyunsaturated ω3 fatty acids

No C18:4ω3 or C20:5ω3 was detected in any of the 16 samples of adipose tissue.

The C18:3ω3 relative weight content averaged 20.36 ± 0.83 and 18.39 ± 0.62‰ (n = 4 in both cases) in the control animals examined at the end of the 3 months initial period and 8 weeks thereafter. In the ω3D rats, it increased ($p < 0.001$) from 0.00 ± 0.00 to 11.67 ± 0.92‰ (n = 4 in both cases) before and 8 weeks after the switch in diet. It thus remained lower ($p < 0.001$) in the ω3D rats than in the control animals even at the end of the experiments.

The C22:5ω3 content of adipose tissue was comparable at the end of the 3 months initial period and 8 weeks thereafter, with overall mean values of 1.21 ± 0.11‰ (n = 8) in control

animals and 0.00 ± 0.00‰ (n = 8) in ω3D rats. Likewise, no C22:6ω3 was detected in any of the eight ω3D rats, as distinct ($p < 0.05$) from an overall mean value of 1.14 ± 0.44‰ in the eight control animals (Table 21).

Rats		C18:3ω3	C20:5ω3	C22:5ω3	C22:6ω3
Control	before	20.36 ± 0.83	0.00 ± 0.00	1.11 ± 0.19	0.00 ± 0.00
	after (8 wk)	18.39 ± 0.62	0.00 ± 0.00	1.32 ± 0.12	2.29 ± 0.21
ω3D	before	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	after (8 wk)	11.67 ± 0.92	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 21. Relative weight content (‰) of long-chain polyunsaturated ω3 fatty acids in visceral adipose tissue lipids

3.7.2 Long-chain polyunsaturated ω6 fatty acids

The C18:2ω6 relative weight content of adipose tissue lipids was comparable ($p > 0.45$ or more) in control animals and ω3D rats, whether at the onset or end of the last 8 weeks period (Table 22).

Rats		C18:2ω6	C20:2ω6	C20:3ω6	C20:4ω6	C22:4ω6	C22:5ω6
Control	before	303.06 ± 13.00	2.52 ± 0.06	0.00 ± 0.00	6.72 ± 1.13	1.28 ± 0.29	0.00 ± 0.00
	after (8 wk)	276.14 ± 3.41	2.57 ± 0.05	0.36 ± 0.36	6.52 ± 0.48	1.50 ± 0.17	0.14 ± 0.14
ω3D	before	324.61 ± 23.71	2.67 ± 0.10	0.00 ± 0.00	8.21 ± 0.93	2.09 ± 0.25	1.45 ± 0.21
	after (8 wk)	288.77 ± 19.45	2.46 ± 0.04	0.36 ± 0.36	6.94 ± 0.35	1.56 ± 0.11	0.63 ± 0.25

Table 22. Relative weight content (‰) of long-chain polyunsaturated ω6 fatty acids in visceral adipose tissue lipids

No C18:3ω6 was detected in any of the 16 samples under consideration. The C20:2ω6 content was comparable in the 4 groups of rats. A sizeable amount of C20:3ω6 (1.46 ± 0.00; n = 2) was only detected in two out of 16 samples. The C20:4ω6 content was not significantly higher ($p > 0.1$) in the ω3D rats examined before the switch in diet (8.21 ± 0.93‰; n = 4) than in the other rats (6.73 ± 0.39‰; n = 12). The C22:4ω6 content was significantly higher ($p < 0.02$), however, in the ω3D rats examined before the switch in diet (2.09 ± 0.25‰; n = 4) than in the other animals (1.45 ± 0.11‰; n = 12). Likewise, the C22:5ω6 content found in the ω3D rats examined before the switch in diet (1.45 ± 0.21‰; n = 4) exceeded ($p < 0.05$ or less) that found in either the control animals (0.07 ± 0.07‰; n = 8) or the ω3D rats examined 8 weeks after the switch in diet (0.63 ± 0.25‰; n = 4).

3.7.3 Saturated fatty acids

The relative weight content of C12:0, C14:0, C16:0 and C18:0 were comparable to one another in the four groups of rats (Table 23). The sole significant difference ($p < 0.02$)

Rats		C12:0	C14:0	C16:0	C18:0
Control	before	0.72 ± 0.09	9.57 ± 0.93	253.51 ± 5.71	30.07 ± 1.20
	after (8 wk)	0.68 ± 0.04	9.52 ± 0.59	264.89 ± 2.80	30.26 ± 0.76
ω3D	before	0.77 ± 0.07	10.57 ± 1.13	253.17 ± 11.65	34.09 ± 1.78
	after (8 wk)	0.74 ± 0.05	10.48 ± 0.59	261.90 ± 7.80	29.79 ± 1.61

Table 23. Relative weight content (‰) of saturated fatty acids in visceral adipose tissue lipids

concerned the comparison between the C18:0 content of the ω3D rats examined before the switch in diet (34.1 ± 1.8‰; n = 4) and the other animals (30.0 ± 0.7‰; n = 12).
No C20:0 or C24:0 was detected in any of the 16 samples of adipose tissue. A sizeable amount of C22:0 (0.55 ± 0.04‰; n = 2) was only found in two out of four ω3D rats examined before the switch in diet.

3.7.4 Monodesaturated fatty acids

The mean values for the relative weight content of C16:1ω7, C18:1ω9 or C20:1ω9 did not differ significantly from one another in the four groups of rats under consideration (Table 24). No C22:1ω9 could be detected in any of the 16 samples of visceral adipose tissue.

Rats		C16:1ω7	C18:1ω9	C20:1ω9
Control	before	39.95 ± 3.73	328.06 ± 7.87	1.83 ± 0.10
	after (8 wk)	40.90 ± 3.13	341.94 ± 3.67	1.77 ± 0.05
ω3D	before	38.02 ± 7.35	321.17 ± 9.80	1.75 ± 0.16
	after (8 wk)	40.07 ± 3.25	342.42 ± 13.01	1.68 ± 0.03

Table 24. Relative weight content (‰) of monodesaturated fatty acids in visceral adipose tissue lipids

3.8 Food intake and body weight

As indicated in Table 25, over the last 7-8 weeks of the present experiments, both the food intake and relative gain in body weight were higher ($p < 0.05$ or less) in the ω3D rats maintained on the sunflower oil diet than in either the control rats maintained on the soya oil diet or the ω3D rats switched to the latter diet. It could be objected that the relative magnitude of the changes in body weight exceeded that of the changes in food intake. However, taking into account the differences in both body weight gain and food intake, the estimated caloric efficiency was close to the expected theoretical value, as documented by the following considerations. In one group of 4 ω3D rats maintained for a further period of 7 weeks on the sunflower diet, the daily food intake (19.61 ± 0.27 g/day per rat; n = 30) exceeded by 1.93 ± 0.51 g/day per rat ($p < 0.001$) that recorded in one group of 4 control animals maintained on the soya oil diet (17.68 ± 0.43 g/day per rat; n = 30). In these two groups of rats, the gain in body weight over the same period of 7 weeks averaged 1.93 ± 0.14 g/day per rat in the ω3D rats as distinct ($p < 0.02$) from 1.09 ± 0.11 g/day per rat in the control animals. The caloric requirement to account for the difference in body weight gain (≤ 7.6 ± 1.6 cal) was thus commensurate with the increase in the caloric supply of the diet (6.2 ± 1.6 cal), which provided about 3.2 cal/g.

Rats		Food intake[a]	Relative gain in body weight[b]
Control	soya oil diet	100.0 ± 1.6 (31)	13.35 ± 1.38 (4)
ω3D	sunflower oil diet	112.1 ± 2.5 (56)	24.67 ± 1.82 (2)
ω3D	soya oil diet	104.4 ± 1.6 (45)	12.97 ± 2.70 (4)

[a]The food intake over the last 8 weeks of the present experiments is expressed relative to the mean value found at the same time after the initial 3 months period in control animals maintained on the soya oil diet
[b]The relative gain in body weight (%) was assessed by comparison of individual measurements made at the end of the initial 3 months period and seven weeks thereafter

Table 25. Food intake and relative gain in body weight

3.9 HOMA index for insulin resistance

At the end of the 3 months initial period, the logarithmic values of the HOMA index for insulin resistance were higher ($df = 4$; $p < 0.02$) in the ω3D rats than in the control animals. In the former ω3D rats, such values progressively decreased after the switch in diet as a function of the length of exposure to the soya oil diet ($r = - 0.5956$; $n = 12$; $p < 0.05$). Eight weeks after the switch in diet, the HOMA index eventually reached in the ω3D rats a mean value (1.16 ± 0.09 U/l.mM; $n = 3$) comparable ($p > 0.7$) to that found at the same time in the control animals also exposed to the soya oil diet (1.20 ± 0.05 U/l.mM; $n = 3$).

4. Discussion

The present experimental design prevents any undesirable aggravation of liver steatosis and visceral obesity, as well as food intake, otherwise resulting from an increase in the lipid content of the diet from 5 to 10% (Malaisse et al., 2009; Sener et al., 2009). It also caused correction of insulin resistance in the ω3D rats.

The exposure of ω3D to the soya oil diet also allowed the repletion of long-chain polyunsaturated ω3 fatty acids in the intestinal tract, liver, red blood cell and brain phospholipids.

The changes in the fatty acid pattern of tissue lipids were not always merely and directly attributable to the respective content of each diet in a given fatty acid, as documented by the accumulation of selected long-chain polyunsaturated ω6 fatty acids in the phospholipids of ω3D rats and the correction of this situation after the switch from the sunflower oil to soya oil diet. Incidentally, before such a switch the total amount of four ω3 fatty acids (C18:3ω3, C20:5ω3, C22:5ω3 and C22:6ω3) and three ω6 fatty acids (C20:4ω6, C22:4ω6 and C22:5ω6) was comparable in the control animals and ω3D rats (Carpentier et al., 2011b).

The present findings also support the view that a transfer of information takes place between the liver and either RBC or brain, as documented for instance by the delayed alignment of the phospholipid fatty acid pattern in the latter two sites on that prevailing at the hepatic level. It was recently proposed that such a process may even be operative in the absence of any dietary deprivation of long-chain polyunsaturated ω3 fatty acids (Malaisse et al., 2010).

Last, the present findings provide further evidence to the fact that changes in the dietary supply of ω3 fatty acids may also affect the relative content of tissue lipids in selected saturated and monodesaturated fatty acids.

In conclusion, the procedure used in the present experiments appears indeed suitable to ensure the repletion of long-chain polyunsaturated ω3 fatty acids in previously ω3-depleted rats.

5. Acknowledgment

We are grateful to C. Demesmaeker for secretarial help.

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4

The Effect of Flavonoids in Soybean Products in Lymphocytes from IBD and Colon Cancer Patients After Treatment with Food Mutagens and Hydrogen Peroxide

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1. Introduction

The generation of DNA damage by environmental, medical or life style factors is considered to be an important initial event in carcinogenesis. At the cellular level, a balance between the production of oxidative radicals and the compensational action of antioxidants, which might become pro-oxidant at high concentrations (Anderson et al., 1994) is crucial for our health. Imbalance on either side, especially towards an increase in oxidative stress, might result in various detrimental effects including cell death and cancer. Despite various cellular mechanisms to counteract these adverse events the sheer number of potentially carcinogenic compounds leading to oxidative stress can negatively affect the DNA integrity of cells.

Dietary flavonoids acting as antioxidants (Rice-Evans, 2001) have been identified to be capable of counteracting these adverse oxidative effects (Ross and Kasum, 2002). They are classified as low-molecular-weight polyphenolic compounds that are ubiquitously present in fruit and vegetables and categorised according to their chemical structure into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Flavonoids such as quercetin and rutin present in soybean products have potent antioxidant properties and mimic oestrogens, hence are being used to ease menopausal symptoms. Soy flavonoids are also believed to lower the blood level of triglicerydes and cholesterol preventing coronary heart disease as well as osteoporosis (Valachovicova et al., 2004). They have a wide variety of biological effects acting either as anti- or pro-oxidants depending on their concentration (Anderson et al., 1997; Duthie et al., 1997) and/or in combination with food mutagens (Anderson et al., 1997). Anderson et al. observed positive responses with flavonoids when lymphocytes were treated with them alone in the Comet assay, and in combination with food mutagens they were showing exacerbating effects at low doses and were protective at high doses (Anderson et al., 1997). The antioxidant potency of several widespread dietary

flavonoids showed a dose-dependent reduction of induced oxidative DNA damage *in vitro*, highlighting an even higher protective effect than vitamin C (Noroozi et al., 1998). It has also been shown that flavonoid intake can lower the mortality rate caused by coronary heart disease (Kaur et al., 2007). Typical flavonoids are kaempferol, quercetin and rutin (the common glycoside of quercetin), belonging to the class of flavonols. The strongest evidence for a cancer-preventive effect shows quercetin with strong antioxidant properties (Chondrogianni et al., 2010; Hollman et al., 1996). It has been reported that the average intake of flavonoids is 23 mg per day with quercetin contributing almost 70% (Hollman et al., 1996; Wach et al., 2007). As it scavenges highly reactive species such as peroxynitrite and hydroxyl radicals (Boots et al., 2008), quercetin protects not only against various diseases such as atherosclerosis, cancer, osteoporosis, pulmonary and cardiovascular diseases but also against ageing (Chondrogianni et al., 2010; Ekstrom et al., 2010; Ossola et al., 2009; Terao, 2009; Zhou et al., 2010).

Lifestyle factors like alcohol intake, physical inactivity, stress, food additives, high animal fat and/or red meat intake and also cooking-derived carcinogens such as heterocyclic amines (HCA), have been identified as having a strong impact on human health and being involved in the aetiology of cancer in general (Adamson et al., 1996; Bogen, 1994). Evidence for a positive association of colorectal cancer and adenomatous polyps with HCA exposure has been provided by several studies (Butler et al., 2003; Felton et al., 2007; Gunter et al., 2005; Knize and Felton, 2005; Murtaugh et al., 2004; Navarro et al., 2004; Nowell et al., 2002; Shin et al., 2007; Wu et al., 2006). HCA are formed by cooking proteinaceous food, mainly seen as heat-induced non-enzymatic browning that involves creatinine, free amino acids and monosaccharides (Schut and Snyderwine, 1999). More than 20 carcinogenic/mutagenic HCA have been isolated so far (Nagao et al., 1997; Wakabayashi et al., 1992). Major subclasses of HCA found in the human diet comprise of aminoimidazoazaarenes (AIA), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Schut and Snyderwine, 1999). It has been estimated that the daily intake of HCA can reach 50 µg (Knize et al., 1995; Krul et al., 2000), depending on the type of meat, temperature and the method of cooking (Messner and Murkovic, 2004; Wu et al., 2001). The heterocyclic amine PhIP is considered to be the most abundant HCA responsible for inducing various types of tumours in rats (Felton et al., 2004).

More than 600 individual compounds and complex dietary mixtures have been studied for protective effects towards HCA (Schwab et al., 2000) and numerous articles have been published regarding mammalian enzymes involved in the bioactivation and detoxification of these compounds (Eisenbrand and Tang, 1993). The genotoxicity of HCA originates from their activation by a series of reactions involving cytochrome P450 when the parent compound is converted to an electrophilic derivative such as a nitrenium ion that covalently binds to DNA resulting in DNA adducts and subsequently in nucleotide alterations and chromosomal aberrations (Goldman and Shields, 2003; Hatch et al., 2001). IQ induces unscheduled DNA synthesis in liver cells and shows strong mutagenic properties in the *Salmonella typhimurium* test system, which contribute to its classification as a potent carcinogen (Maeda et al., 1999; Murata et al., 1999; Weisburger et al., 1986) Also in addition to the formation of DNA adducts, oxidative damage to the DNA itself plays a crucial role in the carcinogenic process of food mutagens (Maeda et al., 1999; Murata et al., 1999). Heterocyclic amines like IQ are able to generate free radicals in the presence of NADPH and cytochrome b5 reductase (Maeda et al., 1999).

Colorectal tissue is constantly exposed to different chemicals and free oxygen radicals formed during metabolic activation. High intracolonic levels of free radicals may form active carcinogens or mitogenic tumour promoters through the oxidation of procarcinogens, either by hydroxyl radicals in faecal water or by secondary peroxyl radicals (Babbs, 1990). Within an inflamed bowel, disproportionate amounts of reactive oxygen species (ROS) can be additionally produced (Loguercio et al., 1996; Simmonds and Rampton, 1993). Ulcerative Colitis and Crohn's disease are inflammatory disorders of the gastrointestinal tract, associated with increased risk for colorectal cancer (Soderlund et al., 2010), which are unevenly distributed within the populations throughout the world. Although the exact cause of inflammatory bowel disease (IBD) remains unknown, the epidemiology of IBD has provided an insight into the pathogenesis of the disease by examining geographic, ethnic and other IBD risk factors (genetic, environmental, etc.) as well as their natural history (Danese and Fiocchi, 2006). Interestingly, reactive oxygen species (Seegert et al., 2001) are produced in abnormally high levels in cells from IBD patients (Rezaie et al., 2007) leading to oxidative stress and thus to DNA damage due to an imbalance between innate and exogenous antioxidants and ROS (Hemnani and Parihar, 1998; Soffler, 2007). Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases (Davies, 1995). Oxidative stress arising from the pathophysiology of cancer, may even serve as a biomarker (Hopkins et al., 2010), when there is an imbalance between production of ROS and their removal by intrinsic antioxidants (catalase) and antioxidant micronutrients.

In the present study, we used the Comet assay, which evaluates direct DNA breaks and is a fast and reliable method to assess DNA integrity in virtually any cell type without the requirement for cell culture (Moller, 2006). Three groups of individuals served as blood donors: healthy volunteers, IBD patients as well as patients with histopathologically confirmed, untreated colon cancer. It is known that lymphocytes from colon cancer patients exhibit higher levels of DNA damage caused by the intrinsic oxidative stress arising from colorectal cancer (Hopkins et al., 2010) and that these lymphocytes may also serve as an early predictive marker of cancer risk (Vodicka et al., 2010). Separated lymphocytes from IBD patients and healthy individuals were treated with H_2O_2 co-treated with quercetin and IQ with epicatechin. Also lymphocytes from colon cancer patients and healthy individuals were treated with IQ and PhIP with and without the supplementation of the antioxidant flavonoids, quercetin and rutin to show that these three flavonoids are able to reliably protect cells against the damaging effects of reactive oxygen species, even in the context of diseases like IBD or colorectal cancer where levels of ROS are already highly increased. Non-physiological doses were used *in vitro* to study the genotoxicological responses where higher, yet non cytotoxic doses are used as a routine procedure.

2. Materials and methods

2.1 Chemicals

The chemicals for the Comet assay were purchased from the following suppliers: RPMI-1640 medium, agarose and low melting point agarose from Invitrogen, Ltd. (Paisley, U.K.); DMSO (dimethyl sulfoxide), ethidium bromide, Trypan blue, EDTA, Trizma base, Triton X-100, quercetin, epicatechin, rutin and hydrogen peroxide H_2O_2, from Sigma Chemical Company (Dorset, U.K.); sodium chloride and sodium hydroxide from BDH Laboratory

Supplies (Poole, England); Lymphoprep cell separation gel from Nycomed Pharma Axis Shield (Oslo, Norway); FCS (foetal calf serum) from Nalgene, Rochester (New York, USA). The food mutagens, IQ (2-amino-3-methyl-3h-imidazo[4,5-f]quinoline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), were obtained from Toronto Research Chemicals, Inc. (Downsview, Ontario, Canada).

2.2 Collection of samples

After informed consent, approximately 10 ml heparinised blood were taken by venepuncture from the IBD and colon cancer patients at the Department of Gastroenterology, Bradford Royal Infirmary (BRI) and St. Luke's Hospital, Bradford, UK. Healthy volunteers were recruited within the Division of Biomedical Sciences at the University of Bradford (West Yorkshire, UK). Ethical permission was obtained from both the BRI Local Ethics Committee (Reference no.: 04/Q1202/15) and the University of Bradford's Sub-Committee for Ethics in Research involving Human Subjects (Reference no.: 0405/8). Over a period of three years, a set of samples from healthy controls, IBD patients and colon cancer patients was obtained.

2.3 Questionnaire for patients and controls

A questionnaire was administered to each donor immediately after taking the blood sample. The completed questionnaire for the patient and control groups provided essential information about lifestyle, endogenous (gender, age) and exogenous factors (intake of medicines and alcohol, smoking habits and diet). There were two different studies performed: study I involving IBD patients and study II – colorectal cancer patients. Both studies had separate treatment regimes.

2.4 Lymphocyte separation for the Comet assay

The heparinised blood was diluted with 0.9% saline in a 50:50 proportion and 6 ml of this dilution was carefully layered on top of 3 ml of Lymphoprep in 15 ml conical tubes followed by centrifugation (20 minutes at 800 g) at room temperature. The buffy coat layer of lymphocytes (above the Lymphoprep layer) was then transferred to another tube pre-filled with 10 ml of saline and centrifuged (15 minutes at 500 g). The supernatant was removed without disturbing the pellet which was then resuspended in PBS or RPMI-1640 medium and used for the *in vitro* experiments. Some of the lymphocytes were frozen in liquid nitrogen for long-term storage. In this case the pellet was resuspended in FCS containing 10% DMSO, and lodged in liquid nitrogen vapour overnight, before final storage after complete insertion in the storage Dewar.

2.5 Cell viability

Cell viability at the concentrations chosen for each experiment was checked after treatment and before performing the Comet assay. Viability was determined by Trypan blue dye exclusion indicating intact cell membranes (Phillips, 1973). 10 µl of 0.05% Trypan blue was added to 10 µl of cell suspension and the percentage of cells excluding the dye was estimated using a Neubauer Improved haemocytometer (Pool-Zobel et al., 1992). Only concentrations with viability over 80% were accepted for use in the studies to avoid artefactual results from cytotoxicity (Henderson et al., 1998).

2.6 Treatment of lymphocytes

2.6.1 Treatment for IBD group of experiments

Isolated lymphocytes (approx. 10^6 cells per ml) from IBD patients (n=10) and healthy controls (n=10) were treated without metabolic activation for 30 minutes in RPMI at 37 °C either with different concentrations of quercetin (0, 100, 200, 250 μM) in the presence of hydrogen peroxide (50 μM) or with different concentrations of epicatechin (0, 25, 50, 100 μM) in the presence of IQ (50 μM). Lymphocytes from healthy individuals served as the control groups. After the treatment, the cells were pelleted (5 minutes at 900 g). For DNA damage studies, the cell suspension was mixed with the same volume of 1% low melting point agarose for the Comet assay.

2.6.2 Treatment for colorectal cancer group of experiments

Lymphocyte suspensions (100 μl, 10^6 cells per ml) from colon cancer patients (n=20) and healthy controls (n=20) were exposed to defined concentrations of food mutagens and/or flavonoids in the presence of RPMI in a total volume of 1 ml. The treatment was for 30 min at 37 °C. As lymphocytes showed little or no difference in response with metabolic activation, the Comet assay was performed in the absence of metabolic activation to avoid any confounding factors (Anderson, 1997, 1998). To investigate DNA damage, the following concentrations were used 10, 25, 50 and 75 μM for PhIP and 25, 75, 100 and 150 μM for IQ based on preliminary studies (data not shown). To investigate the modulatory effect of flavonoids, the highest concentrations of IQ and PhIP were used for simultaneous combination treatment with the flavonoids, quercetin and rutin, supplemented at concentrations of 100 (50 for rutin), 250 and 500 μM.

2.7 Alkaline comet assay

The slide preparation for the Comet assay and the assay itself was carried out as previously described (Tice et al., 2000). An aliquot of 100 μl of lymphocyte suspension was mixed with 100 μl of 1% low melting point agarose (in PBS, <40 °C warm) and 100 μl of this suspension were spread onto each of the two microscope glass slides pre-coated with 1% normal melting point agarose (in water, dried overnight). After cover-slips were applied, the slides were placed on an ice-cold tray. Once the agarose set, the cover-slips were removed and a final third layer of 0.5% low melting point agarose (in PBS) was added and allowed to solidify as well on ice for 5 min. For each concentration, two replicate slides were produced. For cell lysis, the slides were immersed laterally in a container with cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) and incubated at 4 °C overnight. Then, the slides were placed on the tray of an electrophoresis tank, filled with cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH <13) and incubated for 30 minutes at 4 °C in the dark to allow the unwinding of DNA and expression of alkali labile sites. Electrophoresis was conducted at the same temperature for 30 minutes at 0.75 V/cm. The current was adjusted to 300 mA by raising or lowering the buffer level. After electrophoresis, the slides were removed from the tank and soaked three times for 5 minutes each with neutralizing Tris buffer (400 mM, pH 7.4). Cellular DNA was stained with 60 μl of 20 μg/ml ethidium bromide and cover-slips applied. Slides were examined using a fluorescence microscope equipped with a charge couple device (CCD) monochrome camera and a computerised image analysis system, Komet 4.0 (Kinetic Imaging, Liverpool, UK) to measure the comet parameters. All slides were coded by an independent person ensuring that scoring took place completely randomized and in a "blind" manner (Faust et

al., 2004). For each replicate slide, 25 cells were scored (50 cells in total) for each individual in each group making 500-1000 observations per experimental point, allowing a more than adequate statistical power to detect effects (Hartmann et al., 2003).

2.8 Statistical analysis

Data were tested for normality prior to statistical analysis. Normal distributions were checked through the Kolmogorov-Smirnoff and Shapiro-Wilk's Test to assess whether parametric statistics could be used. Study I: Gaussian normality was violated for many of the scale variables as endorsed by the Kolmogorov-Smirnov test. Therefore, non-parametric test procedures were adopted wherever necessary, such as the Kruskal-Wallis (K-W) and the Mann-Whitney (M-W) tests for independent samples. When testing intra-subject differences in DNA damage, the Wilcoxon Signed Rank (WSR) test was applied. For the binary response variables, Fisher's Exact (FE) test was applied. Throughout the analyses, a significance level of 5% was used and unilateral alternative hypotheses preferred to bidirectional tests (wherever appropriate). Study II: differences in measured parameters between healthy and colon cancer subjects were assessed by the parametric One Way ANOVA Test, since data were normally distributed. The relationship between DNA damage and various parameters characterising colon cancer status and unexposed control was analyzed using Post-hoc analysis (Dunnett test). The mean of each set of data was used in the statistical analysis. A probability level at $p<0.05$ was regarded as statistically significant. Differences between two experimental groups were tested by the unpaired Student *t*-test when comparing confounding factors due to gender, diet, smoking and drinking habits. The SPSS package version 16 was used to compare patient and control groups at different doses of H_2O_2, food mutagens and flavonoids.

Statistical analyses were performed on mean values for each cluster group (healthy individuals, IBD and colon cancer patients) for each possible combination of chemicals. The experimental unit was the individual. The Comet data parameters used to measure DNA damage were Olive tail moment (OTM; arbitrary unit, the fraction of DNA in the tail multiplied by the tail length) and % tail DNA (the percentage of DNA in the tail) recommended to be the most reliable comet measurements with OTM being the most statistically significant (Kumaravel and Jha, 2006). Of these two parameters, OTM is one of the most commonly reported measures of DNA damage but is recommended to be provided together with % tail DNA (Tice et al., 2000). Together they clearly define the comets indicating a linear relationship to the DNA break frequency over a wide range of levels of damage and both can be applied for scientific purposes (Hartmann et al., 2003; Kumaravel et al., 2009).

3. Results

Mutagenic effects of food mutagens and H_2O_2 in lymphocytes of all groups, healthy individuals and IBD/cancer patients, were examined *in vitro* by comparing the untreated controls to the different treatment doses. Supplementing the treatment with various concentrations of the flavonoids, quercetin, epicatechin and rutin, the reduction of the genotoxic impact of chemicals was also evaluated. The different combined treatments of H_2O_2/food mutagens and flavonoids were then compared to a positive control being a non-

supplemented high dose of H_2O_2/food mutagens. Subsequently, an intergroup comparison between healthy individuals and IBD/cancer patients was carried out evaluating the baseline DNA damage as well as the difference in sensitivity of lymphocytes of all groups. Also, the contribution of confounding factors was evaluated for all experiments.

3.1 Study I: IBD patients

3.1.1 Patient versus control groups

As shown in Table 1 and Figure 1, there was a significant difference in baseline DNA damage before *in vitro* treatment in lymphocytes of IBD patients when comparing them with healthy individual controls ($p < 0.001$). The study groups as well as the control groups after treatment showed significant increases in DNA damage induced by H_2O_2 ($p < 0.001$) and IQ ($p < 0.001$). The induced damage caused by the *in vitro* treatment with H_2O_2 or IQ decreased significantly in both groups when co-treated with the flavonoids quercetin or epicatechin, respectively.
Flavonoid supplementation at the highest concentration (250 µM quercetin or 100 µM epicatechin) caused an overall significant reduction of the induced DNA damage within the patient group and the control groups. This resulted in a 48.6% ($p < 0.001$) reduction of H_2O_2 induced DNA damage and a 43% ($p < 0.001$) reduction of IQ induced DNA damage within the patient groups. For both control groups, reductions in DNA damage of 35.2% and 57.1%, respectively, were observed (both, $p < 0.001$) (Table 1 and Figure 1). As expected, the two different control groups showed similar baseline DNA damage (M-W, $p = 0.174$).

Different concentrations	H_2O_2 + Quercetin (group one)				Different concentrations	IQ + Epicatechin (group two)			
	Olive tail moment		% tail DNA			Olive tail moment		% tail DNA	
	Study group	Control group	Study group	Control group		Study group	Control group	Study group	Control group
A1	10.9 ± 2.1	4.1 ± 1.3	19.9 ± 1.5	9.2 ± 1.2	A2	17.5 ± 2.2	4.6 ± 2.4	26.8 ± 1.1	9.2 ± 0.8
B1	14.4 ± 3.2	12.8 ± 2.4	24.8 ± 0.2	18.5 ± 0.6	B2	22.2 ± 3.1	15.9 ± 1.7	30.8 ± 1.2	21.4 ± 0.4
C1	5.3 ± 1.8	5.7 ± 1.7	21.1 ± 1.9	9.0 ± 0.6	C2	15.2 ± 1.9	7.8 ± 2.2	23.1 ± 0.5	13.0 ± 0.8
D1	7.1 ± 1.4	6.9 ± 1.2	22.6 ± 1.4	11.8 ± 0.7	D2	16.5 ± 1.8	5.6 ± 2.7	25.6 ± 1.4	9.5 ± 2.1
E1	8.8 ± 3.1	6.2 ± 1.1	21.6 ± 2.1	11.5 ± 1.2	E2	12.7 ± 3.9	6.4 ± 2.6	21.6 ± 0.2	11.5 ± 1.3

Table 1. IBD patient/study and control groups, Olive tail moment (OTM) and % tail DNA after *in vitro* treatment with H_2O_2 and IQ and supplementation with the flavonoids at different concentration levels. A1, A2**:** No treatment; B1: H_2O_2 50 µM + Quercetin 0 µM ; B2: IQ 50 µM + Epicatechin 0 µM; C1: H_2O_2 50 µM + Quercetin 100 µM; C2: IQ 50 µM + Epicatechin 25 µM; D1: H_2O_2 50 µM + Quercetin 200 µM; D2: IQ 50 µM + Epicatechin 50 µM; E1: H_2O_2 50 µM + Quercetin 250 µM; E2: IQ 50 µM + Epicatechin 100 µM;

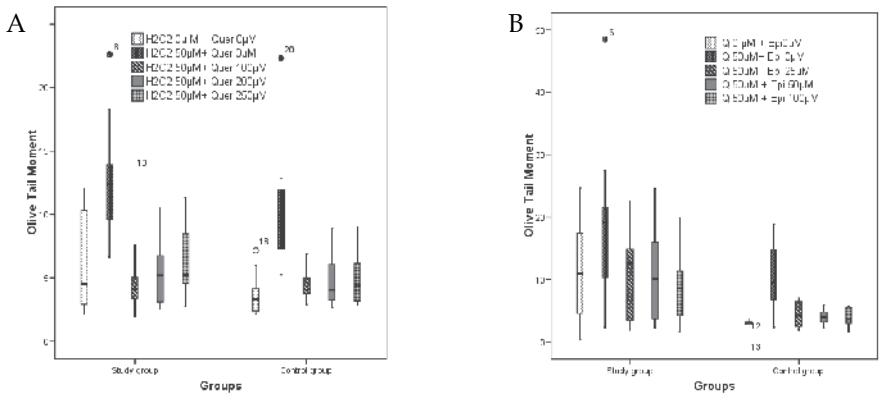

Fig. 1. IBD patient group and control group after *in vitro* treatment with A) H_2O_2 (50 µM) and supplementation with the flavonoid quercetin (Quer) at different concentration levels and B) IQ (50 µM) and supplementation with the flavonoid epicatechin (Epi) at different concentration levels. Pooling the data for each group and comparing median levels of DNA damage and 75% quartiles showed them to be significantly different (Kolmogorov-Smirnov test, $p < 0.001$). The ° symbols followed by a number indicate individual outliers with the respective patient number.

3.1.2 Differences in IBD sub-groups

As shown in Table 2 and Figure 2 in both series of experiments there was less DNA damage in the UC patient group (n = 4) than in the CD group (n = 4) each being significantly different (Kruskal-Wallis (K-W) test, $p < 0.001$) when compared with the combined patient groups, which also included the indeterminate group where it was difficult to differentiate into UC or CD (n = 2). Also there was less induced DNA damage in the study group treated with H_2O_2 and quercetin compared with the study group treated with IQ and epicatechin although the patients were selected randomly.

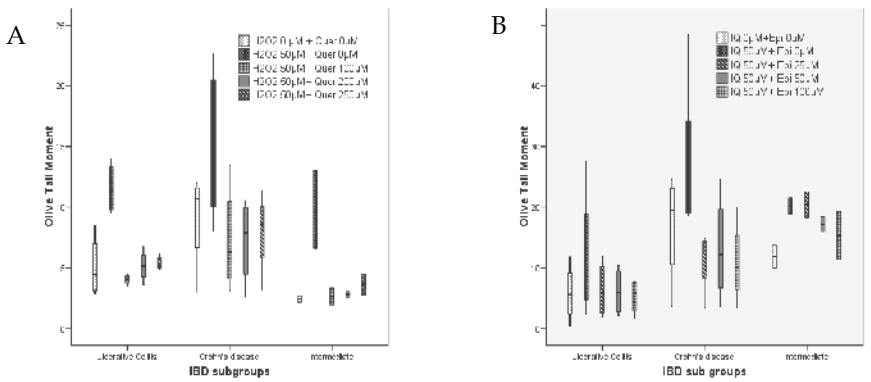

Fig. 2. DNA damage within three IBD subgroups after *in vitro* treatment with A) H_2O_2 (50 µM) and supplementation with the flavonoid quercetin (Quer) at different concentration levels and B) IQ (50 µM) and supplementation with the flavonoid epicatechin (Epi) at different concentration levels. The first two groups were diagnosed with Crohn's disease and Ulcerative Colitis. The third group was an indeterminate subgroup.

3.1.3 Confounding factors

3.1.3.1 Ethnicity, age, gender, smoking and drinking habits

There were small differences of median levels of DNA damage in Caucasians (n = 13) and Asians (n = 7) after treatment with H_2O_2 and quercetin as well as in males and females. A similar effect was observed within groups treated with IQ and epicatechin. However, these differences were not found to be statistically significant. There were also no statistically significant differences in DNA damage in the age distributions between patients as well as between control individuals (H_2O_2 with quercetin experiment: patients' mean age = 42.4 years ± 11.6, control individuals' mean age = 28.9 years ± 9.0; IQ with epicatechin experiment: patients' mean age = 39.2 years ± 10.3, control individuals' mean age = 22.6 years ± 9.2). No major differences were seen due to smoking and/or drinking habits (Table 2).

Confounding factors	H_2O_2 (0 µM) + Quercetin (0 µM)				IQ (0 µM) + Epicatechin (0 µM)			
	Defining factors	N	OTM	p-value	Defining factors	N	OTM	p-value
Type of IBD#	Ulcerative colitis	4	5.3 ± 0.5		Ulcerative colitis	4	3.5 ± 0.1	
	Crohn's disease	4	9.8 ± 0.6	p < 0.001	Crohn's disease	4	7.3 ± 0.7	p < 0.001
	Indeterminate	2	7.3 ± 0.9		Indeterminate	2	7.0 ± 2.2	
Age$^{\Delta}$	0-25	7	7.9 ± 2.3		0-25	7	8.2 ± 0.5	
	26-45	8	11.3 ± 02	p = 0.298	26-45	9	12.4 ± 2.5	p = 0.395
	More than 46	5	13.0 ± 3.3		More than 46	4	10.7 ± 0.1	
Smoking$^{\Delta}$	Active smokers	9	12.4 ± 0.6		Active smokers	9	7.4 ± 3.2	
	Ex-smokers	3	7.3 ± 1.2	p = 0.357	Ex-smokers	2	15.5 ± 0.3	p = 0.090
	Non-smokers	8	9.5 ± 2.1		Non-smokers	9	12.4 ± 1.1	
Drinking habit$^{\Delta}$	Non-alcoholic	7	10.6 ± 1.1		Non-alcoholic	10	12.7 ± 1.0	
	Moderate	8	9.3 ± 0.8	p = 0.704	Moderate	9	7.6 ± 4.2	p = 0.123
	Severe	5	12.2 ± 0.4		Severe	1	15.0 ± 0.7	
Ethnic origin$^{\Delta}$	Caucasian	13	10.6 ± 1.4	p = 0.905	Caucasian	12	9.8 ± 0.9	p = 0.487
	Asian	7	10.3 ± 0.3		Asian	8	11.6 ± 2.7	
Gender$^{\Delta}$	Female	13	10.1 ± 2.2	p = 0.700	Female	10	10.3 ± 3.0	p = 0.838
	Male	7	11.1 ± 0.9		Male	10	9.7 ± 2.3	

Table 2. Details of patient and control groups relating to confounding factors and their significant differences. OTM – Olive tail moment; # - only patient values included, values for healthy individuals shown in Table 1; Δ - patient and control group values combined

3.1.3.2 Previous medication in the IBD group as a confounding factor

Patients had been treated with a range of drugs for IBD, namely, azathioprine, mesalazine and pentasa, asacol, prednisolone, mercaptopurine alone or in combination prior to taking part in the study. Azathioprine & pentasa, azathioprine & mesalazine, mercaptopurine & balsalazide (n = 6); asacol (n = 1); pentasa & prednisolone, prednisolone & mesalazine (n = 3). Within the treatment groups, there appeared to be differences but they were not significant.

3.2 Study II: colon cancer patients

3.2.1 Patient versus control groups

Treating lymphocytes from healthy individuals and cancer patients with food mutagens IQ and PhIP *in vitro* resulted in a dose-dependent statistically significant induction of DNA

damage for both parameters measured, Olive tail moment (OTM) and % tail DNA (Table 3). For the 25µM IQ treatment of lymphocytes from colon cancer patients, the induced DNA damage measured in % tail DNA reached significance while the evaluated OTM did not.

	Healthy individuals	Colon cancer patients	Healthy individuals	Colon cancer patients
	Mean OTM ± SE		% tail DNA ± SE	
Negative control	2.10 ± 0.30	4.10 ± 0.42 §§§	10.95 ± 1.30	18.18 ± 1.25 §§§
25 µM IQ	3.23 ± 0.31 **	4.84 ± 0.47 §§	14.69 ± 1.01 **	20.12 ± 1.26 * §§§
75 µM IQ	5.50 ± 0.38 ***	6.18 ± 0.40 ***	21.82 ± 1.27 ***	24.76 ± 1.23 *** §
100 µM IQ	6.03 ± 0.43 ***	6.97 ± 0.49 ***	23.98 ± 1.28 ***	25.90 ± 1.54 ***
150 µM IQ	8.47 ± 0.58 ***	9.75 ± 0.53 *** §	33.80 ± 1.22 ***	35.52 ± 1.94 ***
150 µM IQ and 100 µM Q	7.67 ± 0.51	7.24 ± 0.37 †††	29.06 ± 1.05 ††	30.11 ± 1.48 †
150 µM IQ and 250 µM Q	5.90 ± 0.46 †††	7.32 ± 0.54 †††	22.53 ± 1.62 †††	28.86 ± 1.52 †† §§
150 µM IQ and 500 µM Q	3.44 ± 0.26 †††	5.81 ± 0.71 † §§	16.13 ± 1.16 †††	24.57 ± 2.39 ††† §§
150 µM IQ and 50 µM R	7.76 ± 0.59	7.21 ± 0.37 †††	27.42 ± 1.59 †	28.95 ± 1.13 ††
150 µM IQ and 250 µM R	7.27 ± 0.47 †	6.63 ± 0.37 †††	26.21 ± 1.32 ††	26.74 ± 1.20 †††
150 µM IQ and 500 µM R	4.12 ± 0.59 †††	5.18 ± 0.50 †††	15.98 ± 1.78 †††	21.82 ± 1.38 ††† §§
Negative control	2.90 ± 0.36	3.55 ± 0.30	13.53 ± 1.30	16.51 ± 0.96 §
10 µM PhIP	3.75 ± 0.41	4.38 ± 0.33 **	16.92 ± 1.42 *	19.95 ± 1.20 ** §
25 µM PhIP	5.57 ± 0.49 ***	5.41 ± 0.33 ***	23.31 ± 1.52 ***	24.15 ± 1.03 ***
50 µM PhIP	5.70 ± 0.54 ***	7.07 ± 0.49 *** §	24.01 ± 1.58 ***	27.79 ± 1.36 *** §
75 µM PhIP	8.58 ± 0.85 ***	8.27 ± 0.71 ***	31.05 ± 2.52 ***	33.79 ± 1.64 ***
75 µM PhIP and 100 µM Q	6.80 ± 0.58 †	6.75 ± 0.53 †	27.16 ± 1.46 †††	27.68 ± 1.36 ††
75 µM PhIP and 250 µM Q	5.99 ± 0.58 ††	5.71 ± 0.49 ††	23.99 ± 1.68 †††	24.35 ± 1.41 ††
75 µM PhIP and 500 µM Q	3.72 ± 0.43 †††	4.17 ± 0.45 †††	16.73 ± 1.41 †††	18.81 ± 1.50 †††
75 µM PhIP and 50 µM R	7.63 ± 0.56	7.25 ± 0.47 †	30.07 ± 1.79	29.28 ± 1.52 †
75 µM PhIP and 250 µM R	6.39 ± 0.41 ††	6.39 ± 0.39 ††	25.38 ± 1.03 ††	27.13 ± 1.02 ††
75 µM PhIP and 500 µM R	4.34 ± 0.30 †††	5.85 ± 0.56 †† §	18.94 ± 0.72 †††	24.36 ± 1.54 ††† §§

Table 3**.** DNA damage induced *in vitro* in lymphocytes from healthy individuals and colon cancer patients by the food mutagens IQ and PhIP and its reduction by flavonoid supplementation with various concentrations of quercetin (Q) and rutin (R).

Significantly different from the negative control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$
Significantly different from highest dose of food mutagen IQ: † $p<0.05$; †† $p<0.01$; ††† $p<0.001$
Significantly different from healthy individuals: § $p<0.05$; §§ $p<0.01$; §§§ $p<0.001$

Different concentrations of the flavonoids quercetin (100, 250 and 500 µM) and rutin (50, 250 and 500 µM) showed modulating effects on human lymphocytes of both donor groups in the presence of high doses of food mutagens, 150 µM IQ or 75 µM PhIP (Table 3). In the majority of the experiments supplementation with flavonoids resulted in a significant dose-dependent reduction of the induced DNA damage ranging from 1.4 to 2.5 times. For lymphocytes from healthy individuals, only the lowest quercetin dose together with IQ and the lowest dose for rutin together with IQ and PhIP measured in OTM, as well as the lowest dose of rutin together with PhIP when evaluating % tail DNA did not reach significant levels. At the highest supplemented flavonoid dose, the DNA damage from a high dose of food mutagen was significantly reduced to levels of damage in lymphocytes (from both donor groups) which was comparable to a treatment with a six times lower dose of the food mutagen IQ and a 7.5 times lower dose of PhIP, respectively.
Intergroup comparisons showed lower basic DNA damage in lymphocytes from healthy individuals (negative control) when compared to those from colon cancer patients (Figure 3 and Table 3). This difference was highly significant ($p < 0.001$ for parameters, OTM and %

tail DNA) for the negative control of the IQ experiment and significant for the PhIP experiment ($p < 0.05$ for % tail DNA; the OTM parameter did not reach significance: $p = 0.085$). Also, after treatment with food mutagens IQ and PhIP this higher baseline damage led to a significantly higher induction of DNA damage in lymphocytes from cancer patients for IQ concentrations of 25 µM ($p < 0.01$ for OTM; $p < 0.001$ for % tail DNA), 75 µM ($p < 0.05$ for % tail DNA) and 150 µM ($p < 0.05$ for OTM) as well as PhIP concentrations of 10 µM ($p < 0.05$ for % tail DNA) and 50 µM ($p < 0.05$ for OTM & % tail DNA).

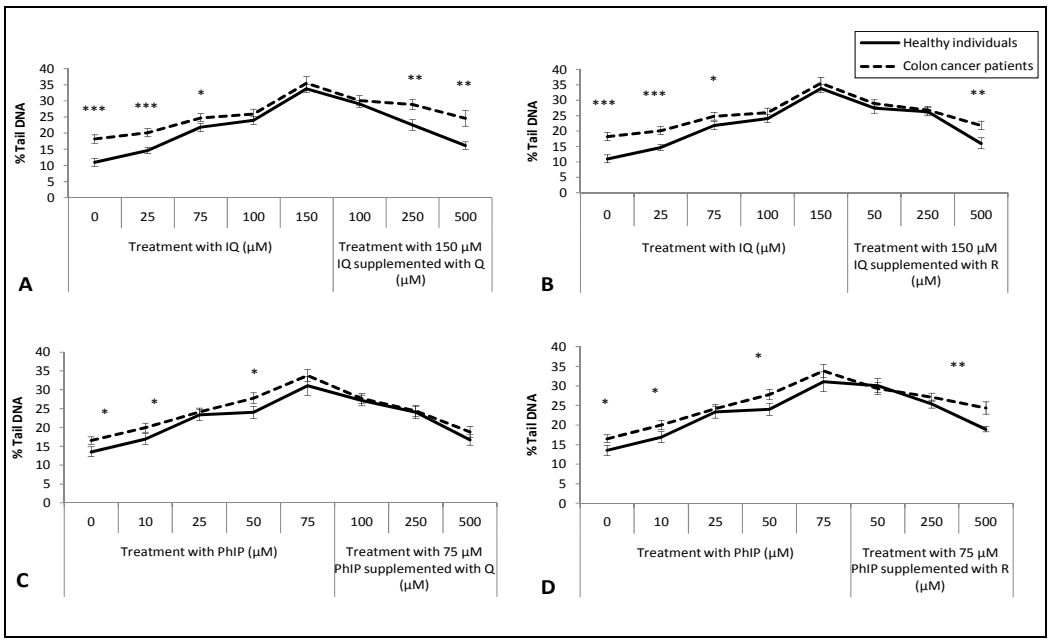

Fig. 3. The Comet parameter % tail DNA is shown indicating DNA damage in lymphocytes after *in vitro* treatment with IQ (25, 75, 100 and 150 µM) and then supplementation of the highest dose with either the flavonoid quercetin (Q) [Panel A] or rutin (R) [Panel B] as well as treatment with PhIP (10, 25, 50 and 75 µM) and then supplementation of the highest dose with either the flavonoid quercetin (Q) [Panel C] or rutin (R) [Panel D]. The flavonoids were used at different concentrations from 50 µM up to 500 µM. Intergroup comparisons of healthy individuals and colon cancer patients revealed significantly increased induction of DNA damage, when treating with heterocyclic amines alone and together with flavonoids, between healthy individuals and the colon cancer patient group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). All other types of data comparisons are shown in Table 3.

When supplementing a single high-dose treatment of either IQ (150 µM) or PhIP (75 µM) with flavonoids (quercetin or rutin), the intergroup comparison showed only at the highest levels of flavonoid supplementation significant differences in the reduction of DNA damage caused by the food mutagen (Figure 3 and Table 3). Except for the supplementation of PhIP with 500 µM of quercetin (Figure 3C), lymphocytes from colon cancer patients showed significantly higher amounts of DNA damage at higher flavonoid concentrations in comparison to healthy volunteers (Figures 3A, 3B & 3D), i.e. less reduction of induced damage by the flavonoid (IQ + 500 µM quercetin, $p < 0.01$ for OTM and % tail DNA; IQ +

250 μM quercetin, $p < 0.01$ for % tail DNA; IQ + 500 μM rutin, $p < 0.01$ for % tail DNA; PhIP + 500 μM rutin, $p < 0.05$ for OTM and $p < 0.01$ for % tail DNA). The parameter % tail DNA for genetic damage was more sensitive compared to OTM.

3.2.2 Confounding factors

Confounding factors such as age, gender, diet, smoking habits and alcohol intake were also investigated (Table 4). A significant higher baseline DNA damage ($p < 0.001$) in lymphocytes from colon cancer patients was observed for parameters OTM and % tail DNA compared to those from healthy individuals. There was also a significant difference between subjects of >50 years of age when compared to those under 50 years of age ($p < 0.01$) showing a 1.80-fold and 1.54-fold increased baseline DNA damage for OTM and % tail DNA, respectively. No statistically significant differences were found when focusing on smoking habits, alcohol intake and diet, although, when comparing Western to Asian/vegetarian type diet the OTM parameter almost reached significance ($p = 0.061$). DNA damage in male lymphocytes was significantly ($p < 0.05$) higher than in lymphocytes from females for the Comet assay parameter % tail DNA but not for the OTM parameter ($p = 0.450$).

Confounding factor	Sub-groups	N	Mean OTM ± SE	% tail DNA ± SE	Description
Diagnosis	Healthy	20	2.10 ± 0.30	10.95 ± 1.30	Colon cancer patients vs. healthy individuals
	Colon cancer	20	4.10 ± 0.42 ***	18.18 ± 1.25 ***	
Age$^{\Delta}$	<50 years	18	2.02 ± 0.32	9.99 ± 1.38	>50 vs. <50 years of age
	>50 years	22	3.64 ± 0.38 **	15.42 ± 1.16 **	
Smoking$^{\Delta}$	Active smokers	7	3.16 ± 0.85	12.12 ± 2.58	Active / ex-smokers vs. non-smokers
	Ex-smokers	16	2.69 ± 0.40	12.27 ± 1.52	
	Non-smokers	17	2.99 ± 0.44	13.80 ± 1.54	
Drinking habit$^{\Delta}$	Severe	15	3.20 ± 0.48	13.69 ± 1.60	Severe / moderate drinking vs. no alcohol
	Moderate	14	2.85 ± 0.47	12.97 ± 1.79	
	No alcohol	11	2.48 ± 0.54	11.66 ± 1.86	
Diet$^{\Delta}$	Western	25	3.22 ± 0.39	13.66 ± 1.38	Western vs. Asian/vegetarian diet
	Asian/vegetarian	15	2.30 ± 0.32	11.58 ± 1.23	
Gender$^{\Delta}$	Female	17	2.50 ± 0.35	10.79 ± 1.07	Female vs. male individuals
	Male	23	3.16 ± 2.97	14.39 ± 1.45 *	

Table 4. Confounding factors for healthy individuals and colon cancer patients and their influence on the baseline DNA damage using the Comet assay. Significantly different from the negative control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. OTM - Olive tail moment; % tail DNA-fraction of DNA in the tail; Δ - patient and control group values combined

4. Discussion

Crohn's disease (CD) and Ulcerative Colitis (UC), known as inflammatory bowel disease (IBD), are fairly common chronic inflammatory conditions of the gastrointestinal tract. Although the exact aetiology of IBD remains uncertain, dysfunctional immunoregulation of the gut is believed to be the main cause. Amongst the immunoregulatory factors, reactive oxygen species (Seegert et al., 2001) are produced in abnormally high levels in IBD (Rezaie et al., 2007). An imbalance between antioxidants and ROS results in oxidative stress, leading to cellular damage (Rezaie et al., 2007) and subsequently cell death or cancer. Colorectal cancer is a heterogeneous neoplasm consisting of cancer cells with various proliferation rates and the potential to metastasise (Ozdemirler Erata et al., 2005). Genetic alterations caused by cellular overproduction of ROS are required for neoplastic progression

(Soderlund et al., 2010). Such changes are based on DNA damage triggered by endogenous but also by environmental and lifestyle genotoxins (Bartsch et al., 2002; Ozdemirler Erata et al., 2005). As the onset of cancer is a prolonged multi-stage process where successive mutations are accumulated, continuous erosion of the genome and defects in repair contribute to this process (Hoeijmakers, 2001; Jiricny and Marra, 2003). Several factors, depending on the socio-economical status, such as large amounts of salted, cured and smoked foods, dietary mutagens, alcohol and obesity may play an important role in increasing mutagenicity leading to an inappropriate stimulation of the immune response or release of ROS subsequently generating further DNA damage (Hursting et al., 2003).

Cooking fish and beef inevitably generate HCA especially at high temperatures (Schut et al., 1999), which are carcinogenic in mice, rats and monkeys producing hepatic, intestinal and mammary tumours (Schoeffner and Thorgeirsson, 2000) and posing a potential risk to humans. HCA have been widely investigated and all of them have so far been described as mutagenic and carcinogenic (Gooderham et al., 2007). Food-derived heterocyclic amines (HCA) like IQ have been shown to be mutagenic in the Ames test inducing gene mutations and tumours *in vivo* (Adamson and Thorgeirsson, 1995; Knize et al., 1995). Food mutagens may cause different types of DNA damage from chromosomal aberrations to subtle nucleotide alterations. Most food mutagens like HCA are able to form reactive DNA adducts by covalently binding to nucleotides. However the effect of food mutagens in carcinogenesis can be modified by heritable traits, namely, low-penetrant genes that affect exposure of the mutagen to DNA through metabolic activation and detoxification or other cellular responses to DNA damage. There is strong evidence that endogenous liver and kidney enzymes or those from commensal bacteria in the intestine are able to metabolically activate HCA which in turn generate DNA damage (El-Zein et al., 2006; Krul et al., 2000). Able to activate and detoxify heterocyclic amines may be enzymes like CYP1A2, *N*-acetyltransferase, sulfotransferase, prolyl tRNA synthetase, phosphorylase and COX isomers (Wolz et al., 2000). In a recent case-control study, no associations were found between colorectal cancer (CRC) risk and polymorphisms within the genes of those enzymes (Sachse et al., 2002). This comprehensive analysis, however, failed to consider commensal bacteria and their potential impact on HCA activation, an effect independent of the host genotype. The pro-carcinogen IQ is predominantly produced through the pyrolysis of creatinine with sugars and becomes significantly mutagenic in the presence of hepatic microsomes (Sugimura and Sato, 1983). Anaerobic colonic bacteria can convert IQ to 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline-7-one (HOIQ), a direct-acting mutagen (Bashir et al., 1987). These commensal bacteria can strongly influence IQ-induced DNA damage in colonic cells and also in hepatocytes as measured by the alkaline comet assay (Knasmuller et al., 2001).

As mentioned before DNA damage seems to be also triggered by oxidative stress. When considering the human diet, it should be recognized that food contains both, mutagens and components that decrease cancer risk such as antioxidants (Goldman et al., 2003; Maeda et al., 1999). Flavonoids are known to have antioxidative properties *in vivo* (Rice-Evans, 2001) and modulate effects of food mutagens *in vitro* in human lymphocytes and sperm (Anderson et al., 1998). Green tea and, to a lesser extent, black tea are a rich source of still another group of flavonoids called catechins. The activity of quercetin is believed to be due to its antioxidative properties, however, it has been suggested that quercetin may also have pro-oxidative activities, which might then directly affect genotoxicity (Lee et al., 2003). Conclusively, quercetin acts as a strong antioxidant and scavenger of free radicals while it

might simultaneously undergo an oxidation process giving rise to the formation of the semiquinone radical (Papiez et al., 2008).

The present studies demonstrate that H_2O_2, PhIP and IQ are capable of inducing significant DNA damage as a result of oxidative stress (Figures 1, 2 and 3, Table 1 and 3). There was a significant increase of DNA damage after treating lymphocytes from healthy controls, IBD and colon cancer patients with H_2O_2, PhIP and IQ, while a significant protective effect was found in the presence of the flavonoids quercetin, rutin and epicatechin (Figures 1, 2 and 3, Table 1 and 3).

In the study I, the protective *in vitro* effect of quercetin and epicatechin against oxidative stress in lymphocytes from IBD patients and healthy individuals (Figure 1) was observed. We were able to show that untreated lymphocytes from IBD patients had significantly increased DNA damage when compared to healthy individuals as shown previously with other IBD patients (Najafzadeh et al., 2007). *In vitro* treatment with H_2O_2 and IQ significantly induced DNA damage by oxidative stress in both groups. Flavonoids reduced the baseline DNA damage in lymphocytes from IBD patients treated with H_2O_2 and IQ. Similar effects were achieved with Chaga mushroom extracts (Najafzadeh et al., 2007). When co-treated with flavonoids, a significant protective effect was shown against free radical damage to the DNA generated by H_2O_2 or IQ (Figure 1). There was a very high level of damage in the patient group without any treatment because of their background inflammation and IBD therapeutic drugs which they had taken, but both patients and controls showed a parallel and gradual reduction in DNA damage after treating with flavonoids (Figure 1 and Table 1). Lymphocytes from CD patients in two series of study groups appeared to have a greater level of baseline DNA damage than those from UC patients when compared to the whole patient group ($p < 0.001$) , suggesting that lymphocytes from CD patients are more exposed to oxidative stress than other IBD subgroups (Figure 2). It becomes obvious that an excessive production of ROS and radical nitrogen metabolites occur during the inflammation of the intestine in IBD patients (Kruidenier et al., 2003). It seems that a misbalanced production of pro-inflammatory and anti-inflammatory cytokines is characteristic of IBD and severely affects the immune homeostasis in peripheral blood cells, even more in CD than in UC patients (Sventoraityte et al., 2008). However, all subgroups react in the same way towards exogenous oxidative stressors as well as towards the inhibition of oxidative stress by flavonoids.

The detrimental effects of two common food mutagens, IQ and PhIP, on the DNA were investigated in study II by treating *in vitro* lymphocytes from healthy individuals and from patients diagnosed with colon cancer. Pool-Zobel *et al.* found lymphocytes responses to be very similar to responses in rectal cells (Pool-Zobel et al., 2004), thus, supporting their role as surrogate cells for biomonitoring and *in-vitro* treatments. Both HCA caused in a dose-dependent manner similar levels of DNA damage in lymphocytes of both groups for the Comet assay parameters OTM and % tail DNA (Table 3), supporting the classification for IQ as "probably carcinogenic to humans" and for PhIP as "possibly carcinogenic to humans" (IARC, 1993). Higher doses significantly increased the induced DNA damage. IQ and PhIP were shown to be potent genotoxins and carcinogens (Adamson et al., 1995; Durling and Abramsson-Zetterberg, 2005; Duthie et al., 1997). Even very low doses (10^{-3} to 10^{-4} μM PhIP) induce expression of the DNA damage response proteins like p53 and increase proliferation in oestrogen receptor (ER)-negative MCF10A cells (Gooderham et al., 2007). Hence, PhIP may induce/enhance carcinogenicity via DNA damage and/or oestrogen receptors (Bennion et al., 2005; Felton et al., 2004). IQ on the other hand can form DNA adducts like N-

(deoxyguanosin-8-yl)-IQ in the presence of nitric oxide constituting a possible cancer risk for individuals with colon inflammation (Lakshmi et al., 2008).

In our study the DNA damage induced in lymphocytes of both donor groups by food mutagens IQ and PhIP was effectively and dose-dependently reduced by supplementation with the flavonoids quercetin and rutin (Table 3). The level of DNA damage from the highest HCA dose reduced by the highest dose (500 μM) of flavonoids was comparable to that of a six times (for IQ) and 7.5 times (for PhIP) lower non-supplemented dose of food mutagen.

Strong antioxidative effects of flavonoids to protect against DNA damage have been known for some time (Anderson et al., 2003; Collins, 2005; Perez-Vizcaino et al., 2009; Rice-Evans, 2001) and *in-vitro* experiments on human colonocytes suggested that especially quercetin plays a crucial role in the defence against oxidative insults (Duthie and Dobson, 1999). In human lymphocytes quercetin and rutin already showed a dose-dependently protective effect against DNA damage caused by the mutagenic anticancer drug mitomycin C (Undeger et al., 2004). However, neither myricetin, quercetin nor rutin increased the rate of DNA strand break repair in various cell types such as Caco-2, Hep G2 and V79 (Aherne and O'Brien, 2000).

We found that the number of individuals in each group was sufficient to establish statistically significant responses ($p < 0.001$) shown in our study for the food mutagens. Our results indicate that the baseline DNA damage was higher for all experiments in lymphocytes from colon cancer patients when compared to healthy individuals (Table 3 and Figure 3). Disease states which involve an overproduction of ROS may therefore inflict significantly higher DNA damage in peripheral lymphocytes from patients when compared to the baseline level of damage in healthy individuals. This has been shown for diseases like Irritable Bowel Syndrome and diabetes (Collins et al., 1998b; Najafzadeh et al., 2009; Wyatt, 2006). It also confirms findings of Vodicka *et al.* who observed increased chromosomal damage in lymphocytes of newly diagnosed cancer patients compared with healthy controls (Vodicka et al., 2010). Similar observations of a higher baseline DNA damage were made for head and neck squamous cell carcinoma patients (Palyvoda et al., 2003) and breast cancer patients (Rajeswari et al., 2000; Smith et al., 2003) in addition to higher levels of cytogenetic damage (Palyvoda et al., 2003). Even the modulating effect of flavonoids in a co-treatment with a high dose of food mutagen (Table 3) seem to be affected by the higher baseline damage as the induced DNA damage in lymphocytes from colon cancer patients was not reduced to the levels of healthy individuals. Except for the supplementation of PhIP with 500 μM of quercetin (Figure 3C), lymphocytes from colon cancer patients showed significantly higher amounts of DNA damage at higher flavonoid concentrations (Figures 3A, 3B & 3D), i.e. less reduction of induced damage, that may suggest that higher concentrations of flavonoids would be required to achieve a protective effect. A possible reason for this finding could be a reduced repair capacity which was found for breast cancer patients after *in-vitro* treatment of lymphocytes with N-methyl-N-nitro-N-nitrosoguanidine or ionising radiation (Smith et al., 2003). The repair capacity of first degree relatives to these patients was also decreased (Rajeswari et al., 2000; Smith et al., 2003). These differences in repair capacity may either be an effect of cancer *per se* due to a changed lymphocyte population alongside the oncogenic process, or a higher DNA damage and slower repair among some individuals who may be more predisposed to develop cancer (Palyvoda et al., 2003).

An analysis of confounding factors such as age, gender, diet, smoking habits and alcohol intake (Table 4) on the baseline level of DNA damage in lymphocytes in Study II showed a

significant higher damage for subjects of >50 years of age ($p < 0.01$) as previously reported (King et al., 1994; Mendoza-Nunez et al., 2001). When examining the confounding effect aspects the numbers of participants were reduced yet we still found statistically significant effects for age (>50. $p < 0.01$) for both OTM and % tail DNA and for gender (males, $p < 0.05$) for % tail DNA only. An increase in DNA damage in lymphocytes has been observed among elderly individuals but was not significant (Betti et al., 1994; Mendoza-Nunez et al., 2001). We are aware our groups are not best matched for age however these were the only individuals available at the time. Despite this fact we were able to detect an age effect in a >50 age group. Although smoking and alcohol consumption is associated with an increased risk for colorectal cancer (Bardou et al., 2002; Emmons et al., 2005) probably due to DNA damage via increased levels of oxidative stress (Lodovici and Bigagli, 2009; Obe and Anderson, 1987; Pool-Zobel et al., 2004), no statistically significant differences were found in our study when focussing on smoking habits, alcohol intake or diet. This was also the case in the IBD study (Study I). DNA damage in male lymphocytes, however, was significantly higher than in lymphocytes from females for the Comet assay parameter % tail DNA but not for OTM. According to the literature, male gender constituted a risk factor for DNA damage (Collins et al., 1998a), where elderly males had more than twice the probability of having DNA damage than females (Mendoza-Nunez et al., 2001). After *in vitro* treatment with IQ and PhIP an increase in DNA damage in lymphocytes was observed in our study but confounding factors such as diet, smoking and drinking habits did not seem to influence this response, even though numerous environmental and lifestyle compounds can have an impact on the exposure, metabolism and cell proliferation response of HCA (Felton et al., 2004). Nevertheless, in most of the cases the damage was similarly distributed and the general response to treatment may still be dependent on possible dietary interactions after exposure to food mutagens and individual factors like individual susceptibility, cellular antioxidant / micronutrient levels or disease states (Airoldi et al., 2004; El-Zein et al., 2006; Ferguson and Philpott, 2008; Han et al., 2008; Pool-Zobel et al., 2004). It has been estimated that up to 90% of the cases of colorectal cancer could be prevented with life style modifications such as balanced diet, avoidance of smoking and alcohol and physical activity (Boursi and Arber, 2007).

5. Conclusion

In conclusion, a significant protective effect of the flavonoids quercetin, epicatechin and rutin against oxidative DNA damage has been demonstrated after oxidative stress has been induced *in vitro* by H_2O_2, heterocyclic amines IQ and PhIP in lymphocytes obtained from IBD and colon cancer patients and healthy individuals. Lymphocytes from IBD, cancer patients and controls had increased DNA damage possibly due to an overproduction of ROS and there was a significant difference in response between all donor groups to treatment with HCA alone and together with flavonoids in both studies. It is believed that flavonoids operate through their antioxidant properties and responses of H_2O_2 and HCA to quercetin, epicatechin and rutin observed throughout the study, support the hypothesis that food mutagens target DNA by generating ROS. Concepts such as chemoprevention specifically focus on the long-term use of protective agents like vitamins (Boursi et al., 2007; Das et al., 2007). As shown, flavonoids have impressive antioxidant properties and are able to effectively protect the integrity of lymphocytes' DNA from ROS induced by HCA, which would make them the ideal supplemental compounds to prevent the onset of cancer

(Dashwood, 1999; Ross et al., 2002). Although the possible health benefits of consuming flavonoids seem to be obvious, there are so many biological activities attributed to them, that further study may be justified.

The flavonoids significantly reduce DNA damage *in vitro* in lymphocytes of IBD and colorectal cancer patients as well as healthy individuals. Thus, a diet containing flavonoids could be very effective in reducing baseline and exogenously induced oxidative DNA damage of IBD and colorectal cancer patients.

6. Acknowledgement

The authors want to thank the clinical staff of Bradford Royal Infirmary Hospital and St. Luke's Hospital for their support in this study.

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5

Effects of Dietary Soybean Trypsin Inhibitors on Detection of Resistance to Pyrethroid and Spinosad Insecticides in *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

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1. Introduction

During the last 50 years, worldwide use of synthetic insecticides to control insect pests has led to both insecticide resistance and environmental problems (Roush and Tabashnik, 1990). *Helicoverpa armigera* (Hübner) is arguably Australia's most important agricultural pest and insecticide resistance remains an enduring threat. Throughout its history, Australian *H. armigera* has been shown to evolve a range of mechanisms to confer resistance to insecticides (Gunning et al., 1991; Gunning et al., 1996a; Gunning et al. 1996b). Therefore it is vital that any resistance monitoring programme must not only be able to detect resistance at low frequencies but also be able to detect resistance resulting from any number of potential mechanisms. Pyrethroid and spinosad resistance in Australia are known to be caused by sequestration and or hydrolysis of non-specific esterases (Gunning et al., 1996b; Gunning et al., 2007; Gunning & Balfe 2002).

Soybean based diets are common artificial diets for *Heliothine* species. To avoid chronic exposure to artificially high levels of protease inhibitors in artificial insect diets, it is usual to heat-treat legume components to denature the inhibitors (Shorey & Hale, 1965; Teakle and Jensen, 1985). However, not all laboratories follow this regime, and while some laboratories heat-treat legume components of diets, other laboratories involved in *Heliothine* rearing, use or have used, raw soybean flour. There are also a variety of procedures used to heat treat soybean trypsin inhibitors in artificial diet preparation, which may result in varying degrees of protease inhibitor degradation.

Leguminous seeds, such as those from soybean, are protected against herbivores, including insects, by anti-nutritional defence proteins- trypsin inhibitors, which are found in the seeds and raw flour made from the seeds. Levels of these plant protease inhibitors are high in legume seeds, comprising 1-5% of total protein (Macintosh et al., 1990; Sastry et al., 1987) Trypsin inhibitors of the Kunitz type (Kunnitz, 1945a; Kunnitz, 1945b) are single chain polypeptides (~ 20 kDa) that act on target serine proteases in the gut forming a 1:1 complex. Perhaps the best known of these inhibitors is Kunitz soybean trypsin inhibitor (KSTI).

Midgut serine protease activity has been found in a wide variety of Lepidopteran pests, including *H. armigera,* where trypsin is a major protease in the *H. armigera* midgut (Macintosh et al., 1990). In *H. armigera*, KSTI acts against gut serine proteases and are larval growth retardants and may cause death through the prevention of protein digestion (Johnston et al., 1993; Wang et al., 1995).

KSTI and other potent trypsin inhibitors are also known to have synergistic effects with delta endotoxins of *Bacillus thuringiensis*. This has been shown in Lepidoptera (*H. armigera*, *Manduca sexta, Heliothis virescens, Trichoplusia ni* and *H. zea*), as well as *Leptinotarsa decemlineata* (Colorado potato beetle) (Macintosh et al., 1990; Shao et al., 1998; Zhang et al., 2000, Zhu et al., 2007; Christtellier et al., 1992; Hubert et al., 2008). A recent study has shown that raw soybean diets can prevent the detection of non-specific esterase mediated resistance to *Bacillus thuringensies* toxins in *H. armigera* (Gunning & Moores, 2010).

In addition to Bt toxin, non-specific esterases in Australian *H. armigera* are also known to confer resistance to pyrethroids and spinosad by sequestration and/or hydrolysis (Gunning et al., 1996b; Gunning et al., 2007; Gunning & Balfe 2002). This work therefore examines the effects of raw or heat denatured soybean flour artificial diets on the detection of spinosad and pyrethroid resistance in *H. armigera*. The efficacy of two commonly used methods of denaturing soybean trypsin inhibitors (dry roasting soybean flour and boiling soybean flour with water prior to diet incorporation) were also compared.

We conclude that detection of spinosad and pyrethroid (bifenthrin) resistance in *H. armigera* can be masked if an artificial diet gives chronic exposure to potent trypsin inhibitors present in raw soybean flour and that boiling soybean flour does not achieve the temperature required to effectively denature soybean trypsin inhibitors.

2. Materials and methods

2.1 Insects

The *H. armigera* strains used were a susceptible strain (susceptible), and pyrethroid and spinosad resistant strains. The resistant strains Spin-R and Pyr-R (derived from survivors of resistance monitoring) were known have non-specific esterase mediated resistance to spinosad and pyrethroids respectively. A multi-resistant field strain (Breeza Field) collected off cotton at Breeza, NSW was also used.

2.2 Rearing methods

Helicoverpa armigera larvae were reared on a diet modified from that of Shorey and Hale (Shorey & Hale, 1965). The diet was altered by the substitution of dry roasted soybean flour for pinto beans and the addition of wheat germ and propionic acid. The artificial diet comprised (A) soybean flour (Allied Mills) (450 g), roasted in a microwave oven on full power for 5 minutes (temperatures in excess of 200^{o}C are achieved), wheat germ (Allied Mills) (120g), brewers yeast (Phytofoods) (105 g), ascorbic acid (Phytofoods) (10.1 g) and nipagen (3.3g), sorbic acid (3.3g), thiabendazole (0.8 g) and streptomycin sulfate (0.2g) (all obtained from Sigma); (B) agar grade J3 (Gelita) (45 g) and H_20 (1200 ml), (C) formaldehyde (40%) (BDH) (6 ml) and H_20 (1500 ml); (D) propionic acid mix (propionic acid (42%) / 4% phosphoric acid (BDH) (8 ml). Ingredients (A), (C) and (D) were blended together, the agar and cold water of (B) were mixed and brought to the boil, cooled to 70^{0} C and then blended with the other ingredients till smooth. The diet was poured into a shallow tray and allowed to set.

Helicoverpa armigera were reared in an insectary at 25^0 C, 70% relative humidity, in natural light. Adults were kept in tall plastic cages with shredded paper at the base and cloth lids (nappy liners), on which the eggs were collected. The moths were fed on a honey solution (5%). The eggs were surface sterilised in a sodium hypochlorite (Coles Supermarkets) solution (0.1%) and allowed to dry. Eggs were sealed into plastic, ventilated containers with a small amount of rearing diet, allowed to hatch and develop to 2nd instar. Second instar larvae were transferred to a block of diet (~2g) in 32 well trays (CD International), sealed with vented, adhesive lids (CD International) and reared to pupation. Pupae were removed from the diet, sterilised in sodium hypochlorite solution (0.1%), dried and transferred to moth cages.

2.3 Diets for bioassays

For pyrethroid and spinosad bioassays, three forms of the artificial diet were prepared. The standard diet (described above) incorporating dry roasted soybean flour, a second diet incorporating raw soybean flour or a third diet in which 450 g soybean flour was first boiled for 4 min in 1500 ml of water before being incorporated with rest of diet ingredients (A, C and D) above.

2.4 Insecticides and bioassay

Insecticides used were technical grade bifenthrin (Crop Care) and spinosad (Dow), dissolved and serially diluted in acetone.

The larval bioassay procedure utilised topical application, similar to that recommended by the Entomological Society of America (Anon, 1970). Technical-grade material was dissolved in acetone and 5 - 6 serially diluted concentrations were prepared. Three replicates of 10, 30-40 mg third instar larva were treated at each dose by applying 1 µl of solution to the dorsal thorax of each with a microapplicator (Hamilton).

Following treatment, the larvae were maintained individually at 25 ± 1^0C in bio-assay trays (Bio-BA-1280©, C-D International, Inc., Pittman, N.J. USA, 609-5832-2392) and supplied with adequate diet. Mortality was assessed at 48 hours and 72 hours after treatment for bifenthrin and spinosad respectively. Larvae were considered dead if they were unable to move in a coordinated manner when prodded with a blunt probe. There was no control mortality. Data were analysed by Probit Analysis (Finney, 1970) and resistance factors calculated from the ratio of the resistant strain LD_{50} and the LD_{50s} / susceptible strain LD_{50} and the $LD_{99.9s}$.

3. Results

3.1 Spinosad

Log dose probit data for spinosad bioassays are shown in Table 1. The treatment of soybean flour in the three rearing diets made no significance to spinosad toxicity in the spinosad susceptible strain. On a diet made from dry roasted soybean flour, the spinosad resistant strain was found to be 62 and 378 fold resistant to spinosad at the LD_{50} and the $LD_{99.9}$ levels respectively. The field strain was 7 and 131 fold resistant at the LD_{50} and the $LD_{99.9}$ levels respectively. Bioassays on the raw soybean flour diet and boiled soybean flour, however, failed to detect resistance, with the LD_{50} and $LD_{99.9}$ levels in the resistant and susceptible strains not being significantly different.

3.2 Bifenthrin

Data for bifenthrin are shown in Table 2. The treatment of soybean flour in the three rearing diets again resulted it no significant effect to bifenthrin toxicity in the pyrethroid susceptible

strain. In the pyrethroid resistant strain, high levels of resistance to bifenthrin were recorded from larvae reared and bioassayed on the diet prepared with dry roasted soybean flour. Resistance factors were 284 and 320 fold at the LD_{50} and the $LD_{99.9}$ levels respectively. Larvae from the field strain were 37 and 154 fold at the LD_{50} and the $LD_{99.9}$ levels respectively. Low levels of bifenthrin resistance (13.3 and 10.4 fold at the LD_{50} and the $LD_{99.9}$ levels respectively) were detected in the bifenthrin resistant strain on a raw soybean flour diet, but significant resistance was not detected in the field strain. Significant bifenthrin resistance was not detected in either the field or pyrethroid resistant strain when soyflour was boiled prior to incorporation into the *Helicoverpa* diet mix.

Strain	Soybean flour treatment	χ^2	Slope	LD_{50} (95 %fiducial limits)	Resistance factor	$LD_{99.9}$ (95 %fiducial limits)	Resistance factor
Susceptible	Dry roasted	0.5	3.1	0.29 (0.16 – 0.34)	1.0	1.6 (0.17 – 3.1)	1.0.
Spin-R	Dry roasted	0.43	2.1	10.0 (13.0– 21.0)	62	605 (153 – 2390)	378
Breeza Field	Dry roasted	5.8	1.5	1.9 (1.1- 3.2)	7	210 (45 – 910)	131
Susceptible	Raw	0.45	3.0	0.28 (0.15 – 0.34)	1.0	1.5 (0.19 – 3.2)	1
Spin-R	Raw	0.5	3.2	0.29 (0.16 – 0.33)	1.0	1.7 (0.14 – 3.2	1.1
Breeza Field	Raw	1.2	3.1	0.29 (0.06 – 0.14)	1.0	1.8 (0.19 – 3.2)	1.2
Susceptible	Boiled	0.4	3.2	0.28 (0.16 -0.33)	1.0	1.5 (0.2 - 3.3	1
Spin-R	Boiled	1.1	3.0	0.29 (0.14 – 0.37)	1.0	1.8 (0.25 – 4.0)	1
Breeza Field	Boiled	0.6	3.0	0.3 (0.13 – 0.39)	1.1	1.7 (0.16 – 3.2)	1.1

Table 1. The effects of differing dietary soybean flour treatments on response of third instar (30 – 40 mg) *Helicoverpa armigera* larvae to topically applied spinosad.

Strain	Soybean flour treatment	χ^2	Slope	LD_{50} (95 %fiducial limits)	Resistance factor	$LD_{99.9}$ (95 %fiducial limits)	Resistance factor
Susceptible	Dry roasted	1.5	3.8	0.03 (0.01 – 0.05)	1	0.125 (0.08 – 1.6)	1
Pyr-Resistant	Dry roasted	0.62	4.6	8.5 (6.7 – 10.1)	284	40.0 (31 -66)	320
Breeza Field	Dry roasted	1.9	1.9	1.1 (0.7 – 1.9)	37	19.2 (6.6 – 56)	154
Susceptible	Raw	1.5	3.8	0.03 (0.01 – 0.05)	1	0.11 (0.07 – 1.6)	1
Pyr-Resistant	Raw	0.8	3.6	0.4 (0.1 – 0.6)	13.3	1.30 (0.5 – 4.2)	10.4
Breeza Field	Raw	1.1	3.5	0.06 (0.01 – 0.07)	2	0.15 (0.05 – 1.6)	1.2
Susceptible	Boiled	1.5	3.6	0.03 (0.01 – 0.05)	1	0.120 (0.06 – 1.9)	1
Pyr-Resistant	Boiled	1.1	3.0	0.12 (0.08 – 0.47)	4	0.5 (0.20 – 3.5)	4.3
Breeza Field	Boiled	0.7	3.3	0.04 (0.01 - 0.07)	1	0.14 (0.07 – 1.9)	1.1

Table 2. The effects of differing dietary soybean flour treatments on response of third instar (30 – 40 mg) *Helicoverpa armigera* larvae to topically applied bifenthrin.

4. Conclusion

The data show that treatment of soybean flour in the preparation of *Helicoverpa* rearing diet greatly influenced the observed mortality following topical application of spinosad and bifenthrin in the *H. armigera* resistant strains. Diet prepared from raw soybean flour and soybean flour that had been boiled prior to diet incorporation, hindered detection of spinosad and pyrethroid resistance in strains known to be highly resistant. In particular, these diets interfered in the detection of non-specific esterase mediated resistance to spinosad and pyrethroids (bifenthrin).

The protease inhibitors present in soybean seeds, including the KSTI-like inhibitors, are reversibly denatured and require prolonged heating at high temperatures to bring about irreversible denaturation (Kunitz, 1948). KSTI is highly resistant to thermal and acidic denaturation (Roychaudhuri, 2003). Thus any resistance to xenobiotics that is conferred by enhanced serine hydrolases (i.e. esterases) could be compromised by the presence of KSTI-like inhibitors in the diet.

A previous study (Gunning & Moores, 2010), showed that a raw soybean flour diet prevented detection of non-specific esterase mediated resistance to *Bacillus thuringensis* toxins in *H. armigera*, whereas resistance was detectable in larvae reared on a roasted soybean flour diet. The difference was attributed to synergism by dietary KSTI or other inhibitors in the raw flour diet. These data are consistent with that of the present study, where there were observed differences between ability to detect an esterase mediated

resistances (spinosad and pyrethroid) in *H. armigera* on raw soybean flour and roasted soybean flour diets.

Given that KSTI is remarkably resistant to thermal denaturation and that denaturation is readily reversible at lower temperatures, it is likely that failure to detect resistance on a diet made from boiled flour is due inadequate denaturation of KSTI and/or other trypsin inhibitors. We observed that a soybean flour/ water mix boiled at 40^{o}C, which is far below the temperature required to permanently denature KSTI (Kunitz, 1948). It is also possible that boiling the soybean flour in water solubilises the tyrpsin inhibitors.

KSTI and other trypsin inhibitors are known to have synergistic effects with delta endotoxins of *Bacillus thuringiensis* against *H. armigera* and other insects (*Manduca sextai, Heliothis virescens, Trichoplusia ni, Helicoverpa zea*) and *Leptinotarsa decemlineata* (Colorado potato beetle) (Macintosh et al., 1990; Shao et al., 1998; Zhang et al., 2000, Zhu et al., 2007; Christtellier et al., 1992; Hubert et al., 2008). It was suggested that the mechanism of synergism might be prevention of activation of the toxin by gut serine proteases due to KSTI action (Zhu et al., 2007). In Australian *H. armigera,* differences in detection of resistance to Bt toxin between raw and dry roasted soybean flour were attributed to synergism by dietary KSTI in the raw flour diet. It was also suggested that greater susceptibility of cotton populations of *H. armigera* to Cry1Ac, compared to *Helicoverpa punctigera*, might have been due to dietary STI inhibition of detoxificative enzymes in *H. armigera* (Bird & Akhurst, 2007). The present data suggest that KSTI and/or other trypsin inhibitors in soybean flour may also act as spinosad and pyrethroid synergists against non-specific esterase mediated resistance in *H. armigera*.

Non-specific esterases, derived from cell adhesion proteins, are serine hydrolases with an ability to sequester, pyrethroids, spinosad and Bt toxins in Australian *H. armigera* (Gunning et al., 1996b; Gunning et al., 2007; Gunning & Balfe, 2002, Gunning et al., 2005;). These resistances can be synergised by esterase inhibitors (Gunning et al., 1999, Young et al., 2006, Gunning et al., 2007). It is possible that these non-specific esterases, found in the *H. armigera* midgut, are inhibited by dietary protease inhibitors such as KSTI, found in a raw or incompletely denatured soybean flour diet.

This study has indicated that rearing and bioassay of *H. armigera* on artificial diets that have been inadequately treated and thus give chronic exposure to abnormally high levels of active soybean protease inhibitors may be unsuitable for detection of metabolic-based resistance to spinosad or pyrethroids (bifenthrin).

5. Acknowledgements

The authors would like to thank, Industry and Investment NSW for supporting this work. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

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6

Conjugated Linoleic Acid: A Milk Fatty Acid with Unique Health Benefit Properties

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1. Introduction

Human diet comprises of milk and milk products in both developed and developing parts of globe. Milk fat is the major energy source in Indian diet but due to the fear of hypercholesterolemia, saturated fats have lead to avoidance of dietary fats especially of animal origin. However, milk contains a number of components with beneficial properties, one such compound associated with the fat phase is Conjugated Linoleic Acid (CLA) which has potential health benefits towards human beings.

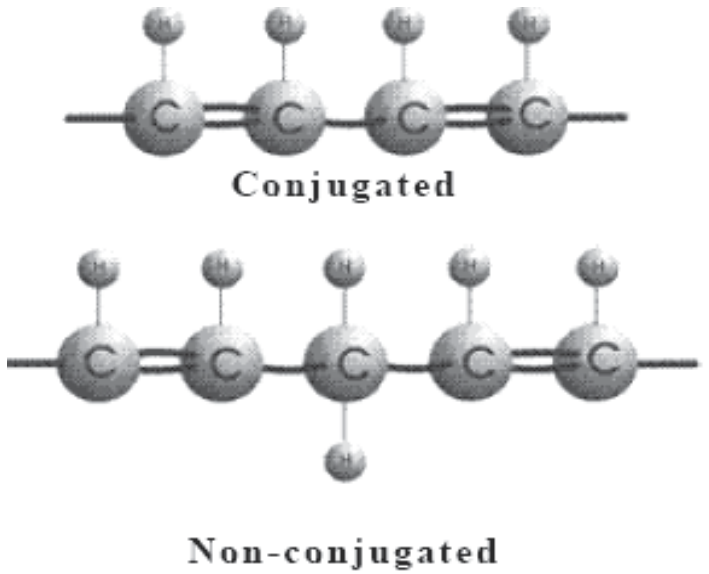
Conjugated Linoleic Acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12, $C_{18:2}$) with two conjugated double bonds at various carbon positions in the fatty acid chain. It is formed as an intermediate during the biohydrogenation of linoleic acid by linoleic acid isomerase from the rumen bacteria *Butyrivibrio fibrisolvens* (Kritchevsky, 2000) or from the endogenous conversion of *trans*-11, $C_{18:1}$ (Transvaccenic Acid) another intermediate of linoleic or linolenic acid biohydrogenation by Δ^9-desaturase in the mammary gland (Corl *et al*., 2001).

Milk fat is the richest natural dietary source of CLA. Milk contains an average 4.5mg CLA/g of fat (Kelly *et* al., 1998). Recent studies have shown that the CLA content of milk fat can be markedly enhanced by dietary manipulation especially those involving dietary addition of plant oils which are high in unsaturated fatty acids (Griinari and Bauman, 1999). Dietary increase of linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) is one of the feeding strategies for increasing the CLA concentration in milk which is the main precursor of CLA. The main sources of linoleic acid for feeding animals are cereals, oil seeds, oils etc (Kelly *et al*., 1998)

There is an increasing research interest towards the CLA and its potential health benefits such as anticarcinogenic, antiatherogenic, antidiabetic and immunomodulatory effects (Belury, 2002; Tyagi and kathirvelan, 2006). The potential anti-cancer effect of CLA is well documented, with the majority of experimental work conducted *in vitro* or in animal models (Ip et al., 1994). It has been demonstrated that CLA has the ability to affect mammary cancer, stomach cancer, skin cancer and prostate cancer. Most of the anticarcinogens are of plant origin but CLA is unique, it is present in food from animal sources and its anti-cancer efficacy is expressed at concentrations close to human consumption level. The unique structural and functional properties of CLA appear to modulate cellular process involved in carcinogenesis.

1.1 Chemical structure of CLA

CLA is a term given to a group of positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12, $C_{18:2}$ LA) in which double bonds are conjugated, instead of being in the typical methylene interrupted configuration. Each double bond can be *cis or trans* configuration giving rise to possible CLA isomers (Kelly *et al.*, 1998). Although conjugation of double bonds occurs as part of free radical mediated oxidation of linoleic acid. CLA is a true isomer of LA, in that it does not possess additional oxygen (Vandenberg *et al*., 1995).

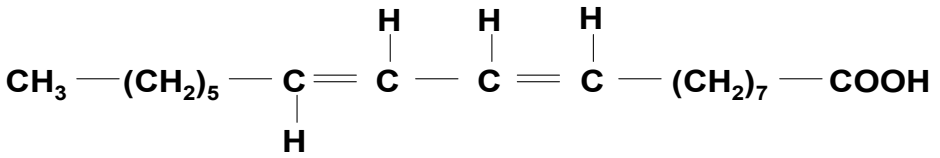


1.2 Isomers of CLA

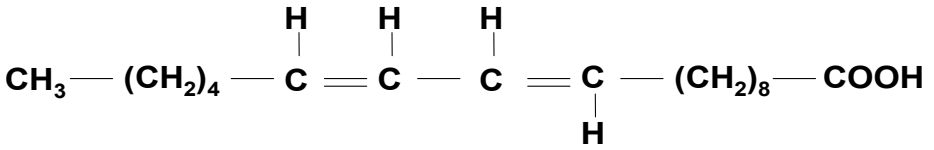
CLA therefore, includes 28 positional and geometrical isomers of which only *cis*-9, *trans*-11 and *trans*-10, *cis*-12 have thus far been proven to have biological activities (Park *et al*., 2003). Of the two physically important isomers *cis* -9, *trans* -11 (rumenic acid) is the most prevalent comprising 80-90% of the total CLA in food products from ruminants where as *trans*-10, *cis*-12 is present in small amounts at 3-5% of total CLA (Parodi, 2003). Analysis of cheese using silver ion- high performance chromatography and G-C electron ionization mass spectrometry showed that isomers of CLA present (in fat percent) total CLA were *cis*-9, *trans*-11 (78-84%); *trans*-7, *cis*- 9 plus *trans* -8, *cis* -10 (8-13%); *trans*-11,*cis*-13 (1-2%); *cis*-12,*trans*-14 (<1%) and the total *trans*/*trans* isomers (5-9%) (Parodi, 2003)

1.3 Sources of CLA

CLA occurs in many foods, however, the principal dietary sources are dairy products and other foods derived from ruminants. The CLA content of some common foods is shown in (Table 1).



cis-9, trans-11 CLA



trans-10, cis-12 CLA

Foodstuff	Total CLA (mg/g of fat)	Foodstuff	Total CLA (mg/g of fat)
Dairy products		*Meats*	
Homogenized milk	4.5	Ground beef	4.3
Condensed milk	7.0	Lamb	5.6
Butter milk	6.1	Pork	0.6
Mozzarella cheese	4.9	Chicken	0.9
Plain yogurt	4.8	Salmon	0.3
Ice-cream	3.6	Ground turkey	2.5

Table 1. CLA content of common foods (Chin *et al.*, 1992)

2. Biosynthesis of CLA

CLA found in milk and meat of ruminants originates from two sources (Griinari and Bauman, 1999). CLA is formed during ruminal biohydrogenation of linoleic acid and the second source is CLA synthesized by animal tissues from *trans*-11 $C_{18:1}$, another intermediate in the biohydrogenation of unsaturated fatty acid. Hence, the uniqueness of CLA in ruminant edible products relates to incomplete biohydrogenation of dietary unsaturated fatty acids in the rumen.

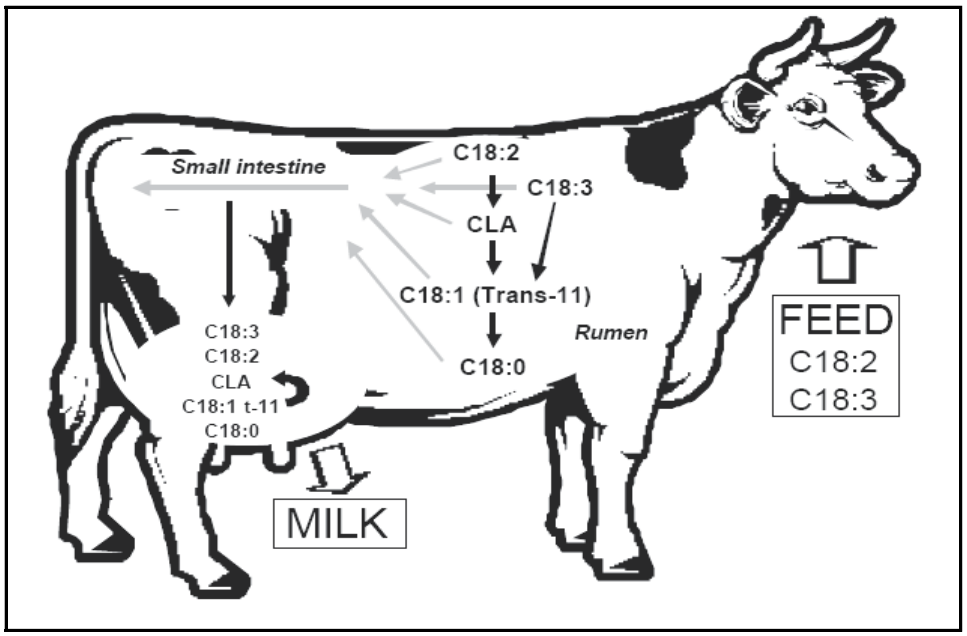
2.1 CLA synthesis in the rumen

Dietary lipids undergo two important transformations in the rumen (Devillard *et al.*, 2004). First, lipolysis - hydrolysis of ester linkages catalyzed by microbial lipase and second, biohydrogenation of unsaturated fatty acid.

The first reaction in linoleic acid (*cis-9*, *cis-12*) biohydrogenation is isomerization of the *cis*-12 double bond. Linoleate isomerase is the enzyme responsible for forming conjugated double bonds from the *cis*-9, *cis*-12 double bond structure of linoleic as well as α and γ linolenic acids. The enzyme is bound to the bacterial cell membrane and demonstrates an absolute

substrate requirement for a *cis*-9, *cis*-12 diene system and a free carboxyl group. The second reaction is a reduction in which *cis*-9, *trans*-11 CLA is converted to *trans*-11 $C_{18:1}$(*trans*-vaccenic acid). Isomerization of the *cis*-12 double bond was followed by rapid conversion of *cis*-9, *trans*-11 CLA to *trans*-11 octadecenoic acid. Hydrogenation of the *trans*-11 monoene occurred less rapidly, and it increased in concentration (Qiu, 2004). Therefore, *trans*-11 $C_{18:1}$ reduction seems to be rate-limiting in the biohydrogenation sequence of unsaturated C_{18} fatty acids. As a consequence, this penultimate biohydrogenation intermediate accumulates in the rumen and is, therefore, more available for absorption.

Similar to biohydrogenation of linoleic acid, biohydrogenation of linolenic acid begins with an isomerization followed by a sequence of reductions and terminates with the formation of stearic acid. Rumen biohydrogenation of α-linolenic acid produces *cis*-9, *trans*-11, *cis*-15 conjugated octadecatrienoic acid as the predominant initial isomerization product, and this is followed by reduction of the *cis*-double bonds. As a consequence, *trans*-11 octadecenoic acid is a common intermediate in the biohydrogenation of both α-linolenic acid and linoleic acid (Fig.1)



2.2 Endogenous synthesis

Griinari and Bauman (1999) proposed that a major proportion of CLA in tissue and milk lipids synthesized endogenously from *trans* vaccenic acid (TVA) by Δ^9 – desaturase, in mammary gland (Fig.1). Griinari *et al* (2000) examined the potential for endogenous synthesis of CLA by infusing TVA abomasally and measuring the changes in milk fat CLA. By day three it resulted in a 31% increase in milk fat CLA indicating that an active pathway for endogenous synthesis existed in the mammary gland.

Griinari *et al.* (2000) reported that the contribution of endogenous synthesis to the over all CLA content in milk fat was 84%, making it the primary source. Lock and Garnsworthy (2002) estimated the endogenous synthesis of CLA to be more than 80% of total collected

duodenal samples and estimated the rumen synthesis of CLA to be 4-7%. Endogenous *cis*-9, *trans*-11 CLA would originate from desaturation of *trans*-11 $C_{18:1}$ by delta-9 desaturase (Fig. 1). The enzymatic reaction introduces a cis double bond between carbons 9 and 10 of fatty acids.

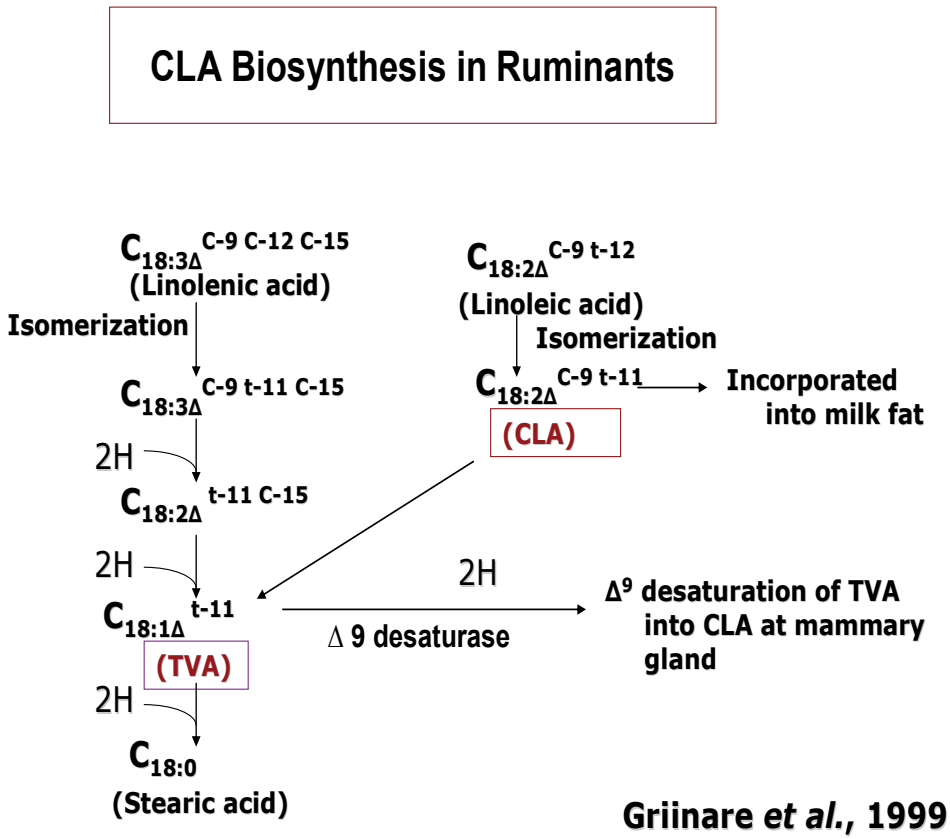


Fig. 1. CLA Biosynthesis in Ruminants

3. Role of rumen bacteria in CLA synthesis

Ruminants rely on microbial digestion of forages and supplementary feed materials in the rumen to provide nutrients that would otherwise be unavailable to the host animal. Ruminant diets typically contain polyunsaturated fatty acids, which are toxic to many rumen microorganisms (Harfoot and Hazlewood, 1997). However, to protect themselves against these toxic effects, rumen microorganisms have mechanisms to hydrolyse and biohydrogenate dietary lipids (Kemp and Lander, 1984).

Rumen bacteria play a major role in biohydrogenation. They convert C_{18} unsaturated fatty acids to stearic acid, via a number of intermediates. Biohydrogenation intermediates have received a lot of attention recently because of their potential effects on human health. CLA and *trans* vaccenic acid are one of the intermediates of fatty acid biohydrogenation in the rumen.

3.1 Biohydrogenation of unsaturated fatty acid by rumen bacteria

Rumen fluid contains bacteria, fungi and protozoa. Small amount of contribution is made by fungi and protozoa in biohydrogenation. Among which the major source of biohydrogenation is bacteria when compared to fungi and protozoa (Nam and Garnsworthy, 2007). Bacteria involved in biohydrogenation process can be divided into two groups i.e., A and B

Group A bacteria: They hydrogenate linoleic and linolenic acid to *trans*-11 octadecadienoic acid, and are not able to or incapable of hydrogenating octadecadienoic acid. Examples are *Butyrovibrio*, *Micrococcus*, *Ruminococcus* and *Lactobacillus.*

Group B bacteria: They are capable of hydrogenating a wide range of octadecadienoic acids, including *cis*-9 (oleic) and *trans*-11 (transvaccenic) acids as well as linoleic acid to stearic acid. Examples are *Fusocillus S*p and gram negative rods.

More than 250 bacterial strains from 14 genera were examined and strains belonging to the genera *Enterococcus*, *Micrococcus*, *Propionobacterium* and *Lactobacillus* were found to produce considerable amounts of CLA from linoleic acid.

Butyrivibrio fibrisolvens

CLA formation in rumen has mainly been associated with bacterial activity. The ruminal bacterium *Butyrivibrio fibrisolvens* has been used as a model for *cis*-9, *trans*-11 CLA production (Grinarii *et al.,* 2000). *Butyrivibrio fibrisolvens* a gram negative curved rod, strict anaerobic bacteria of moderate butyric acid producers with potent linoleic acid isomerase activity by using roll tube technique with ATCC media from rumen of cross bred cattle (Fig 2; Fig 3),has been isolated in our lab and research work is under publication.

Fig. 2. Translucent colony of *Butyrivibrio Sp* in roll tube.

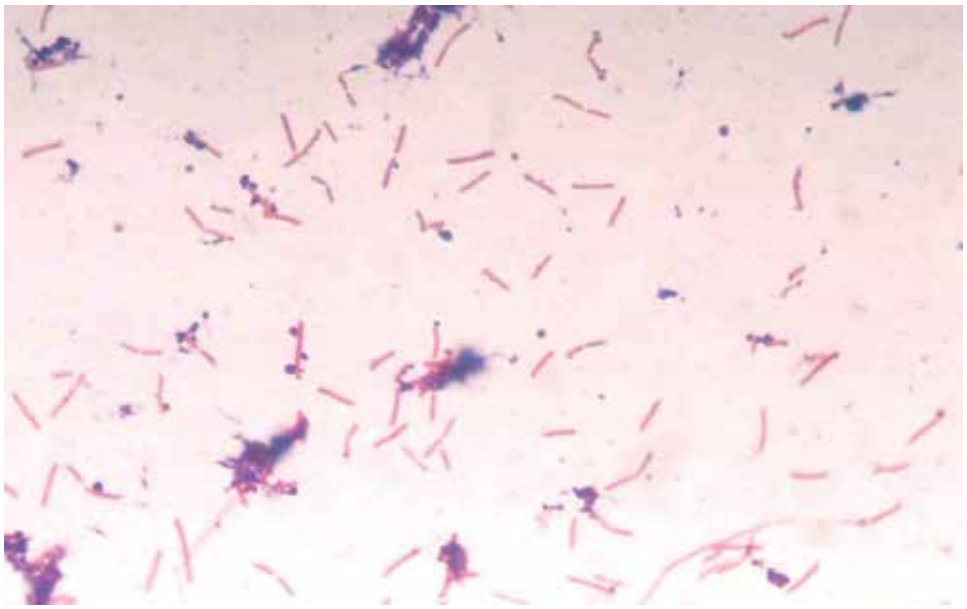


Fig. 3. Gram – ve, curved/straight rods of *Butyrivibrio Sp.*

3.2 Bacteria other than rumen origin involved in CLA synthesis

In addition to rumen microflora, microbial CLA production has also been reported in *Propionibacteria freudenreichii* used as dairy starter cultures (Jiang *et al*., 1998). Ogawa *et al*., (2005) reported the production of CLA from free linoleic acid by *Lactobacillus acidophilus*. Kishino *et al*., (2002) found that *Lactobacillus plantarum* formed high levels of CLA from free linoleic acid upon extended incubation. *Bifidobacteria* also produce CLA, mainly the *cis*-9, *trans*-11 isomer (Coakley *et al*., 2003).

4. Factors affecting CLA content in milk

A cow's diet, breed, age, non-nutritive feed additives, such as ionophores (Table 2) can affect the CLA content in milk fat. Among these factors, the diet is known to strongly influence the CLA content of milk and includes feedstuffs such as pasture, conserved forages, plant seed oils, cereal grains, marine oils and feeds and animal fat.

4.1 Lipolysis and biohydrogenation in rumen

Any change in the process of lipolysis or biohydrogenation will influence the supply of their intermediate and end products, including CLA, to the small intestine and ultimately their contents in the milk and meat. Replacement of forages with grain in the diet reduced the rates of lipolysis and biohydrogenation (Gerson *et al*., 1985). Increased proportion of nitrogen in the diet resulted in increased rates of lipolysis and biohydrogenation by rumen contents *in vitro* (Gerson *et al*., 1983). Lipolysis and hydrogenation reactions were more rapid with feed particles ranging from 1-2 mm size than from 0.1 to 0.4 mm size and this was due, at least in part, to microbial population density (Gerson *et al*., 1988). Biohydrogenation of fatty acids averaged 47% in the rumen of cows fed diets containing calcium salts of palm oil

and 71% with diets containing fat from animal or vegetable sources (Wu and Palmquist, 1991). Factors affecting ruminal fermentation and microbial population are undoubtedly the keys to control the regulation of biohydrogenation and CLA synthesis.

4.2 Diet, feed to the animal

CLA are highly correlated with either linoleic or alpha linolenic acid intake. It is well known that linoleic and linolenic acid are both indirect precursors of the CLA isomer *cis*-9, *trans*-11 (Aii *et al.,* 1999) but the highest concentration of this CLA isomer is generally obtained with linolenic acid rich diets (Dhiman *et al*., 2000). Dhiman *et al*., (1999) reported that cows grazing pasture had 500% higher CLA content in milk fat compared to cows fed a diet containing 50% conserved forage (hay and silages) and 50% grain. Tyagi *et al* (2007) reported that three fold (187 %) increase in total CLA content in milk of cow and buffaloes by feeding berseem fodder than the concenrtae feeding. About 48 to 56% of the total fatty acids in fresh forages consist of $C_{18:3}$ (Bauchart *et al*., 1984) and form substrate for ruminal biohydrogenation.

Dietary Factor	**Effect on CLA Content of Milk Fat**
Lipid Substrate	
Unsaturated vs saturated fat	Increased by addition of unsaturated fat
Type of plant oil	Greatest with oils high in $C_{18:2}$
Level of plant oil	Dose dependent increase
Calcium salts of plant oils	Increased as with free oils
High oil plant feeds	
High oil corn	Minimal effect
Soybeans	Heat processing will increase
Rapeseed vs soybean	Similar effect
Modifiers of Biohydrogenation	
Forage: concentrate ratio	Increased with high ratio
Fish oils	Greater increase than with plant oils
Monensin - ionophore	Variable effect
Dietary buffers	Little effect
Combination	
Pasture vs conserved forages	Higher on pasture

Source: Bauman *et al.,* (2001)

Table 2. Different substrate (dietary factors) that affect CLA content in milk fat

4.3 Rumen pH

Rumen pH has an important role in maintaining a viable rumen environment suitable for *Butyrivibrio fibrisolvens* involved in the biohydrogenation of linoleic and linolenic acid. Ruminal pH of 6.0 or above has a positive effect on TVA and CLA content in rumen cultures (Troegeler-Meynadir *et al.,* 2003). Qiu *et al.* (2004) observed that reduced ruminal pH reduces total and cellulolytic bacterial number and thus reduce biohydrogenation which in turn

increase CLA. Martin and Jenkins (2002) from their continuous culture data suggest that culture pH seems to have most influence on the production of *trans*-$C_{18:1}$ and CLA isomers by mixed rumen bacteria. When mixed ruminal bacteria were maintained in continuous culture on mixed soluble carbohydrate at a dilution rate of 0.05/hr, concentration of *trans*-$C_{18:1}$ were significantly reduced at a culture pH of 5.5. Because *trans*-$C_{18:1}$ monoenes serve as a precursor of CLA, to maximize CLA synthesis in rumen diet need to be formulated to maintain ruminal pH above 6.0.

5. Feeding strategies to enhance the CLA content in milk

Milk fat is the richest natural dietary source of CLA. Milk contains an average 4.5 mg CLA/g of fat (range 3-6 mg/g). The level of CLA in milk reflects the quantity, which is available for intestinal absorption (Loor and Herbein 1997). Therefore, there is a need to manipulate the feed in such a way to have higher CLA output in the reticulo-rumen for its increased absorption from the intestinal tract and eventually its secretion in the milk.

Dietary factors that affect CLA content have been grouped into four categories related to the potential mechanisms through which they act (Bauman *et al.*, 2001).

1. The first category includes dietary factors that provide PUFA substrates for rumen production of CLA and *trans*-11 18:1. This typically corresponds to increasing the dietary supply of plant and/or fish oils.
2. The second group consists of dietary factors that affect rumen bacteria involved in biohydrogenation, either directly or via changes in rumen environment. For example, modifying the forage: concentrate ratio of the diet, inclusion of ionophores and fish oil typically alters the biohydrogenation of PUFA.
3. The third category includes dietary factors that involve a combination of lipid substrates and modification of rumen biohydrogenation. For example, several investigations have demonstrated that feeding fresh grass to dairy cows doubles the CLA content of milk fat (Lock and Garnsworthy, 2002) and this cannot be fully explained in terms of PUFA supply to the rumen. Other factors or components of grass must promote the production of CLA in the dairy cows.
4. The fourth category is dietary supplements of CLA or *trans*-11 18:1 fatty acids. These must be protected from rumen biohydrogenation, typically with calcium soaps or formaldehyde.

5.1 Vegetable oil – An effective feeding strategy

Manipulation of animal diet primarily involves supplying linoleic acid or linolenic acid as substrates for rumen biohydrogenation. The vegetable/plant oil rich in these fatty acids seems to be an effective strategy for milk CLA manipulation in ruminants.

Feeding plant seed oils, such as sunflower, soybean, peanut, canola, and linseed also increases CLA content in milk (Dhiman *et al.*, 1999). There are a number of other research reports suggesting that feeding processed soybeans, canola, or flax seeds to dairy cows was more effective in increasing milk CLA content than feeding unprocessed seeds (Stanton *et al.*, 1997; Chilliard *et al.*, 2003).

Kathirvelan (2007) reported that buffaloes fed with three different concentrate mixtures (Table 3) with different fatty acid composition (Fig 4) had different level of CLA (Table 4 & Fig 5) and concluded that milk CLA increased 185 percent in buffaloes fed on mustard oil

(2%) plus mustard cake containing rations (19.50 mg/g fat) as compared to GNC fed buffaloes (6.84mg/g fat). Tyagi *et al. (*2006), reported higher level of total CLA (7.12 mg/ g fat) on feeding 30 percent mustard oil cake in concentrate mixture to lactating buffaloes than those fed on GNC based concentrate mixture (6.74 mg/g fat). In another experiment, the same author reported (Table 5) that feeding berseem fodder to goats resulted in high milk CLA content (13.3 mg/g fat) than concentrate plus berseem fed goats (8.5 mg/g fat).

Ingredients	**concentrate mixture (GNC)**	**concentrate mixture (Mustard cake)**	**concentrate mixture (Mustard cake+2% mustard oil)**
Groundnut cake (Expeller)	27	-	-
Mustard cake (Expeller)	-	39	39
Mustard oil	-	-	02
Maize	50	41	41
Wheat bran	20	17	17
Mineral mixture	02	02	02
Salt	01	01	01

Table 3. Ingredients in concentrate mixture (parts)

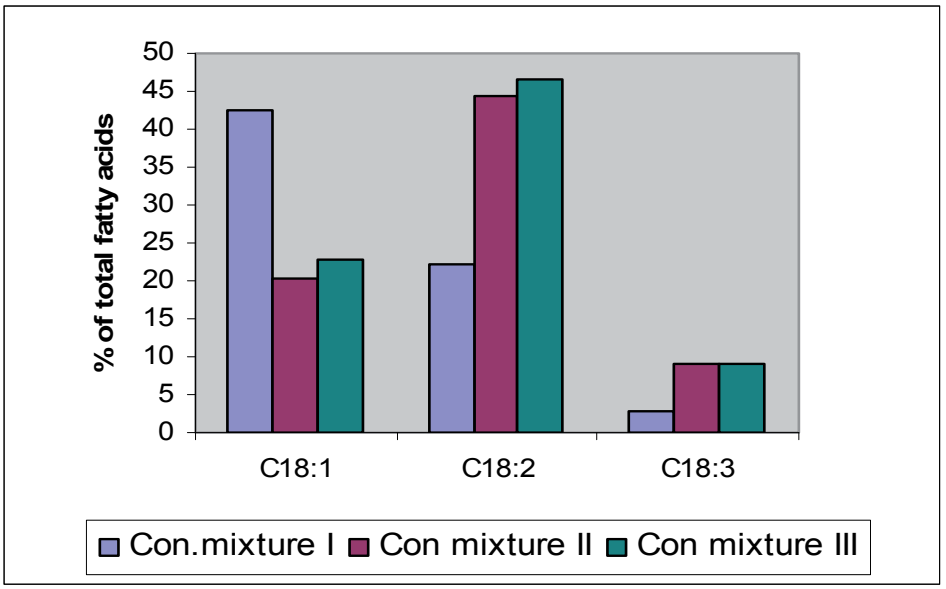


Fig. 4. The levels of $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ in three different concentrate mixtures

Fatty acid	concentrate mixture (GNC)	concentrate mixture (Mustard cake)	concentrate mixture (Mustard cake+2% mustard oil)
C4:0	30.99 ± 0.50	30.95 ± 0.32	30.72 ± 0.26
C6:0	15.63 ± 0.45	16.72 ± 0.45	14.84 ± 0.28
C8:0	8.27± 0.08	9.88 ± 0.64	9.21 ± 0.34
C10:0	16.17± 0.37	18.01± 0.74	17.38 ± 0.52
C12:0	23.13± 0.59	24.21 ± 0.35	22.75 ± 0.50
C14:0	105.20± 0.47	106.82 ± 0.84	105.23 ± 0.41
C14:1	5.36± 0.10	4.87 ± 0.24	5.63 ± 0.32
C16:0	265.12 ± 0.79	265.98± 1.55	271.42 ± 1.75
C16:1	13.40 ± 0.36	13.44 ± 0.34	14.77 ± 0.71
C18:0	144.43^{a} ± 2.19	139.33^{b} ± 0.82	133.06^{c} ± 1.06
C18:1 9-t	1.73 ± 0.17	1.96 ± 6.03	1.94 ± 0.12
C18:1 9-c	238.41 ± 1.68	238.27 ± 0.89	239.98 ± 1.16
C18:1 11-c (VA)	9.55^{a}± 0.45	13.01^{b} ± 0.06	16.98^{c} ± 0.04
C18:2 9-c,12-c	11.80± 0.25	11.38 ± 0.28	12.17 ± 0.34
C20:0	11.94 ± 0.43	9.27 ± 0.59	10.1 2 ± 0.20
C18:3 6-9-12-c	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.04
C18:3 9-12-15-c	3.23 ± 0.16	9.37 ± 0.17	9.09 ± 0.03
Total C18:3	3.23± 0.16	9.37 ± 0.17	9.17 ± 0.01
C18:2 9-c,11-t	6.17^{a}± 0.25	10.51^{b}± 0.23	16.94^{c}± 0.30
C18:2 10-t,12-c	0.67^{a} ± 0.08	1.61^{b} ± 0.16	2.56^{c}± 0.05
Total CLA	6.84^{a} ± 0.33	12.12^{b} ± 0.04	19.50^{c} ± 0.32
Total Omega 3 FA	4.12^{a} ± 0.16	10.36^{b}± 0.25	10.50^{b}± 0.06
Total Omega 6 FA	13.02 ± 0.34	12.64± 0.38	12.47± 0.36
Omega 6 : Omega3	3.18^{a} ± 0.15	1.22^{b}± 0.02	1.19^{b} ± 0.03

Values are Mean ± SE for n=5
Values with different superscript across a row differ significantly ($P<0.05$)

Table 4. Fatty acid composition (mg/g fat) in milk

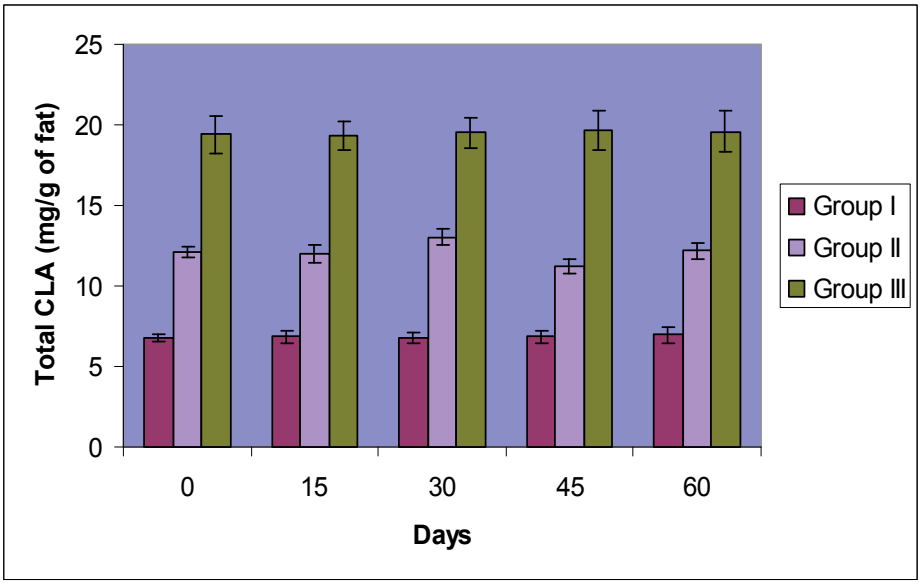


Fig. 5. Total CLA in milk (mg/g of fat) at fort night intervals (Zero day or first sampling after 30 days of adaptation period

Attribute	Treatment	
	Group I	Group II
Fat (%)	3.7 ± 0.15	3.8± 0.08
SNF (%)	6.6± 0.19	7.0 ± 0.16
Total solids (%)	10.3 ± 0.43	10.8 ± 0.26
CP (%)	3.5 ± 0.12	3.5 ± 0.19
	Fatty acids (mg per g)	
C4-14	203.0a ±7.21	227.8 [b] ±5.23
C15:0	18.7±0.65	21.4±0.53
C16:0	253.54[a] ±7.21	255.4 [b] ±6.14
C18:0	134.0 [a] ±4.25	148.9 [b] ±4.28
C18:1 11-trans (TVA)	50.8 [a] ±1.52	35.4 [b] ±0.85
C18:1 9-c	200.1 [a] ±5.24	231.2 [b] ±4.56
C18:2 9-c, 11-t	18.5 [a] ±1.12	10.5 [b] ±0.98
C18:2 10-t, 12-c	0.5±0.08	0.4±0.04
Total CLA	18.9 [a] ±1.30	10.94 [b] ±1.15
C18:3	13.3 [a] ±0.80	8.5 [b] ±1.30

Values with different superscripts across a row differ significantly (P<0.01)

Table 5. Milk composition of goats fed with berseem and berseem plus concentrate

Besides, the feeding of fish oil has been shown to enhance the CLA contents of milk fat. In some studies, fish oil/ fish meal was more effective at enhancing the CLA content of milk than adding similar amounts of soybean oil or combinations of fish oil and soybean oil through extruded soybeans or soybean meal (Ramaswamy *et al.*, 2001) since fish or marine oils are usually rich in long chain PUFA. The inclusion of marine feeds, such as fish meal or sea algae, into dairy cow diets has also been shown to enhance the CLA content of milk (Abu-Ghazaleh *et al.*, 2002)

5.2 Fatty acid composition of different vegetable oils

Dietary effect on CLA content is related probably more to the fatty acid composition of the diet/ feed used than any other factor. Poly unsaturated fatty acid composition of different oils (Table 6) and fatty composition of feeds and fodders used at NDRI cattle yard are reported in Table 7 (Kathirvelan, 2007)

Plant oil	Oleic acid (%)	Linoleic acid (%)	Linolenic acid (%)
Sunflower oil	22.8	67.0	0.45
Soybean oil	19.5	53.2	9.1
Corn oil	27.3	57.5	1.0
Cotton seed oil	16.5	57.4	0.33
Mustard oil	24.12	41.16	9.54
Ground nut oil	46.77	35.67	0.58
Linseed oil	17.7	15.4	57.2

Table 6. Oleic, Linoleic and Linolenic fatty acids concentration in different vegetable oils

Fatty acid	Maize Fodder	Wheat straw	Wheat bran	Maize grain	Groundnut cake	Mustard cake/oil
$C_{14:0}$	1.82	1.26	0.89	0.42	0.53	0.45
$C_{16:0}$	11.24	10.91	20.12	15.14	14.72	8.33
$C_{16:1}$	0.00	0.00	0.00	0.11	0.00	0.24
$C_{18:0}$	5.41	0.00	4.26	3.25	7.28	1.45
$C_{18:1}$	6.62	7.15	16.35	28.42	46.77	24.12
$C_{18:2}$	12.69	10.15	48.90	47.07	25.67	41.16
$C_{18:3}$	45.26	0.00	4.06	1.02	0.96	9.54
$C_{20:0}$	0.00	0.00	0.00	0.00	0.00	0.36
$C_{22:1}$	0.00	0.00	0.00	0.00	0.00	17.82

Table 7. Fatty acids composition of feeds and fodders (% of total fatty acids)

6. Effect of milk process on CLA content in dairy products

Processing of milk into a number of dairy products under normal conditions has no influence on the CLA contents (Shantha *et al.,* 1995). Use of different starter culture, processing conditions and aging periods had negligible effect on the total CLA concentration in dairy products (Shantha *et al.,* 1995). Kathirvelan (2007) reported that when milk was converted into ghee by creamery method, similar level of CLA was found in ghee

(19.54 mg/g fat) as in milk (19.50mg/g fat). Tyagi *et al* (2007) reported an increased level of CLA in ghee prepared by the indigenous method of ghee preparation due to lactic culture fermentation but no such increase was found in cheese and paneer (Table 8). However, Aneja and Murthi (1990) reported substantial increase in CLA content in ghee during its clarifying process at 120°C.

Milk	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	Total C18:3	t10, c12 CLA	C18:2 c-9t11 CLA	Total CLA	Total Ω-3	Total Ω-6	Ω-3: Ω-6
Group I	18.1±1.9	20.2±1.7	102.7±4.2	281.6±5.6	167.2±6.3	296.5±11.5	13.9±0.7	3.9±0.3	0.8±0.03	7.0±0.3	7.7±0.4	4.5±0.2	16. ±0.7	1:3.6
Group II	15.9±0.6	22.6±0.7	112.3±2.2	292.2±6.2	160.6±8.0	289.7.±6.7	13.1±0.4	9.1±0.4	1.7±0.01	11.7±0.7	13.4±0.7	9.9±0.4	16.0±0.5	1:1.6
Group III	14.0±0.8	20.8±0.9	110.1±3.1	317.9±6.1	124.9±4.9	293.7±10.8	13.8±0.6	13.5±0.8	2.5±0.02	14.4±1.1	17.0±1.2	14.2±1.0	16.0±0.6	1:1.1
LSD	3.3*	3.4	7.5*	14.5*	16.8*	8.9	2.5	4.2*	0.14*	2.2*	2.5*	1.8*	1.9	
						Cheese								
Group I	13.8±0.9	19.5±1..2	101.8±5.2	287.0±7.2	169.5±2.8	293.8±13.8	13.4±0.4	7.4±1.9	0.9±0.02	7.1±0.2	8.0±0.4	8.2±1.9	16.2±0.3	1:1.19
Group II	15.7±0.4	21.8±0.8	111.3±1.0	297.7±7.2	160.7±2.0	284.5±11.6	11.1±1.3	7.1±1.0	1.6±0.01	11.1±1.0	12.0±1.0	7.6±1.1	13.0±1.9	1:1.7
Group III	13.7±0.6	20.2±0.9	110.8±4.2	323.1±5.9	134. ±6.8	284.3±14.2	12.9±1.0	12.2±0.8	2.3±0.03	14.1±1.0	16.3±1.3	13.1±0.7	14.3±1.1	1:1.1
LSD	1.1*	1.6*	5.6*	9,6*	6.7*	9.7	2.6	2.2*	0.13*	1.1*	1.4*	1.6*	1.5*	
						Paneer								
Group I	14.0±1.2	20.2±1.8	103.9±6.6	285.5±6.2	167.8±4.2	296.2±22.1	13.3±0.6	6.4±0.9	0.8±0.02	8.3±1.2	9,1±1.3	7.3±1.0	16.1±0.5	1:2.2
Group II	14.1±0.3	19.7±0.5	104.9±0.8	297.3±3.3	151.8±10.9	293.5±15.0	14.2±0.9	10.4±2.8	1.8±0.02	11.5±2.1	13.4±2.6	11.6±3.1	16.5±0.6	1:1.4
Group III	14.4±0.7	21.0±0.8	110.7±1.9	308.1±10.3	132.3±12.5	292.8±10.5	13.3±0.3	11.8±1.6	2.3±0.04	16.0±2.5	18.3±3.2	13.1±2.0	15.2±0.7	1:1.1
LSD	1.1	1.4	5.8*	8.4*	6.1*	8.8	1.8	0.5*	0.14*	1.8*	2.1*	0.6*	0.7*	
						Ghee								
Group I	13.8±0.9	19.6±1.1	102.3±5.5	288.2±6.3	170.9±3.6	302.4±11.1	13.4±0.3	6.0±0.1	0.8±0.02	7.5±0.2	8.2±0.1	6.6±0.2	16.3±0.2	1:2.5
Group II	13.9±0.6	20.5±0.6	107.1±3.7	290.2±2.3	161.7±2.2	301.5±14.5	13.3±0.1	9.1±0.5	1.2±0.04	11.5±0.2	12.7±0.6	10.2±0.6	16.0±0.2	1:1.5
Group III	13.6±0.6	19.7±1.0	106.2±3.5	308.7±5.1	125.4±4.7	301.1±10.5	14.2±0.8	14.1±0.5	2.3±0.02	16.5±2.0	18.8±2.1	15.7±0.4	16.0±0.9	1:1.0
LSD	1.2	1.4	5.5	9,6*	14.5*	8.6	1.7	2.8*	0.13*	3.1*	3.9*	2.9*	0.9	

*P<0.01 The nutritional requirements of Group-I buffaloes were fulfilled through concentrate mixture, In Group- II concentrate mixture + Berseem and in Group –III, Berseem only.

Table 8. Level of CLA (mg/g) in buffalo milk and the dairy products prepared through different processing treatments

7. Potential health benefits of CLA

The biological properties of dietary CLA are currently attracting considerable interest because of its diverse physiological outcomes in animal studies. Beyond its nutritional value, dietary CLA is effective in suppressing tumor development during initiation, promotion and progression phases of carcinogenesis (Belury, 2002). Not only CLA is a powerful anticarcinogen, but it also has antiatherogenic, immunomodulating, growth promoting, lean body mass enhancing and antidiabetic properties. Hence CLA is considered being a functional food.

Growing evidences suggest that CLA has numerous health benefits towards human being. Milk and meat from ruminants are richest natural source of CLA, which has been shown to have anticancer properties (Parodi, 1994). CLA reduced plasma lipoproteins and early atherosclerosis in animals (Lee *et al.,* 1994). CLA was able to normalize impaired glucose tolerance in diabetic rats (Houseknecht *et al.,* 1998). CLA has been shown to have immunomodulatory properties by enhancing mitogen induced lymphocyte blastogenesis, lymphocyte cytotoxic activity and macrophage killing ability (Wong *et al.,* 1997)

7.1 Anticarcinogenic property of CLA

The recent interest in CLA began with the isolation from hamburger meat as an anticarcinogenic factor. Partially purified extracts from fried ground beef was shown to contain mutagenic modulator activity inhibiting the initiation of mouse epidermal carcinogenesis by 7, 12-dimethylbenz[a]anthracene, a pro-carcinogen (Ha *et al*. 1987). CLA was repeatedly shown to have anticarcinogenic effects in animal models for stomach neoplasia (Ha *et al*. 1990), mammary tumors (Ip *et al*., 1997), and skin papillomas (Belury *et al*. 1997). As low as 0.05% level of CLA is enough to significantly decrease the induced mammary tumors in rodents (Ip *et al*. 1997). CLA is effective in reducing the size and metastasis of transplanted human breast cancer cells and prostate cancer cells in severely compromised immunodeficient mice. CLA-enriched butterfat was reported to alter mammary gland morphogenesis and also to reduce the risk of cancer in rats (Ip *et al*., 1997).

Kathirvelan (2007) reported that feeding CLA enriched ghee (composition of rat diet given in Table 9) lowered around 37 percent gross tumors incidence (Table 10) in 7, 12 dimethyl benz (a) anthrazene induced mammary gland carcinogenesis in female Wistar rat than the control (soybean oil based diet) group. The mean tumour weight was high in soybean oil fed group (3.30 g) (Fig 6 & Fig 7) than the high CLA ghee fed group (1.29 g) (Fig 8). The common type of tumour (benign) found in the three groups were fibroma, adenoma and fibroadenoma but adenocarcinoma (malignant) type (Fig 9) was found only in soybean oil based diet group. Thus, CLA feeding not only inhibited the benign type tumour but malignant tumour as well.

7.2 Cancer modulation mechanism of CLA

There are various intriguing possibilities regarding its anti-carcinogenic action. However, the concrete anti-carcinogenic mechanism still is unclear.

7.3 CLA acts as an antioxidant

This is the first theory proposed in support of its anticarcinogenic action. CLA through dietary fat gets incorporated into neutral lipids and phospholipids, preferably in the former.

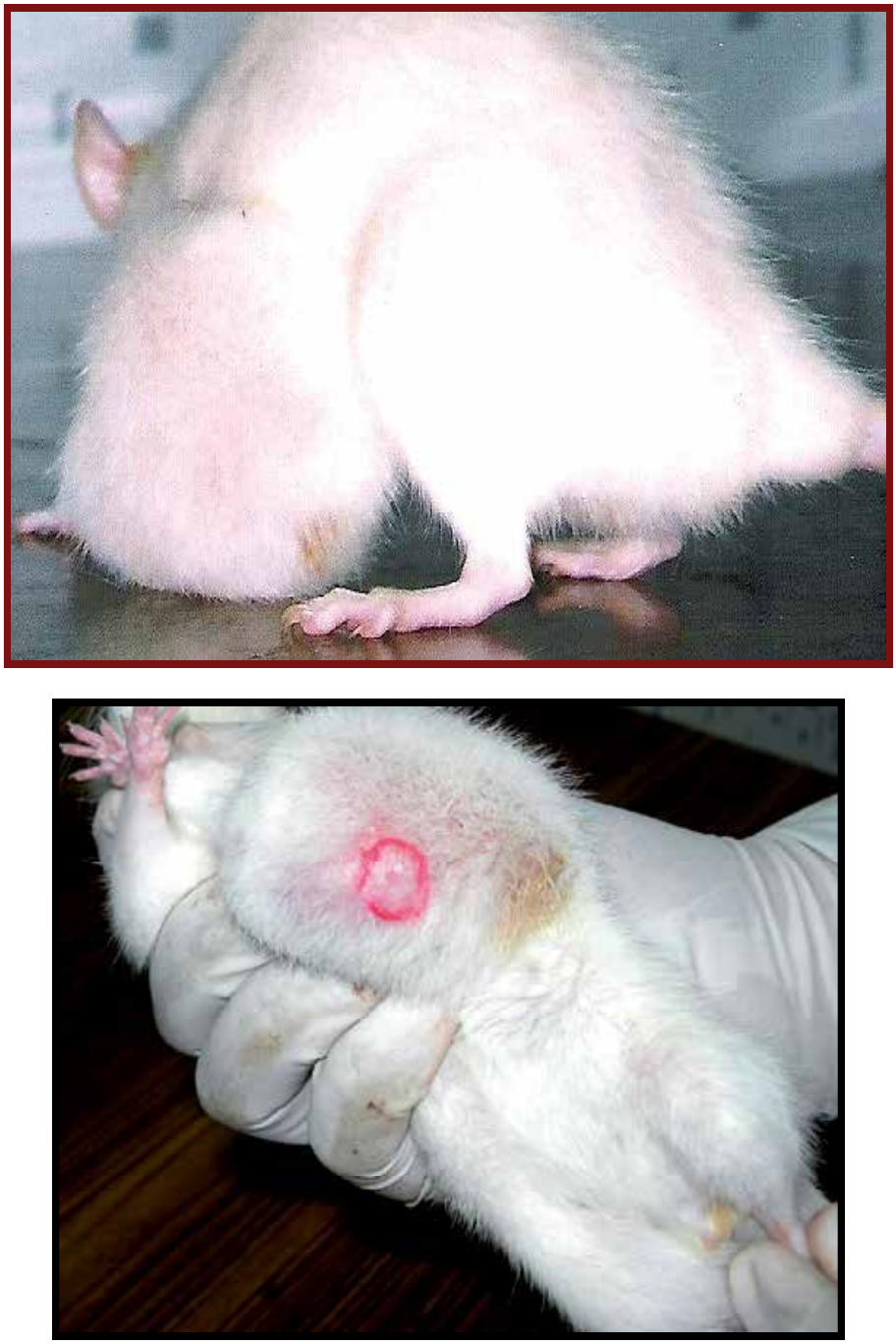
They act as free radical scavengers thereby providing an *in situ* 'defence mechanism' against membrane attack by dangerous oxygen free radicals (Ha *et al*., 1990).

Component	Soybean oil based diet	Low CLA (12.12 mg/g fat) ghee based diet	High CLA (19.54mg/g fat) ghee based diet
Bengal gram	540.0	540.0	540.0
Wheat	130.0	130.0	130.0
GNC	60.0	60.0	60.0
Soybean oil	200.0	-	-
Low CLA ghee	-	200	-
High CLA ghee	-	-	200
Skimmed milk powder	44.4	44.4	44.4
Mineral mixture	21.6	21.6	21.6
Vitamin mixture	2.0	2.0	2.0
Choline chloride	2.0	2.0	2.0

Table 9. Composition of rat diets (g/kg of diet)

Groups	**Percent tumour incidence***	**Percent Individual tumour incidence**				**Mean tumour weight (g)**
		Fibroma	**Adenoma**	**Fibro adenoma**	**Adenoma sarcoma**	
Soybean oil based diet	83.33 (25/30)	36.00 (9/25)	24.00 (6/25)	28.00 (7/25)	12.00 (3/25)	3.30 ± 1.39
Low CLA (12.12 mg/g fat) ghee based diet	63.33 (19/30)	42.11 (8/19)	21.05 (4/19)	36.84 (7/19)	0.00	2.42 ± 0.88
High CLA (19.54mg/g fat) ghee based diet	46.70 (14/30)	57.14 (8/14)	14.29 (2/14)	28.57 (4/14)	0.00	1.29 ± 0.27

Table 10. Effect of feeding CLA enriched ghee on mammary gland tumour incidence in 7, 12 DMBA administered rats



Rats were killed 32 weeks after DMBA administration
* Includes both palpable and histo pathological examination

Fig. 6. Rat showing the mammary tumour with the diameter approximately 3.4 cm (Soybean oil based diet)

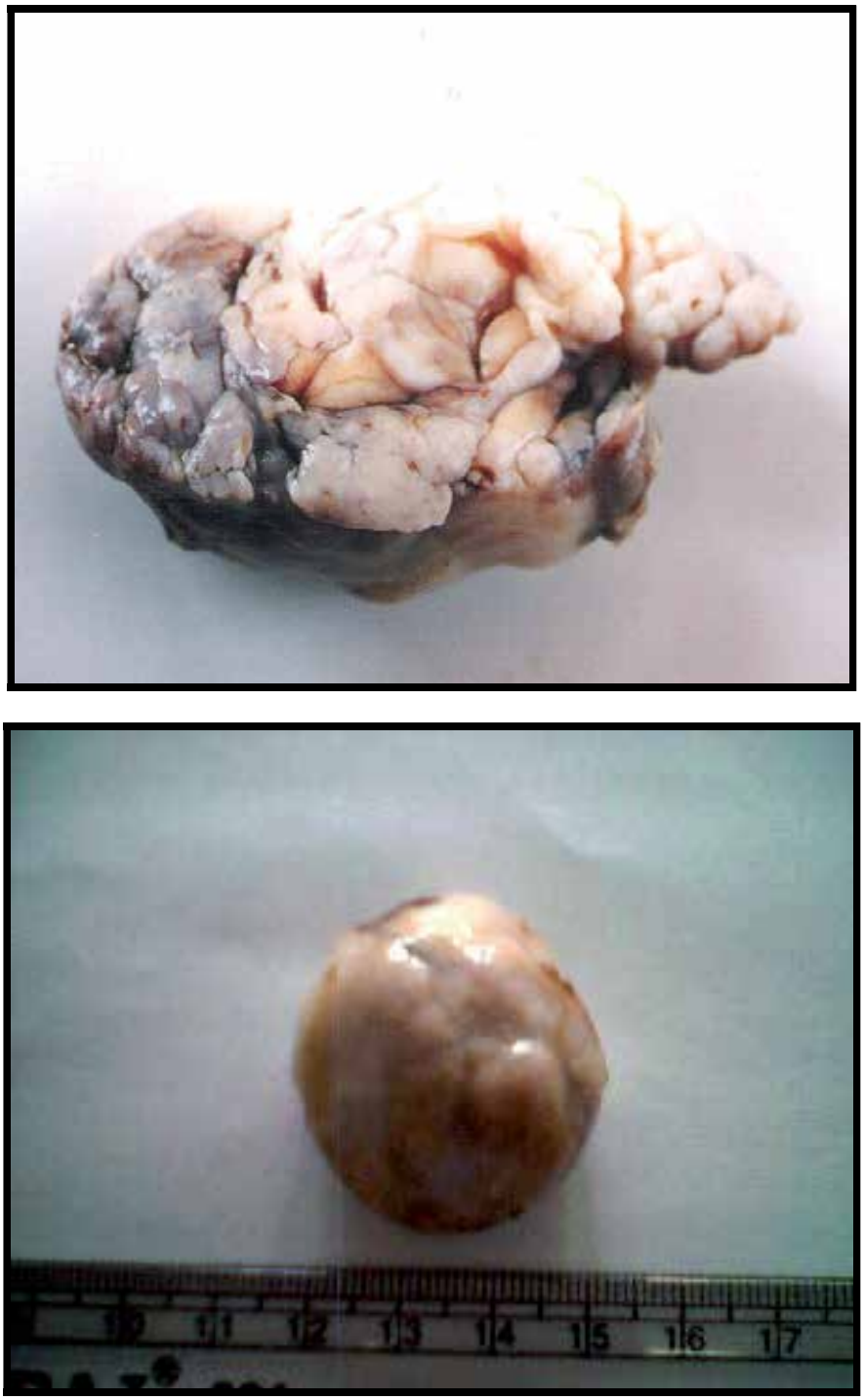


Fig. 7. Excised tumour showing 3.0 cm diameter (Soybean oil based diet)

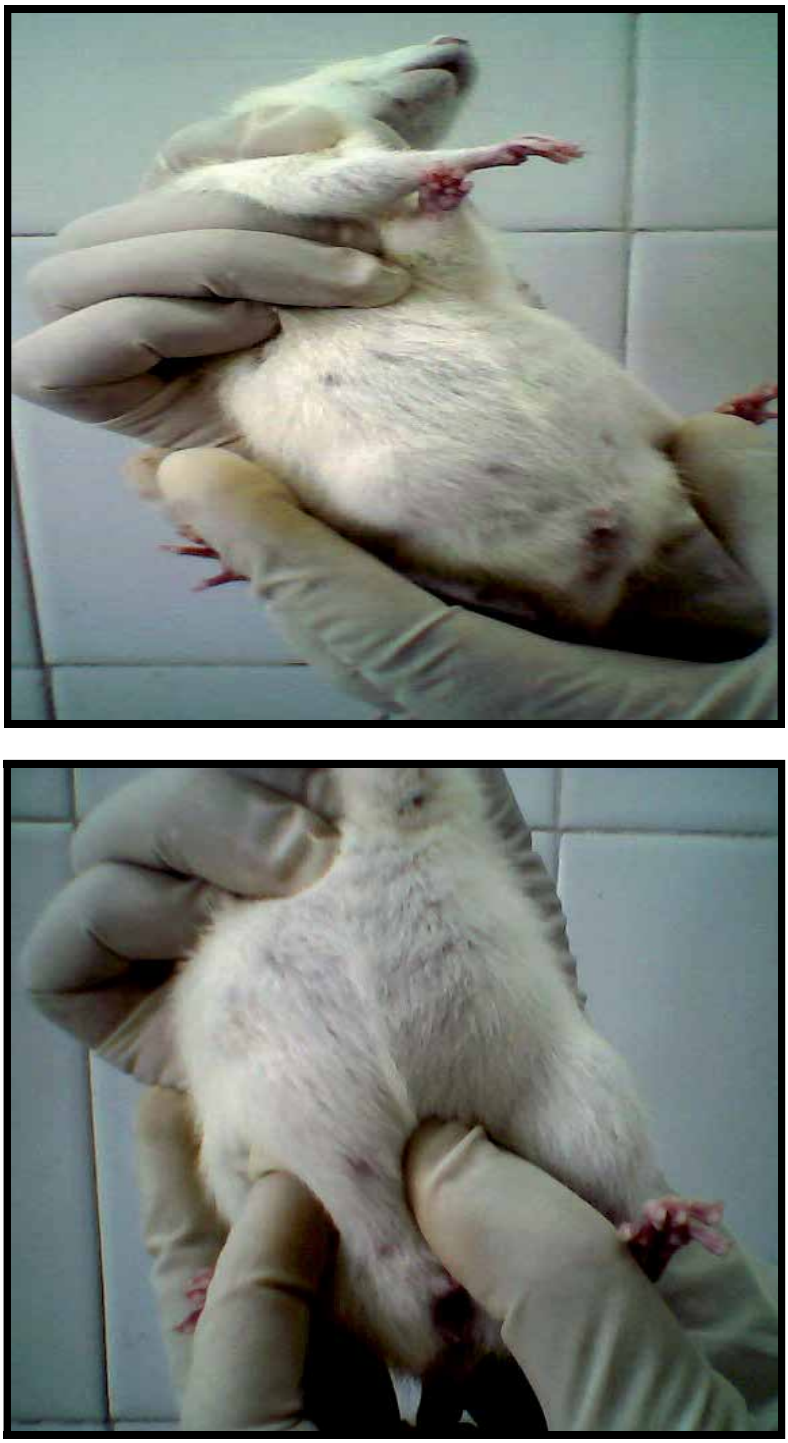
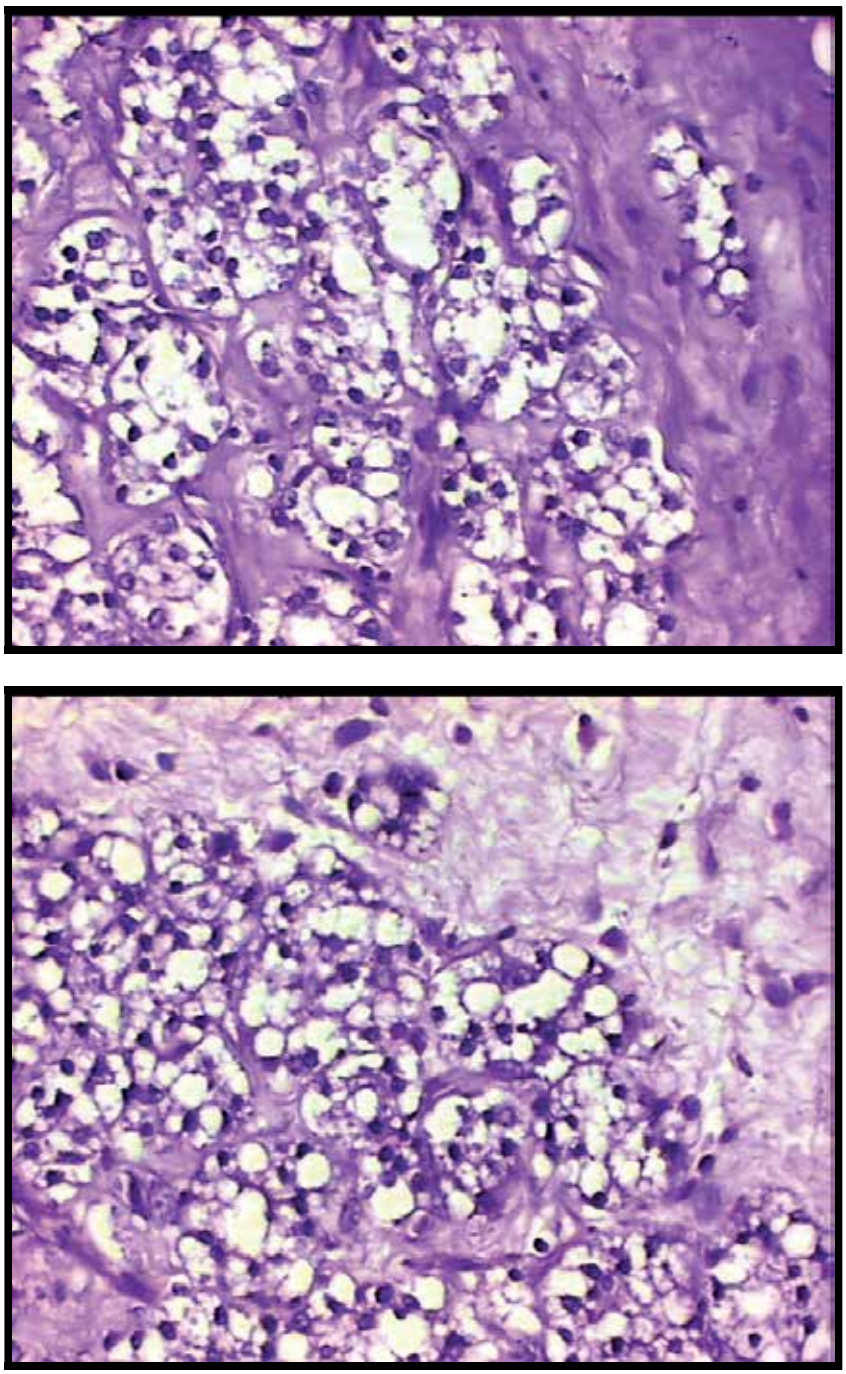


Fig. 8. Rats showing tumour with diameter of 1.1 cm (CLA ghee based diet)



(Soybean oil based diet) H&E x 400

Fig. 9. Adenocarcinoma showing stromal invasions with tumor cells

Compared with other antioxidants, CLA has been shown to be more potent antioxidant. It is more potent than alpha-tocopherol and as effective as butylated hydroxytoluene (BHT) in inhibiting iron-thiocyanate induced peroxide formation. In addition, CLA was shown to be as effective as vitamin E and butylated hydroxyanisole in inhibiting the formation of thio barbituric acid reactive substances (TBARS), as a bio marker often used to assess oxidation in biological systems.

Liver is the major organ in which most of the chemicals, drugs and carcinogens undergo metabolism (Krishnaswamy and Raghuramulu, 1999). Several environmental carcinogens have been reported to elicit hepatic oxidative stress and alter the activities of detoxifying and antioxidant enzymes during extra hepatic tumorigenesis (Aruna and Sivaramakrishnan, 1996). Antioxidant and detoxification enzymes can block carcinogenesis by acting as inhibitors of environmental carcinogens or mutagens (Cunningham and Lokesh, 1983).

Catalase and superoxide dismutase are the primary antioxidant enzymes involved in direct elimination of toxic free radicals which may result in amelioration of oxidative damage. Amelioration of oxidative damage by scavenging of free radicals formed during oxidative stress may protect cell against carcinogenicity and toxicity (Ketterer *et al.,* 1990). Glutothione-S-transferase is a biotransformation enzyme involved in the detoxification of xenobiotics, carcinogens, free radicals and peroxides by conjugating these toxic substrates with GSH (Glutathione reduced) thus ultimately protecting cells and organs against induced toxicity. Hence, enhancement of these enzymes by a natural or synthetic component may result in the amelioration or inhibition of extra hepatic tumorigenesis and CLA acts as chemopreventive agent by counteracting the carcinogen induced oxidative stress and elevating the levels of detoxifying enzymes (Ip *et al*., 1999). Ip *et al*. (1999) have clearly established a relationship between dietary CLA, oxidation and mammary carcinogenesis. Dietary CLA 0.25% or higher reduced the formation of TBARS in non initiated mammary tissue *in vivo* in parallel with CLA inhibition of mammary carcinogenesis. Kathirvelan (2007) observed that feeding CLA enriched ghee resulted in increased antioxidative as well as detoxifying enzymes in liver and mammary gland than the soybean oil based diet fed rats (Table 11).

Liver enzymes	**Cancer groups**		
	Soybean oil based diet	**Low CLA ghee based diet**	**High CLA ghee based diet**
Catalase (U/mg protein)	$479.91^{a} \pm 11.9$	$609.60^{b} \pm 7.62$	$730.67^{c} \pm 11.36$
SOD (U/mg protein)	$9.63^{a} \pm 0.79$	$15.86^{b} \pm 0.84$	$20.60^{c} \pm 1.41$
GST (U/mg protein)	$0.79^{a} \pm 2.50$	$1.19^{b} \pm 3.77$	$1.42^{c} \pm 4.51$
Mammary gland			
Catalase (U/mg protein)	$22.26^{a} \pm 1.71$	$35.66^{b} \pm 1.41$	$47.52^{c} \pm 1.43$

Values are Mean ±SE for n=12, One CAT Unit (U) is equivalent to 1µmol of H_2O_2 consumed /minute/ mg of protein at 25°C, One SOD unit is equivalent to the amount of enzymes that inhibit the reaction 50% , One GST unit is expressed as mmoles of cDNB conjugated /minute/mg protein.
Values with different superscript across a row differ significantly at ($P<0.05$)

Table 11. Effect of feeding CLA enriched ghee on liver and mammary gland enzymes activities in cancer induced groups

7.4 Inhibit arachidonic acid ($C_{20:4}$) derived eicosaonoid metabolism in the target organ (Belury et al., 1997)

Many types of cancer cells have an enzyme called cycloxygenase (Cox-2) and use it as a biological fuel to propagate rapidly. CLA inhibits the enzyme thereby modulation the prostaglandin biosynthesis from linoleic acid in normal course (Urquhart *et al*, 2002). The prostaglandins (PGE_2) secreted by cancer cells under the activity of Cox-2 acts as a tumor promoter. Unusual PUFAs ($C_{18:3.}$ $C_{20:3}$) derived from CLA leads to the formation of arachidonic acid which behaves differently than that of its usual nature when derived from linoleic acid in normal course (Iwakiri *et al*, 2002). This cerueval behavior leads to eicosaonoid inhibition viz. inhibition of prostaglandins and leukotrienes which further leads to reduction of diacylglycerol (Tumor promoting factor) upto 50% (Kritchersky et al, 2000). The cancer preventive activity of CLA is unlikely to be indicated by interference with the metabolic cascade involved in converting linoleic acid to eicosaonoid.

7.5 Reduce the formation of carcinogen – DNA adducts

CLA leads to a reduced formation of a carcinogen and DNA adducts (Moon *et al*, 2003) in a similar manner to substrate legend binding. This may be due to CLA check at the initiation point of carcinogenesis further preventing the binding of electrophile intermediates of DNA thereby causing permanent DNA lesion. This DNA containing cell when allowed to proliferate under promotion stage may lead to neoplastic cell.

7.6 Stimulate the lymphocyte proliferation

The lymphocytes had enhanced proliferation when stimulated with CLA, *in vivo* and *in vitro* (Miller *et al*, 1994) when a blend of isomers (cis-9, *trans*-11 and *trans*-10 *cis*-12) in the ratio 80:20 are given through the diet, the lymphocyte proliferation is observed (Majumdar *et al,* 2002). This in turn enhances cellular immunity by modulating phenotype and effect or functions of CD^{8+} lymphocytes (cells involved in both adaptive and innate immunity) involving T-cell receptors. The CD^{8+} lymphocytes are essential for the development of cell mediate protection against neoplastic cells. CLA enhanced the cytotoxic potential of peripheral blood lymphocytes by inducing the proliferation of cytotoxic CD^{8+} cells. Further, CLA was found to increase CD^4 lymphocyte population and NK cell function and number (Ochoa *et al*, 2004). CD^4 cells get differentiated into cytokines TH_1 cells and TH_2 cells (Thymus responsive cells). The TH_1 cells produce interleukin 1, 2 and γ-interferon, activate macrophages and further delayed hypersensitivity reaction whereas TH_2 cells produce interleukin -4, 5, 10 induces esinophiles decrease in number (CLA has anti allergic effect) and is more specialized in inducing B cells for immunoglobin production (CLA has immune stimulation effect) interleukin 1, 2 so derived by induction of TH_1 responsive cells further stimulate growth of γ-interferon in T-cells and NK cells. The γ-interferon inhibits PGE_2 synthesis which acts as a tumor promoter factor. Gamma interferon kill tumour either by usual mechanism of secretion of tumour necrosis factor (TNF) or by activated macrophages mediated selective cellular toxicity. NK cells in addition to direct lysis of tumour cells can also participate in antibody dependant cellular cytotoxicity.

7.7 Modulate the activity of phase-1 enzymes of Mixed Function Oxidase system (Benjamin et al, 2003)

CLA has been found to modulate the activity of Cytochrome-P450 (Cyt.P) and reduce the induction of ornithine decarboxylase (ODC) and GTPase activating protein kinase C (PKC).

This facilitates the oxidation and reduction of Cyt.P and hydroxylation as a result of which activated toxicant-Cyt.P complex and subsequently hydroxylated toxicant is available in the end of phase-1 reaction. The enzymes ODC+PKC play key roles in the activation of carcinogens and therefore are tumor production indicators.

7.8 Modulate gene expression (Carta et al, 2002)

The potential molecular mechanism by which CLA shows its anti-carcinogenic activity says that CLA acts as a legend for peroxisomes proliferation receptor (PPAR) which is a steroid hormone receptor (nuclear receptor) . This binding after the transcription process through sequential steps. The regulatory gene is lost and so the target genes are altered. This activates the endonuclease action; influx of Ca^{2+} takes place in the cytoplasm which leads to DNA fragmentation. The cell growth and differentiation is arrested and thus induce apoptosis.

7.9 CLA and atherosclerosis

While considerable research has focused on a potential anticarcinogenic effect of CLA, there are few studies indicating that CLA may also reduce the risk of cardiovascular diseases in animal models. Unlike linoleic acid, there is a paucity of information regarding the effect of dietary conjugated linoleic acid on plasma lipoproteins and aortic atherosclerosis.

Lee *et al.*(1994) first tested whether CLA might affect the initiation and progression of atherosclerosis lesions in rabbits through its effect on lipid peroxidation. They reported that rabbits fed an atherogenic diet and supplemented with 0.5g CLA per day for 22 weeks had significantly less plasma triglyceride, plasma LDL-cholesterol (LDL-C) and LDL-C/HDL-C ratio than control animals. CLA feeding also resulted in fewer aortic fatty lesions. Subsequently, (Nicolosi *et al*., 1997) reported that hamsters fed CLA collectively had significantly reduced levels of plasma total cholesterol, non-high density lipoprotein cholesterol, (combined very-low and low-density lipoprotein) and triglycerides with no effect on high density lipoprotein cholesterol, as compared to controls. Kathirvelan (2007) reported that low and high CLA feeding reduced total cholesterol by 52.17 and 26.06 percent, triglycerides by 23.00 and 11.00 percent, however, HDL-c increased by 17.82 and 33.26 percent than soybean oil fed rats (Table 12). He concluded that, antiatherogenic property of CLA was proved by decreasing the total cholesterol, triglycerides, LDL-cholesterol (Fig 10) and atherogenic index (Fig. 11) and increasing the HDL-cholesterol blood plasma. In addition to this, the level of cholesterol and triglyceride deposition in liver and aorta were lower in CLA fed groups (Table 13).

In the literature, dietary CLA has been reported to have controversial results on atherosclerosis in mice. Noone *et al*., (2002) reported that CLA actually did not reduce the incidence of atherosclerosis, but increased the incidence of fatty acid streaks in mice compared to control-fed mice. Study conducted by Munday *et al.* (1999) contradicts the finding of studies conducted in rabbits and hamsters (Lee *et al*., 1994: Nicolosi *et al*., 1997). It is possible that dietary CLA has different influences on the fatty acid and cholesterol metabolism in different animal species. More research is necessary to elucidate the effects of dietary CLA on lipid metabolism and atherogenesis in animal models and eventually human beings.

Since it is difficult to study the effect of CLA on atherosclerosis in humans, an indirect approach by measuring various potential heart disease markers is required (Belury and Kempa-steczko, 2000). Lipid atherogenic risk markers were more favorably influenced by *cis*-9, *trans*-11 isomer than a mixture of CLA or fish oil (Valeille *et al*., 2004) when healthy human subjects were used in a double-blind placebo controlled intervention trial. Noone *et*

al. (2002) demonstrated that CLA isomers improved very low-density lipoprotein cholesterol and plasma triacylglycerol metabolism suggesting that some of the cardio-protective effects of CLA shown in animal studies were relevant to humans as well.

Parameters	Base line levels	Groups		
		Soybean oil based diet	Low CLA ghee based diet	High CLA ghee based diet
Total Cholesterol (mg/dL)	58.09 ± 1.24	81.33^{a} ± 2.91	74.40 ^{b} ± 1.68	69.93^{c} ± 1.44
HDL-C (mg/dL)	34.72 ± 0.78	35.51^{a} ± 0.91	41.84^{b} ± 0.54	47.32^{c} ± 0.96
Triacylglycerol (mg/dL)	40.87 ± 0.77	73.23^{a} ± 1.08	65.01^{b}± 1.16	56.21^{c} ± 0.42
LDL-C (mg/dL)	14.26 ± 1.72	26.79^{a} ± 3.53	19.57^{b} ± 1.86	14.38^{c} ± 1.62
Atherogenic Index (AI)	0.410 ± 0.06	0.971^{a} ± 0.12	0.570^{b} ± 0.05	0.244^{c}± 0.04

Values are Mean±SE for n=8
Values in rows with different superscript differ significantly (P<0.05)

Table 12. Plasma Lipids profile of Wistar rats at baseline and after 16 weeks of CLA enriched ghee feeding.

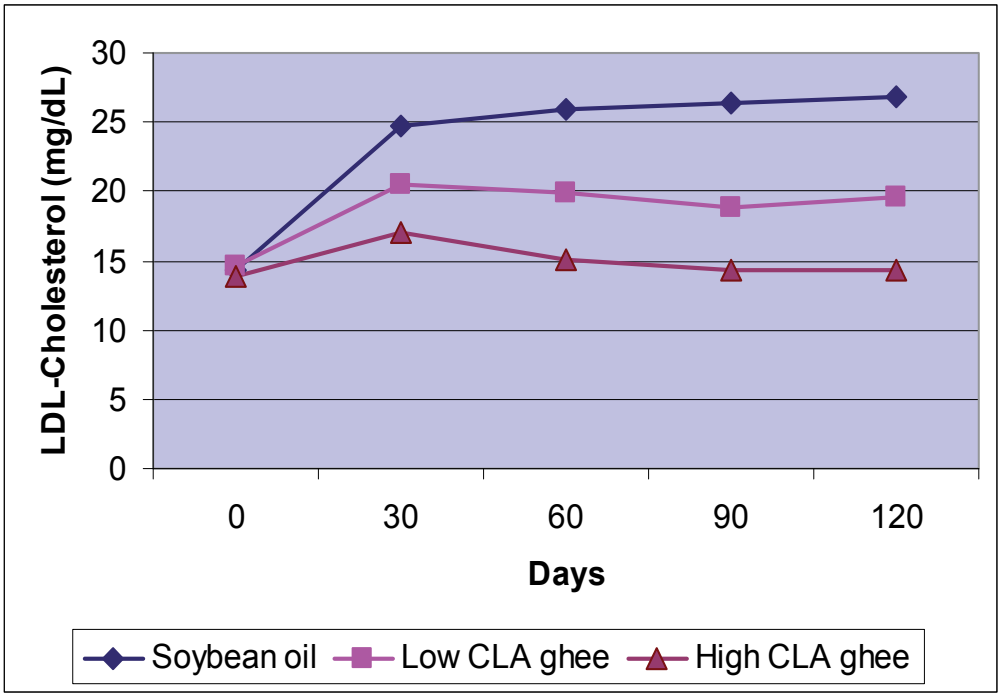


Fig. 10. Plasma LDL (mg/dL) levels in rats fed on CLA enriched ghee diet

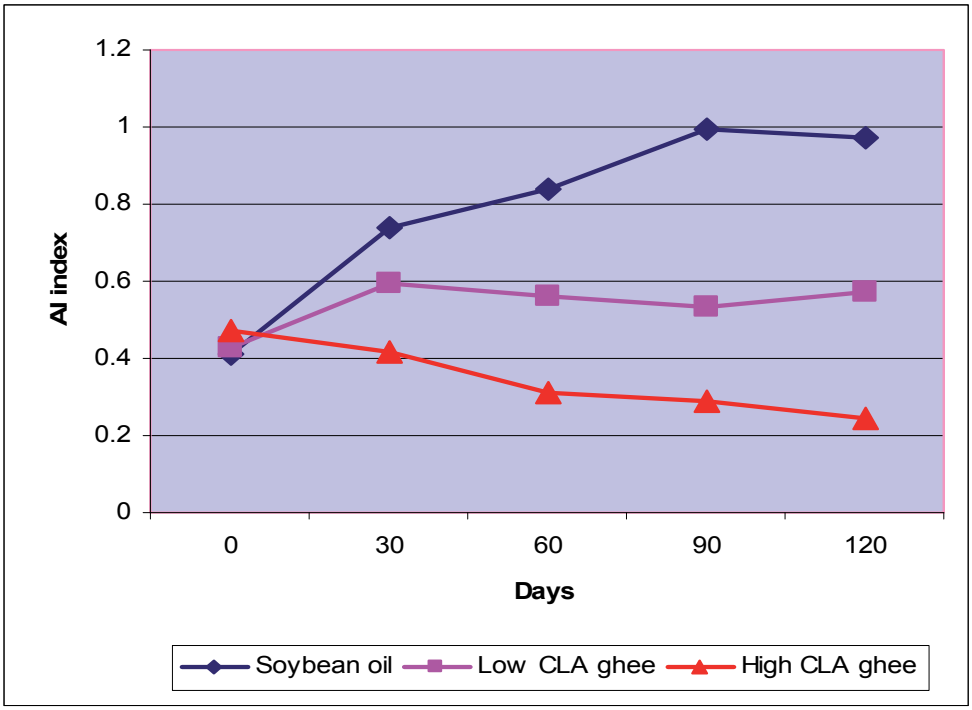


Fig. 11. Atherogenic index in rats fed on CLA enriched ghee diet

Groups	Liver		Aorta	
	Cholesterol	Triglycerides	Cholesterol	Triglycerides
Soybean oil based diet	1.05^{a} ± 0.029	10.08^{a}± 0.145	9.46^{a} ± 0.029	7.06^{a} ±0.073
Low CLA ghee based diet	0.84^{b} ± 0.013	7.12^{b} ± 0.218	6.95^{b} ± 0.015	5.09^{b} ± 0.123
High CLA ghee based diet	0.53^{c} ± 0.008	4.50^{c} ± 0.143	3.64^{c} ± 0.009	2.49^{c} ± 0.109

Values are Mean ± SE for n=8
Values in rows with different superscript differ significantly (P<0.01)

Table 13. Lipid profile (mg/g tissue) in liver and aorta tissue of rats fed on CLA ghee diet

7.10 CLA and lipid metabolism

A major effect of CLA in this respect is the reduction in lipid uptake by adipocytes (Pariza *et al*., 2003) which leads to the reduction in body fat gain (Kim *et al*., 2002). Azain *et al.* (2000) reported that the reduction in body fat mass in rats was the result of reduced adipose tissue size rather than cell number. Similarly Poulous *et al.* (2001) found a reduction in cell size, but not the cell number in rats which had less body fat in response to CLA supplementation.

Carta *et al.* (2002) concluded that a regular intake of CLA and or TVA as its precursor should work as an excellent preventive agent that would modulates lipid metabolism. Kathirvelan (2007) found that feeding CLA ghee (20 % in the diet) to male wistar rats doesn't increase the body weight as compared to the control group (Table 14)

week	Average weekly body weight (gram)		
	Soybean oil diet (Control)	Low CLA ghee diet	High CLA ghee diet
0	131.13 ± 4.70	125.00 ± 2.85	128.57 ± 3.45
2	169.29 ± 5.09	165.01 ± 4.03	163.14 ± 4.68
4	187.86 ± 6.04	185.08 ± 6.22	185.71 ± 7.02
6	203.57 ± 3.45	198.52 ± 5.59	195.29 ± 4.17
8	215.00 ±5.34	214.29 ± 7.58	212.86 ± 4.93
10	231.69 ± 8.55	228.33 ± 7.42	227.51 ± 6.95
12	243.33 ± 7.42	240.00 ± 6.35	239.17 ± 4.47
14	254.00 ± 5.88	257.50 ± 8.14	252.51 ± 6.32
16	263.33 ± 4.81	265.00 ± 6.67	262.12 ± 4.21
Over all mean	211.02 ±13.51	208.81±14.23	207.43 ±13.73

Values are Mean ±SE for n=8

Table 14. Average weekly body weight of rats fed on CLA enriched ghee diet

Studies in rats and mice have shown that feeding CLA at the level of 0.5% in total diet produced small reduction in body fat gain in growing animals (Pariza *et al.,* 1997; Park *et al.*, 1997). Whilst the actions of CLA in inhibiting body fat accumulation have received considerable attention because of increasing concern for marked increases in obesity in western societies, care should be taken in extrapolating these findings to man until more information is available. The specific mechanism by which dietary CLA reduces body fat content is likely to vary from one animal species to another. The results of animal studies are also not conclusive. The mechanism by which CLA alters lipid metabolism and body composition in animals is not fully elucidated. It may be tissue and species-specific. In rodents, CLA-induced alteration of lipid metabolism appears to involve increase in rates of lipolysis and fatty acid oxidation. Support for this mechanism comes from the observation of increased hormone sensitive lipase activity and enhanced carnitine palmitoyl transferase activity in several tissues of mice fed CLA (Pariza *et al.,* 2003).

Peroxisome proliferators activated receptor alpha (PPAR∝), one of the nuclear receptors related to the modulation of environmental and dietary stimuli (Schoonjan *et al.,* 1996), is likely to be involved in the regulation of the gene expression of fatty acid beta oxidation enzymes by dietary CLA. It has been demonstrated that CLA is a potent legend and activator of PPARα (Belury *et al.,* 1997) but Peterson *et al.* (2003) observed that a mixture of CLA isomers increased the gene expression of hepatic fatty acid beta oxidation enzymes through both PPARα dependent and independent mechanisms. The activity of fatty acid

synthatase (FAS), a key enzyme of fatty acid synthesis, was also significantly decreased by dietary CLA as compared to the control diet in the liver of rats.

7.11 CLA and diabetes

Feeding of CLA to rats prone to developing diabetes normalized glucose tolerance and improved hyperinsulinemia as effectively as currently used medications (Houseknecht *et al*., 1998). CLA was fed at 1.5% (by weight) of the diet for 2 week in diabetes induced rats. The study was short-term and needs to be replicated and extended before the results can be applied to human health. Nonetheless, if CLA can improve glucose homeostasis and inhibit body fat accretion as demonstrated in mice, rats, and pigs, then CLA may be beneficial to humans prone to diabetes (Ip *et al*., 2003)

7.12 CLA and immune system:

Cook *et al* (1993) showed that CLA not only enhances immune response, but also protects tissues from collateral damage. Sugano *et al* (1999) proposed that the immune enhancing effect of CLA was by modulating eicosanoid and immunoglobin production. CLA also diminished lipopolysaccharide induced inflammatory events in macrophages through reduced mRNA and protein expression of nitric oxide synthatase and cyclooxigenase-2 as well as subsequent production of nitric oxide and prostaglandin E2 (Cheng *et al*., 2004). Kathirvelan (2007) observed that feeding of CLA enriched ghee to mammary gland cancer induced female wistar rats reduced level of plasma nitric oxide than the non CLA fed rats (Table 15). Cook *et al* (1993) suggested that CLA prevents immune associated wasting by

Days	Cancer groups		
	Soybean oil based diet	Low CLA ghee based diet	High CLA ghee based diet
0	9.25^{a} ±0.31	8.59^{a} ±0.17	8.41^{a} ±0.18
30	18.52^{a} ±0.33	16.45^{b} ±0.41	15.45^{c} ±0.38
60	25.70^{a} ±0.50	19.44^{b} ±0.20	18.38^{c} ±0.23
90	30.28^{a} ±0.49	25.43^{b} ±0.22	23.43^{c} ±0.45
120	38.18^{a} ±0.33	33.16^{b} ±0.53	28.20^{c} ±0.28
150	40.92^{a} ±0.50	35.34^{b} ±0.31	32.54^{c} ±0.25
180	42.46^{a} ±0.46	35.83^{b} ±0.54	32.24^{c} ±0.37
210	43.26^{a} ±0.29	38.50^{b} ±0.28	33.20^{c} ±0.15
240	43.00^{a} ±0.41	37.56^{b} ±0.34	33.07^{c} ±0.47
Over all mean	32.40 ± 3.87	27.81 ± 3.39	24.99 ± 2.86

Values (μmol/ml) are Mean □ SE for n=12
Values in a row with different superscript differ significantly ($P<0.05$)

Table 15. Effect of feeding CLA enriched ghee diet on plasma nitric oxide (μmol/ml) level in mammary gland cancer induced rats

protecting nonlymphoid tissues from the adverse effects of cytokines, which are growth suppressants, because CLA influences the immune system by altering the effects of cytokine, interleukin, leukotriene and many immunoglobulin. Sebedio *et al*., (2000) hypothesized that CLA indirectly affects the function/production of tumor necrosis factor-α

(TNF-α) and interleukin-1 (IL-1). Yu *et al*. (2002) has shown that CLA exhibits anti-inflammatory effects by negatively regulating the expression of certain pro-inflammatory genes. By inducing the activity of peroxisome proliferators activated receptor gamma (PPAR-γ) via CLA, there is a decrease in the production of pro-inflammatory products such as nitric oxide and TNF-α (Yu *et al*., 2002).

7.13 CLA and bone metabolism

Watkins *et al* (1999) found a higher rate of bone formation in chicks fed butterfat, which was suggested to be due probably to increased CLA intake. Dietary CLA led to differences in CLA enrichment of various organs and tissues, bone marrow and periosteum containing the highest concentration of CLA and brain the lowest. Enrichment of chondrocytes with CLA affected collagen synthesis in a dose dependent fashion (Watkins *et al.,* 1999). Reduced production of arachidonic acid and PGE_2 in the chondrocytes was suggested to be the possible mechanism. Such changes in bone biomarkers and bone formation rates in rats were associated with increased *cis*-9, *trans*-11 CLA in bone tissue lipids. McDonald (2000) suggested that increased ash content in CLA fed animals (Park *et al*, 1997) is due to protection of bone loss from cytokines. Further investigation is needed as to how bone metabolism is affected by CLA and mechanism of action related with it.

8. Conclusion

Conjugated linoleic acid is unique; unlike the most naturally occurring anticarcinogenic substances found mainly in plants, it is present in food from animal sources. Milk fat is the richest natural source of CLA and could be increased by manipulation of the nutritional regimes of the animals. Diverse biological roles of CLA in mediating cancer, diabetes, lipid metabolism, atherosclerosis, immune function, bone modeling etc. observed in animal models are quite compelling. Although there have been a few attempts at verifying the positive effects of CLA in human health through case control studies, it is not yet possible to clearly state that CLA supports all the those benefits in humans as well. Limited available literature in humans, however also supports the findings observed in animals and tissue culture models even though the results are inconclusive and even conflicting at times. As a result, natural enrichment of food products through manipulation of animal diet may contribute to the overall goal of obtaining the positive health benefits associated with CLA in the immediate future.

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7

Lunasin, a Cancer Preventive Seed Peptide

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1. Introduction

Cancer has become one of the most common causes of death in industrialized countries and has been defined as the medical challenge of our times. In 2008, 12.7 million new cancer cases and 7.6 million deaths from cancer worldwide have been reported (Ferlay et al., 2010). These authors also estimate that by 2020, approximately 17 million new cancer cases will be diagnosed, and 10 million cancer patients will die. Evidences have shown that as many as 35% of these cases may be related to dietary factors (Manson, 2003), and thus, cancer can be prevented by modifications of nutritional and lifestyle habits. The rising prevalence of cancer worldwide and the corresponding rise in health care costs is propelling interest among researchers and consumers for multiple health benefits of food compounds, including reduction in cancer risk and modification of tumour behaviour (Béliveau, 2007; Kaefer and Milner, 2008). Epidemiological evidence, cell culture and animal tumour model studies have demonstrated that a large number of natural compounds present in the diet could lower cancer risk and even sensitize tumour cells in anti-cancer therapies (de Kok et al., 2008). Daily intake of food rich in anticancer molecules could be compared to a preventive, non-toxic version of chemotherapy that is harmless to the physiology of normal tissue and stops microtumours (Béliveau, 2007).

Phytochemicals are compounds present in plant foods with capacity to affect and regulate multiple key proteins involved in regulation of cellular proliferation, differentiation, apoptosis, angiogenesis or metastasis, and thus, to affect the different carcinogenesis stages (Fimongnari et al., 2008; Ramos, 2008; van Breda et al., 2008). Chemopreventive and chemotherapeutic properties against human cancers have been revealed for different phytochemicals, such as epigallocatechin gallate [(-)-EGCG] (green tea polyphenol), genistein (soybean), apigenin (celery, parsley), isothiocyanates (broccoli), anthocyanins (berries), quercetin (onions), kaempferol (broccoli, grapefruits), curcumin (turmeric), diallyl trisulfide (garlic), and lycopene (tomatoes) (Chen and Dou, 2008; Yang et al., 2008; Fimognari et al., 2008; Singh and Goyal, 2008; Ramos, 2008; Seki et al., 2008). These

molecules have become an invaluable treasure in cancer prevention and chemotherapy, and further research is currently under way to explore their properties and mechanisms of action.

2. Soybean and cancer

Soybean (*Glycine max*) is an ancient legume consumed worldwide, but most commonly in Asian countries, such as China, Japan, Korea, Taiwan and Indonesia. Populations from these countries consume an average of 20 to 80 g of traditional soy foods daily in many forms, including soybean, soybean sprouts, toasted soy protein flours, soy milk, tofu, and fermented soy products, such as tempeh, miso, natto, soybean paste and soy sauce (Coward et al., 1993; Wang and Murphy, 1994). This intake equates to a daily intake of between 25 and 100 mg total isoflavones (Messina et al., 2006) and between 8 and 50 g soy protein (Erdman Jr. et al., 2004). Western populations consume much less soy, only about 1 to 3 g daily, and this is mostly in processed forms, such as soy drinks, breakfast cereals, energy bars and soy "burgers" (Fournier et al., 1998).

Epidemiological evidences have demonstrated an association between the consumption of soybean and improved health, particularly as a reduced risk for cardiovascular diseases (Anderson et al., 1999) and cancer, such as breast, prostate, endometrial, lung, and bladder cancer (Swanson et al., 1992; Wu et al., 1996; Goodman et al., 1997; Jacobsen et al., 1998; Zheng et al., 1999; Kolonel et al., 2000; Sun et al., 2002). Moreover, a number of animal models support anticancer properties of soy which constituents have been shown to suppress tumour growth in a variety of tissues including skin, bladder, mammary and prostate (Messina and Flickinger, 2002). In last decades, studies have isolated and identified an array of biologically active compounds or phytochemicals contained in soybean with cancer preventive effects. Genistein, daidzein and glycitein are the three major isoflavonoids found in soybean and soy products which properties have been extensively studied (Park and Surh, 2004). Large bodies of epidemiological studies have shown people consuming high amounts of these soy isoflavonoids in their diets have lower rates of several cancers, including breast, prostate and endometrial cancer (Lof and Weiderpass, 2006). In animal models, these compounds have been reported to inhibit the development of different types of tumors (Barnes et al., 1990; Li et al., 1999), but the results are not completely conclusive. Isoflavones exert both hormonal and non-hormonal action in the prevention of cancer, but the mechanism by which these compounds exert these chemopreventive properties is not yet clear and is currently a hot topic for research. The hormonal action of isoflavones has been postulated to be through a number of pathways, which include the ability to inhibit many tyrosine kinases involved in regulation of cell growth, to augment transformation growth factor-β which inhibits the cell cycle progression, as well as to influence the transcription factors that are involved in the expression of stress response-related genes involved in programmed cell death (Akiyama et al., 1987; Zhou and Lee, 1998). Other non-hormonal mechanisms by which isoflavones are believed to exert their anticarcinogenic effects are via their anti-oxidant, anti-proliferative, anti-angiogenic and anti-inflammatory properties (Gilani and Anderson, 2002).

During last years, soybean proteins and peptides have attracted attention as drug candidates owing to their possession of certain key advantages over alternative chemotherapy molecules. Soy protein itself, which is lower in sulfur amino acid content than animal protein, has been shown to inhibit the development of carcinogen-induced

tumors in animals (Koski, 2006). Soybean proteins also can be a source of bioactive peptides with diverse and unique health benefits that can be used in the prevention of age-related chronic disorders, such as cardiovascular disease, obesity, decrease immune function and cancer. In contrast to most small-molecule drugs, peptides have high affinity, strong specificity for targets, low toxicity and good penetration of tissues (Bhutia and Maiti, 2008). Soy proteins and peptides have become one group of nutraceuticals that shows potential results in preventing the different stages of cancer including initiation, promotion, and progression (Table 1) (de Mejia and Dia, 2010a). Bowman–Birk protease inhibitor (BBI) is a polypeptide with 71 amino acids which chemopreventive properties have been demonstrated in both *in vitro* and *in vivo* bioassay systems (Losso, 2008). As a result of this evidence, BBI acquired the status of "investigational new drug" from the FDA in 1992, and large-scale human trials are currently undergoing to evaluate its use as an anticarcinogenic agent in the form of BBI concentrate (BBIC) (Armstrong et al., 2000, 2003; Meyskens, 2001). These studies have shown that BBIC is well-tolerated by the patients and led to promising results for prostate and oral carcinomas. BBIC is also being used to investigate its efficacy in the treatment of benign prostatic hyperplasia and ulcerative colitis (Kennedy, 2006). Kunitz trypsin inhibitor (KTI) is another protease inhibitor originally isolated from soybean. The biological significance of KTI in carcinogenesis is mainly attributed to its ability to suppress invasion and metastasis of cancer cells (de Mejia and Dia, 2010a). Recently, there has been increased interest in the potential health benefits of other bioactive polypeptides and proteins from soybean, including lectins and lunasin. Soy lectins are a significant group of biologically active glycoproteins that have been shown to possess cancer chemopreventive activity *in vitro*, *in vivo* and in human case studies (de Mejia et al., 2003). The suggested mechanisms of action for lectins include their effect on tumoral cell membranes, the reduction in cell proliferation, the induction of tumor-specific cytotoxicity of macrophages and the induction of apoptosis. Another suggestion is that lectins could have a strong effect on the immune system by altering the production of various interleukins (de Mejia and Prisecaru, 2005). There is still much to learn about the effects of soybean lectins on cancer risk. However, they are currently being used as therapeutics agents in cancer treatment studies and this area of research holds considerable potential. Lunasin is a novel peptide, identified in soybean, which chemopreventive activity has been recently reported. The purpose of this work is to summarize the possible benefits of lunasin as a chemopreventive agent as well as its demonstrated mechanisms of action.

Soybean component	**Cell line/Mouse model/Human model**	**Chemopreventive activity**	**Reference**
Bowman-Birk protease inhibitor	Human osteosarcoma U2OS cells	↓ cell proliferation, arrest cell cycle, apoptosis induction, ↑ expression of C×43 mRNA	Saito et al., (2007)
	Human ovarian sarcoma M5067 cells in mice	↓ cell proliferation, ↑ expression of C×43 mRNA	Sakurai et al., (2008)
	Human breast cancer MCF-7 cells	Arrest cell cycle, ↓ proteasome chymotrypsin-like activity, ↑ expression of p27 and p21, inactivation of ERK1/2	Chen et al., (2005)

Soybean component	Cell line/Mouse model/Human model	Chemopreventive activity	Reference
		phosphorylation ↓ cell proliferation	Ho and Ng, (2008)
	DMBA-induced carcinogenesis in cultured mouse mammary gland	Inhibition of breast carcinogenesis	Du et al., (2001)
	Human prostate cancer cells	↓ cell proliferation, invasion, and clonogenic survival	Kennedy et al., (2002)
		Induction of apoptosis, ↑ expression of C×43, cleavage of caspase-3	Tang et al., (2009)
	Prostate TRAMP mice model	Inhibition of tumour development	Tang et al., (2009)
	Dimethylhydrazine-induced rat colon cancer	Inhibition of colon carcinogenesis	Kennedy et al., (2002)
	Induced rat prostate carcinogenesis	Reduction of incidence of invasive and in situ prostate neoplasms	McCormick et al., (2007)
	Hepatocellular carcinoma HepG2 cells	↓ cell proliferation	Ho and Ng, (2008)
	Radiation-induced skin fibroblasts	Blocking activation of tyrosine kinase and ↓ epidermal growth factor	Dittmann et al., (1998)
	NB4 promyelocytic leukemia cells	↓ cell proliferation, arrest cell cycle, apoptosis induction, activation of the pathway of caspase-3 and -8 cascades	Huang et al., (2004)
	Human colon HT-29 cells	↓ cell proliferation, inhibition of serine proteases	Clemente et al., (2010)
	Oral leukoplakia in human trials	Inhibition of oral carcinogenesis	Armstrong et al., (2000, 2003); Meyskens et al., (2001)
Kunitz trypsin inhibitor	Ovarian cancer cells	Suppression of cell invasion	Kobayashi et al., (2004b)
	Ovarian HRA cells implanted in C57BL/6 mice	↓ total tumour burden in peritoneal metastasis	Kobayashi et al., (2004b)
	Lewis lung carcinoma cells in C57BL/6 mice	Inhibition of spontaneous metastasis	Kobayashi et al., (2004a)
	Nasopharyngeal CNE-1 and CNE-2 cells	↓ cell growth, apoptosis induction	Fang et al., (2010)

Soybean component	Cell line/Mouse model/Human model	Chemopreventive activity	Reference
	Hepatocellular carcinoma HepG2 cells	↓ cell growth, apoptosis induction	Fang et al., (2010)
Lectins	Different tumour cell lines	↑ sensitivity to be attacked by macrophages	Ganguly and Das (1994)
	Human colon cancer SW1222 and HT29 cells	↓ cell proliferation, blocked aggregation	Jordinson et al., (1999)
	T-cell and B-cell leukemia	Interaction with tumour cells	Reysner (1983)
	Human gastric carcinomas	Direct adhesion to cell membranes and receptors	Terashima et al., (1997)
	Human melanoma A375 cells	Induction of apoptosis by a caspase-dependent pathway	Liu et al., (2009)

Table 1. Effects of soybean Bowman-Birk protease inhibitor (BBI), Kunitz trypsin inhibitor (KTI) and lectins against cancer.

2.1 Lunasin: discovery and beyond

A novel peptide, which sequence is **SKWQHQQDSCRKQKQGVNLTPCEKHIMEKIQG RGDDDDDDDDD,** was originally isolated from soybean cotyledon (Galvez et al., 1997). Because of the properties initially discovered, this peptide was termed lunasin from the Tagalog word "lunas" for cure. Lunasin is composed of 43 amino acid residues with a unique sequence including the presence of a cell adhesion motif composed of RGD and a carboxylic acid tail composed of nine aspartic acid residues. Galvez and de Lumen (1999) reported the biological property of lunasin, formerly known as a soybean cDNA encoding small subunit peptide of 2S soy albumin. They showed that transfection of lunasin plasmid into different mammalian cells caused cell division arrest, abnormal elongation of spindle fiber, chromosomal fragmentation, and cell death or apoptosis. Moreover, treatment of synthetic lunasin showed preferential adherence of this peptide to chromatin, leading to disruption of kinetochore and inhibition of mitosis. This activity seems to be due to the binding of its negatively charged poly-D carboxyl end to the highly basic histones found within the nucleosomes of condensed chromosomes, probably to regions that contain more positively charged, such as the hypoacetylated chromatin found in telomeres and centromeres. Lunasin's sequence also contains the motif RGD known to allow tumour cell attachment to the extracellular matrix (Ruoslahti and Pierschbacher, 1986). Peptides containing this motif have been reported to prevent metastasis of tumour cells by competitive adhesion to extracellular matrices (Akiyama et al., 1995). In the case of lunasin, this motif has been found to be essential for lunasin's internalization into the nucleus of C3H10T1/2 cells, but it is completely unnecessary for internalization into the nucleus of NIH3T3 cells, suggesting that the role of RGD motif might be cell-line specific (Galvez et al., 2001). These initial observations suggested a potential and promising chemopreventive role of lunasin in cancer prevention and led to successive studies designed and conducted to verify this hypothesis.

Lunasin concentration in soybean depends mainly on cultivar and environmental factors including temperature and soil moisture. Analysis of five soybean cultivars grown at three

different temperatures and two soil moisture conditions showed that cultivar and temperature but not soil moisture significantly affected lunasin concentration in soy (Wang et al., 2008b). These authors reported that lunasin concentration of the five cultivars ranged from 7.5 to 10.4 mg/g flour, in which US cultivars Loda, Jack and Dwight showed higher values than French cultivars Queen and Imari. Cultivars grown at higher temperature of 28 °C showed lowest lunasin concentration (8.1 mg/g flour) than those grown at intermediate temperature of 23 °C (9.2 mg/g flour) and low temperature condition (8.8 mg/g flour). A significant interaction was found between cultivar and temperature as well as soil moisture and cultivar in affecting lunasin concentration in soybean. Determination of lunasin concentration in 144 selected, diverse soybean accessions from the U.S. Department of Agriculture by enzyme linked immunosorbent assay showed that lunasin concentration ranged from 0.1 g to 1.3 g/100 g flour (de Mejia et al., 2004). Soybean seeds devoid of BBI also showed varying concentrations of lunasin ranging from 3.3 to 16.7 ng lunasin/mg seed indicating that lunasin is still produced on soybean seed even in the absence of BBI (de Mejia and Dia, 2010b). Analysis of lunasin in Korean soybean cultivar Taekwongkong showed that lunasin appears at 6 weeks after flowering (0.01 μg/g seed) with its highest level found at maturity with concentration of 0.12 μg/g seed (Park et al., 2005). Processing also affects lunasin concentration in soybean and soybean products. For instance, sprouting of soybean seed by soaking showed that lunasin starts decreasing 2 days after soaking and completely disappears after 7 days under both light and dark conditions (Park et al., 2005). de Mejia and co-workers (2004) showed that lunasin concentration of commercially available soy protein is in the range of 13 to 44 mg lunasin/g flour. Lunasin-enriched soy flour showed a concentration of 27.3 mg lunasin/g flour while isoflavone-enriched soybean products extracted with ethanol showed almost no lunasin which might be attributed to poor solubility of lunasin in ethanol. Another study conducted by Jeong et al. (2003) reported that defatted soybean flour had the lowest concentration of lunasin (5.5 mg lunasin/g protein) when compared with soy isolate (6.9 mg/lunasin/g protein) and soy concentrate (8.7 to 16.5 mg lunasin/g protein). They also showed that water-washed soy concentrate had higher lunasin concentration than alcohol-washed soy concentrate. These reports indicate that lunasin concentration in soybean products is affected by processing conditions. Recently, it has been found that environmental factors, such as germination time and temperature have a significant influence on the composition and concentration of bioactive compounds in germinated soybean flour from the Brazilian soybean cultivars BRS 133 and BRS 258 (Paucar-Menacho et al., 2010b, c). These authors reported that protein concentration also affects the final distribution of nutrients and bioactive components in soybean, including lunasin (Paucar-Menacho et al., 2010a).

The first study has demonstrated lunasin's presence in US commercially available soy foods, including soy milk, infant formulas, tofu, bean curd, soybean cake, tempeh, and su-jae (Hernández-Ledesma et al., 2009a). As an example, Table 2 shows type, composition, origin and lunasin and BBI concentrations of different soy milk samples analyzed by these authors. Concentrations of two peptides in soy milk and other soybean products seem to be determined by the soybean variety and the process used during manufacturing, indicating that these two parameters can be used to control contents of these two peptides. Previously it had been demonstrated that large-scale processing of soy to produce different protein fractions influences lunasin concentration. This content varied from 12 to 44 mg lunasin/g of flour when different commercially available soy proteins were analyzed (de Mejia et al., 2004; Jeong et al., 2003).

Sample No.	Type of sample	Composition-main ingredients	Country	Lunasin (mg/100 g product)	BBI (mg/100 g product)
Soy milk-1	Enriched soymilk	Soybeans	USA	15.7 ± 1.3	33.1 ± 4.2
Soy milk-2	Organic original soymilk	Soybeans, malted wheat and barley extract	USA	18.9 ± 2.6	27.1 ± 3.4
Soy milk-3	Organic fortified soymilk	Soybeans, malted wheat and barley extract	USA	14.2 ± 1.1	24.7 ± 4.3
Soy milk-4	Organic plain soymilk	Soybeans	USA	13.8 ± 2.6	45.7 ± 7.2
Soy milk-5	Organic unsweetened soymilk	Soybeans	USA	14.4 ± 2.4	55.9 ± 5.0
Soy milk-6	Organic plain soymilk	Soybeans	USA	14.7 ± 0.8	40.0 ± 5.5
Soy milk-7	Organic original soymilk	Soybeans, rice syrup	USA	13.7 ± 0.9	30.3 ± 3.7
Soy milk-8	Organic plain soymilk	Soybeans, soy protein isolate	USA	13.9 ± 1.0	25.9 ± 4.2
Soy milk-9	Organic original soymilk	Soybeans, malt syrup	USA	18.3 ± 2.4	23.1 ± 3.0
Soy milk-10	Organic original soy drink	Soybeans, barley extract	USA	10.7 ± 0.8	7.2 ± 1.5
Soy milk-11	Fortified soymilk	Soybeans	USA	12.3 ± 0.8	18.8 ± 2.7
Soy milk-12	Unsweetened soymilk	Soybeans	Singapore	11.8 ± 1.3	n.d.
Soy formula-1	Soy-based formula	Corn syrup, soy protein isolate	USA	4.1 ± 0.4	n.d.
Soy formula-2	Organic soy formula	Corn syrup, soy protein	USA	2.8 ± 0.2	n.d.
Soy formula-3	Organic soy formula	Rice syrup, soy protein concentrate	USA	1.5 ± 0.1	n.d.
Tofu-1	Soft Tofu	Soybeans	USA	9.62 ± 0.87	4.63 ± 1.76
Tofu-2	Soft Tofu	Soybeans	USA	7.34 ± 1.04	3.72 ± 0.21
Tofu-3	Silken Tofu Kinugoshi	Soybeans	USA	9.60 ± 0.74	12.41 ± 2.11
Tofu-4	Silken Tofu	Soybeans	USA	4.41 ± 0.49	11.87 ± 1.53
Tofu-5	Silken Tofu	Soybeans	USA	3.69 ± 0.49	5.92 ± 1.15
Tofu-6	Medium firm Tofu	Soybeans	USA	14.30 ± 1.80	4.91 ± 0.53
Tofu-7	Organic Medium firm Tofu	Soybeans	USA	6.66 ± 1.29	4.19 ± 0.54
Tofu-8	Firm Tofu	Soybeans	USA	3.50 ± 0.23	8.23 ± 0.58

Sample No.	Type of sample	Composition-main ingredients	Country	Lunasin (mg/100 g product)	BBI (mg/100 g product)
Tofu-9	Extra firm tofu Chinese style	Soybeans	USA	3.66 ± 0.12	4.57 ± 0.55
Tofu-10	Baked tofu	Soybeans, soy sauce (wheat)	USA	5.47 ± 0.34	2.94 ± 0.48
Tofu-11	Fried tofu	Soybean, soybean oil, soy sauce	USA	0.37 ± 0.05	n.d.
Tofu-12	Dry tofu	Soybeans	Taiwan	2.50 ± 0.27	n.d.
Natto-1	Natto	Fermented soybeans (*Bacillus subtilis* natto)	Japan	n.d.	n.d.
Miso-1	Organic Miso	Soybeans, rice, *Aspergillus oryzae*	Japan	n.d.	n.d.
Tempeh-1	Organic soy tempeh	Soybeans, *Rhizopus oligosporus*	USA	n.d.	n.d.
Tempeh-2	Organic soy tempeh	Soybeans, brown rice, *R. oligosporus*	USA	8.19 ± 0.42	n.d.
Tempeh-3	Organic soy tempeh-flax	Soybeans, flaxseed, brown rice, *R. oligosporus*	USA	6.12 ± 0.40	n.d.
Tempeh-4	Organic soy tempeh-rice	Soybeans, brown rice, *R. oligosporus*	USA	n.d.	n.d.
Bean curd-1	Marinated bean curd	Soybeans, soy sauce	Taiwan	9.53 ± 1.01	14.65 ± 0.60
Bean curd-2	Soybean curd noodle	Soybeans	Taiwan	n.d.	n.d.
Soybean cake-1	Deep fried soybean cake	Soybeans, soybean oil	USA	1.91 ± 0.25	6.57 ± 0.41
Soybean cake-2	Baked soybean cake	Soybeans, soy sauce, sesame oil	USA	1.14 ± 0.15	0.73 ± 0.05

Table 2. Type, composition, country of origin and lunasin and BBI concentrations ofcommercial soy food products

2.1.1 Lunasin's bioavailability

An important property of an ideal cancer preventive agent is its ability, after being orally administrated, to escape gastrointestinal digestion and to be absorbed through the bloodstream reaching the target tissues and organs in an intact and active form. To date, different lunasin's bioavailability studies conducted in both animals and humans have shown promising results. First studies carried out in mice and rats fed lunasin-enriched soy protein found that 35% of ingested lunasin reaches the target tissues and organs in an intact and active form (Jeong et al., 2007a). It has also been reported that BBI and KTI contained in soybean protect lunasin against gastrointestinal digestion, making this peptide bioavailable to exert its chemopreventive properties (Park et al., 2007). Hsieh and co-workers reported that synthetic ^{3}H-labeled lunasin was bioavailable after its oral administration to CD-1 mice, reaching different tissues, including lung, mammary gland, prostate, and brain (Hsieh et al., 2010a). These authors also found that lunasin extracted from the blood and liver of lunasin-

enriched soy flour-fed rats was bioactive and able to suppress foci formation in the same concentration as synthetic lunasin. Lunasin from other seeds have also shown stability towards pepsin and pancreatin *in vitro* digestion (Jeong et al., 2009; Jeong et al., 2010a). These authors demonstrated lunasin's presence in different organs, such as liver, kidney, and blood of rats fed with lunasin-enriched rye. First bioavailability study conducted in humans has demonstrated that 4.5% of lunasin ingested in the form of soy protein reaches plasma of healthy volunteer men (Dia et al., 2009a). Results from this study are relevant in supporting future clinical trials to demonstrate lunasin's cancer preventive properties.

2.1.2 Lunasin against cancer: *in vitro* and in *vivo* evidence

Cell culture experiments have demonstrated that lunasin prevent mammalian cells transformation induced by chemical carcinogens without affecting morphology and proliferation of normal cells. Galvez and co-workers (2001) found that lunasin suppresses foci formation in 7,12-dimethylbenz[a]anthracene (DMBA) and 3-methylcholanthrene (MCA)-induced C3H10T1/2 cells. Similar results have been found by Hsieh and co-workers (2011) in DMBA-induced NIH3T3 cells. This suppressive effect is significantly higher than that exerted by the well-known cancer preventive BBI on an equimolar basis. Lunasin's inhibitory effects have been also found in C3H10T1/2 and NIH3T3 cells transformed by oncogenes and genes that inactivate tumor suppressor proteins (Galvez et al., 2001; Jeong et al., 2003; Lam et al., 2003). Ras-oncogenes are frequently activated in human cancers, playing a central role in the ras/mitogen activated protein kinase (MAPK) signalling cascade, which has a pivotal role in cell proliferation, differentiation, survival and cell death (Barbacid, 1987; Malumbres and Barbacid, 2003). Lunasin has been shown to prevent transformation of NIH3T3 cells transfected with an inducible form of ras-oncogene (Jeong et al., 2003). Moreover, addition of lunasin to mouse fibroblasts NIH3T3 stably transfected with the viral oncogene E1A has been reported to suppress foci formation and to increase protein p21 level (Lam et al., 2003). Oncogene E1A has been associated with human tumours because of its ability to inactivate the tumour suppressor retinoblastoma protein (RB) causing cell cycle arrest and cells transformation (Helt and Galloway, 2003).

These first results made lunasin to be considered as a "watchdog" agent that sits in the nucleus of the cells and effectively does nothing when there is no transformation event. When a transformation event occurs, lunasin is triggered into action (de Lumen, 2005). However, recent studies carried out in our laboratories have revealed that lunasin also acts on established cancer cell lines. Lunasin purified from defatted soybean flour by combination of ion-exchange chromatography and size exclusion chromatography (Dia et al., 2009b) showed potent activity against different human colon cancer cells. Lunasin caused cytotoxicity in four different human colon cancer cell lines with IC_{50} values of 13.0 μM for KM12L4 cells, 21.6 μM for RKO cells, 26.3 μM for HCT-116 cells and 61.7 μM for HT-29 cells (Dia and de Mejia, 2011). These values showed that lunasin is most potent in killing the highly metastatic KM12L4 colon cancer cells than any other colon cell lines used in this study. Crystal violet staining of HT-29 colon cancer cells showed that starting at 10 μM, lunasin caused changes in the morphology and number of viable cancer cells (Dia and de Mejia, 2010). Also, treatment of the oxaliplatin-resistant variants (OxR) of these colon cancer cells showed IC_{50} values of 34.7 μM for KM12L4OxR, 38.9 μM for RKOOxR and 31.6 μM for HCT-116OxR while HT-29OxR was not affected by lunasin treatment. It has been also demonstrated that lunasin causes a dose-dependent inhibition of the growth of estrogen-independent breast cancer MDA-MB-231 cells, with an IC_{50} value of 181 μM (Hsieh et al.,

2010b). Studies carried out to establish a structure/activity relationship showed an IC_{50} value of 138 μM for the 21 amino acid sequence localized at the C-terminus of lunasin, thus being the main responsible for lunasin's inhibitory effect on breast cancer cells proliferation (Hernández-Ledesma et al., 2011). Lunasin's suppressive effect on cell growth has been also found in L1210 leukemia cells, with an IC_{50} value of 14 μM (Wang et al., 2008a).
Chemopreventive properties of lunasin have been also demonstrated *in vivo*. First animal model used to demonstrate these properties was a chemical carcinogen induced SENCAR skin cancer mouse model (Galvez et al., 2001). These authors demonstrated that 250 μg lunasin/week, topically administered to SENCAR mice treated with DMBA and tetradecanoylphorbol-13-acetate (TPA), suppresses skin tumor incidence, decreases tumor yield/mouse and increases the tumor latency period by 70% compared with the untreated control. Moreover, lunasin was found to delay the appearance of papilloma by slowing down epidermal cell proliferation in mouse skin in the presence of DMBA (Hsieh et al., 2004). Promising results have been also found when lunasin acts *in vivo* against breast cancer. Our first findings show a relevant inhibitory effect on mammary tumours development when a lunasin-enriched diet is administered to DMBA-induced mice (Hsieh et al., 2010c). Moreover, lunasin reduces tumour incidence and generation, as well as tumour weight in a xenograft mouse model using human breast cancer cells. Tumour incidence was reduced by 49% and 33%, in nude mice transplanted with MDA-MB-231 cells and administered i.p. injections of lunasin, at 20 mg/kg and 4 mg/kg body weight, respectively, compared with the vehicle-treated group, while no effects were observed when mice were treated with BBI (Hsieh et al., 2010a). In the breast tumour histological sections, lunasin was found to inhibit cell proliferation and to induce cell apoptosis. These studies make lunasin a promising alternative to prevent and/or treat skin and breast cancer. Further research should be needed to demonstrate chemopreventive role of this peptide against other types of cancer, as well as to elucidate its *in vivo* mechanism of action.

2.1.3 Mechanism of lunasin's action

Initially, lunasin was found to act through an epigenetic mechanism of action. This peptide has been demonstrated to compete with different histone acetyltransferase enzymes (HATs), such as yGCN5 and PCAF, inhibiting the histones acetylation and repressing the cell cycle progression (Jeong et al., 2002, 2007b, c). Acetylation has been linked to chromatin disruption and the transcriptional activation of genes, being thus considered one of the most important epigenetic modifications acting on signal transduction pathways including those involved in cancer development (Dwarakanath et al., 2008; Dalvai & Bystricky, 2010). Mistargeted and deregulated HATs activities, as well as their over-expression have been reported to play an important role in several human cancers (Gayther et al., 2000). Lunasin has been reported to inhibit histone acetylation in mammalian cells induced by chemical carcinogens and/or viral oncogenes that provoke inactivation of tumor suppressor proteins, such as RB, p52 and pp32. When lunasin is present in the cell nucleus, it acts as a surrogate tumor suppressor and tightly binding to deacetylated core histones and disrupting the balance between acetylation-deacetylation, which is perceived by the cell as abnormal and leads to cell death (de Lumen, 2005). Recently, studies carried out with human breast cancer MDA-MB-231 cells have demonstrated that lunasin inhibits histones H3 and H4 acetylation in these cells (Hernández-Ledesma et al., 2011), being more potent than other compounds which HATs inhibitory activity has been associated with their chemopreventive role (Balasubramanyam et al., 2003, 2004a, b). Structure/activity relationship have demonstrated,

in one hand, that the percentage of inhibition caused by lunasin is specific of lysine position sensitive to be acetylated, and in another hand, that lunasin's sequence is essential for inhibiting H4 acetylation whereas poly-D sequence is the main active sequence responsible for H3 acetylation inhibition (Hernández-Ledesma et al., 2011). Figure 1 shows the activity of each lunasin-related fragment on histone binding and/or acetylation at different Lys positions.

Studies conducted with different tumour cell lines have demonstrated new mechanisms for lunasin's chemopreventive action. Studies with colon cancer HT-29 and KM12L4 cells have shown that lunasin caused a G2/M phase arrest in the cell cycle and induction of the mitochondrial pathway of apoptosis (Dia and de Mejia, 2010). The G2/M phase cell cycle arrest was attributed with concomitant increase in the expression of the p21 protein in HT-29 colon cancer cells while both p21 and p27 protein expressions were up-regulated by lunasin treatment in KM12L4 colon cancer cells. Measurement of protein expressions associated with mitochondrial pathway of apoptosis showed that lunasin treatment affected the ratio of Bax to Bcl-2 by up-regulating the pro-apoptotic Bax and down-regulating the expression of the anti-apoptotic Bcl-2. This might be attributed to increase in the expression of the pro-apoptotic form of clusterin known as nuclear clusterin which is positively affected by increase in p21 expression. Translocation of Bax into the mitochondrial membrane resulted in the release of cytochrome c as shown by increase in the expression of cytosolic cytochrome c in KM12L4 colon cancer cells treated with lunasin. This resulted in an increase in the activity of caspase-9 and the activity of the executioner of apoptosis caspase-3 in both HT-29 and KM12L4 colon cancer cells treated with lunasin when compared to untreated cells. We proposed the mechanism by which lunasin can induce apoptosis in human colon cancer cells as shown in Figure 2.

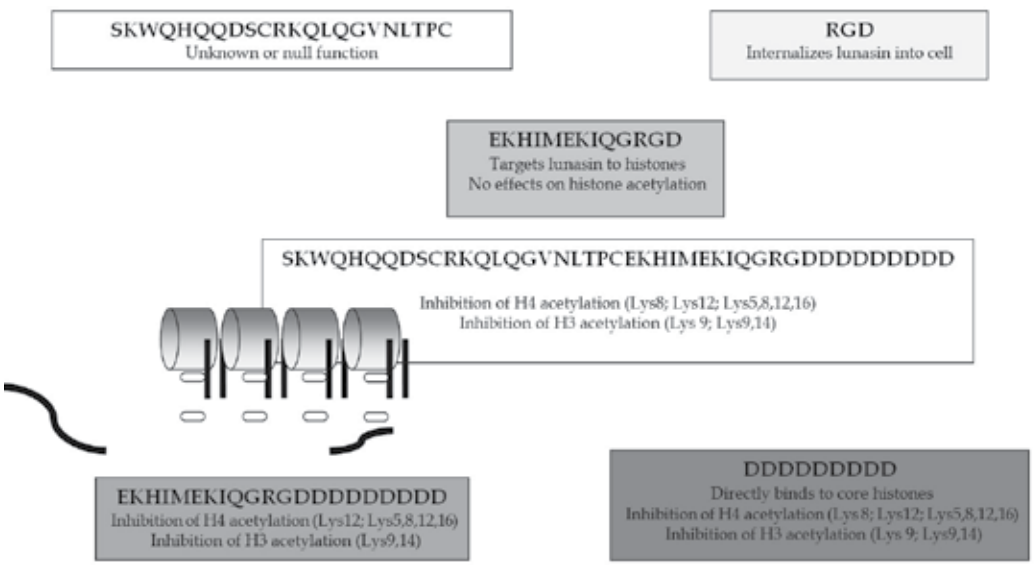

Fig. 1. Amino acids sequence of lunasin peptide and demonstrated histones H3 and H4 acetylation inhibitory activity of the different fragments of its sequence

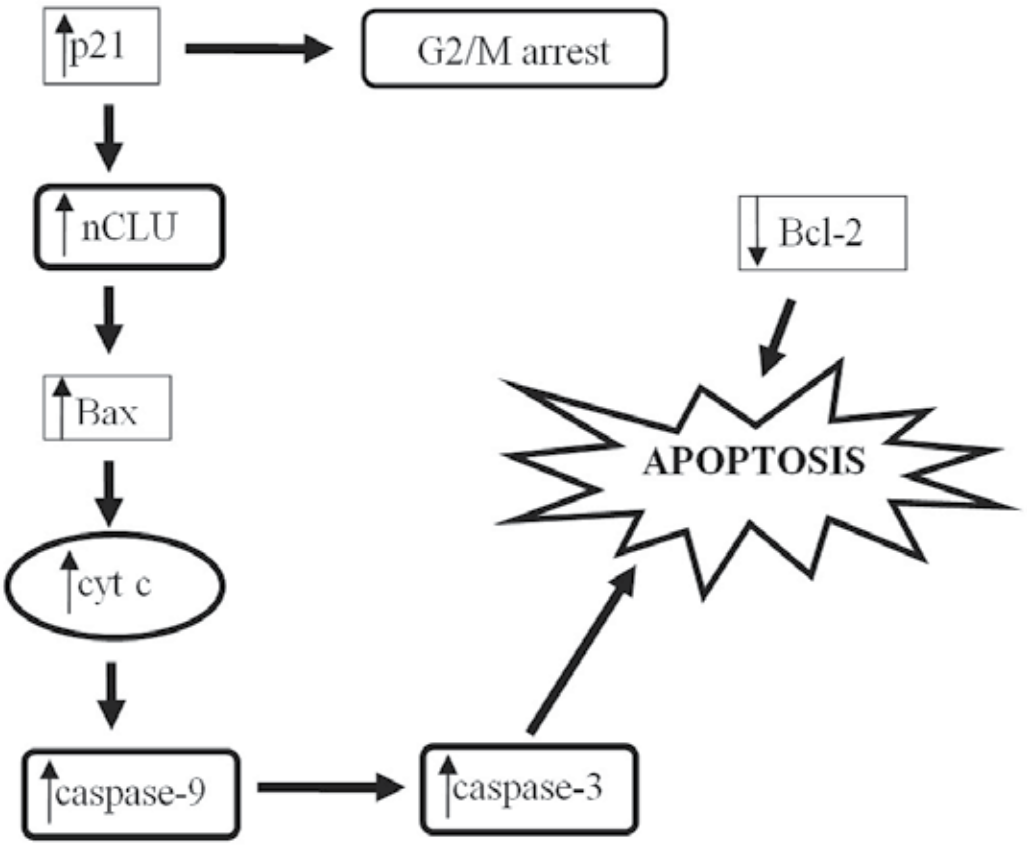

Fig. 2. Proposed mechanism by which lunasin induces apoptosis in human colon cancer cells.

The RGD motif present in the lunasin structure is a recognition site for integrin receptors present in the extracellular matrix (ECM). Integrins are heterodimeric receptors associated with cell adhesion, ECM and cancer metastasis. Treatment of KM12L4 colon cancer cells with lunasin resulted in the modification on the expression of 62 genes associated with ECM and cell adhesion, of which 48 genes were up-regulated and 14 genes were down-regulated (Dia and de Mejia, 2011a). Lunasin down-regulated the gene expression of collagen type VII α1, integrin β2, matrix metalloproteinase 10, selectin E and integrin α5 by 10.1-, 8.2-, 7.7-, 6.5- and 5.0-fold, respectively compared to the untreated KM12L4 colon cancer cells. On the other hand, the expression of collagen type XIV α1 was up-regulated upon lunasin treatment by 11.6-fold (Dia and de Mejia, 2011). These results suggest a potential role of peptide lunasin in cancer metastasis.

Lunasin has been also demonstrated to arrest cell cycle and to induce apoptosis in breast cancer MDA-MB-231 cells (Hsieh et al., 2010b). These authors demonstrated that lunasin modulates expression of different genes and proteins involved in cell cycle, apoptosis and signalling transduction. Inhibition of deregulated cell cycle progress in cancer cells is being considered an effective strategy to delay or halt tumour growth. It is well established that cyclins play a positive role in promoting cell cycle transitions via their ability to associate

with their cognate cyclin-dependent kinases (CDKs) and to activate them (Kato et al., 1993). Over-expression of cyclins D1 and D3 is one of the most frequent alterations present in breast tumours (Sutherland and Musgrove, 2004). Cyclins D interacts with CDK4 or CDK6 to form a catalytically active complex, which phosphorylates RB to free active E2F (Qu et al., 2003). Up-regulation of RB gene expression (Hsieh et al., 2010b), as well as inhibition of RB phosphorylation (Jeong et al., 2007b) have been linked to lunasin's arresting effect on breast cancer cells cycle progress. Down-regulation of cyclins D1 and D3, and CDK4 and CDK6 protein expression as well as modulation of expression of CDC25A, Caspase 8, and Ets2, Myc, Erbb2, PIK3R1 and Jun signaling genes in breast cancer cells might also contribute on this lunasin's suppressive effect (Hernández-Ledesma et al., 2011; Hsieh et al., 2010b).

Lunasin also showed cytotoxic effect in L1210 leukemia cells with an IC_{50} value of 14 μM. The mechanism involved was through arrest of cell cycle at G2/M phase with concomitant pro-apoptotic inducing property. The expressions of caspases-3, -8 and -9 were up-regulated by 12-, 6- and 6-fold respectively which resulted in the increase of percentage of L1210 leukemia cells undergoing apoptosis from 2 to 40% (de Mejia et al., 2010).

Lunasin has been found to exert anti-inflammatory and antioxidant activities that might contribute to its chemopreventive properties. First studies demonstrated that lunasin potently inhibits lipopolysaccharide (LPS)-induced production of pro-inflammatory mediators interleuquine (IL-6), tumor necrosis factor (TNF-α), and prostaglandin E2 (PGE2) in RAW 264.7 cells through suppression of nuclear factor (NF)-κB pathways (Hernández-Ledesma et al., 2009b; de Mejia and Dia, 2009). It has been also reported that this peptide exert its anti-inflammatory activity through modulation of cyclooxygenase-2 (COX-2)/PGE2 and inducible nitric oxide synthase (iNOS)/nitric oxide pathways (Dia et al., 2009b). Moreover, lunasin has been found to exert potent antioxidant properties, reducing LPS-induced production of ROS by macrophage cells, and acting as a potent free radical scavenger (Hernández-Ledesma et al., 2009b). Recently, lunasin purified from *Solanum nigrum* L. has been found to protect DNA from oxidative damage by suppressing the generation of hydroxyl radical via blocking fenton reaction (Jeong et al., 2010).

Silva-Sánchez and co-workers, (2008) reported for the first time the presence of a lunasin-like peptide in amaranth protein fractions. Glutelin fraction in amaranth seeds had the highest lunasin concentration (3.0 g/g). Lunasin was also identified in albumin, prolamin and globulin amaranth protein fractions.

Maldonado-Cervantes and co-workers, (2010) found that the amaranth lunasin-like peptide inhibited the transformation of NIH-3T3 cells to cancerous foci. The open reading frame of amaranth lunasin corresponds to a bifunctional inhibitor/lipid-transfer protein (LTP). There are many new intriguing questions about the function of lunasin in plants and its health-promoting benefits that need further investigations.

3. Conclusion

Epidemiological evidence has demonstrated an association between the consumption of soybean and improved health, particularly reduced risk for cardiovascular diseases and cancer. *In vitro* as well as *in vivo* studies support the cancer preventive properties of soy and soy compounds responsible of these properties. This review has summarized the chemopreventive activity of proteins and peptides that contribute to reported cancer preventive effects of soybean. Among them, peptide lunasin holds a considerable potential.

This peptide, administered in soy proteins, has been demonstrated to be bioavailable after resisting gastrointestinal and serum degradation, and reaching blood and target organs in an intact and active form. Lunasin's efficacy against leukemia, breast and colon cancer using cell culture models has been recently revealed. Animal experiments are being conducted to verify these *in vitro* properties, and to date, a promising role of lunasin against skin and breast cancer has been reported. Moreover, genomics, proteomics and biochemical tools are being applied to complete elucidate its molecular mechanism of action. Obtained results from all these studies make lunasin a good candidate for new generation of cancer preventive agents derived from foods. However, there is still much to be learned about lunasin's effects on cancer prevention. The major challenge on the use of lunasin in treating cancer would be the conversion of *in vitro* and *in vivo* results into clinical outcomes. Therefore, it should be needed to design clinical trials that confirm lunasin's chemopreventive properties against different types of cancer. Other aspects, such as searching for lunasin in other seeds, optimization of techniques to enrich products with this peptide and studying lunasin's interactions with other food constituents affecting its activity should also be conducted.

4. Acknowledgment

B.H.-L. thanks Spanish National Research Council (CSIC) for her post-doctoral research JAE-Doc contract.

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8

Insights into the Pharmacological Effects of Soy Isoflavones on Catecholamine System

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1. Introduction

Natural estrogens have a wide array of biological actions not only on the female reproductive system but also on metabolic homeostasis, cell proliferation and differentiation. The long-term genomic effects of estrogens are known to be mediated through classical nuclear receptors such as estrogen receptor-α (ER-α) and -β (ER-β) (Green et al., 1986; Krust et al., 1986; Mosselman et al., 1996). In addition to this established mechanism of action, much evidence has been accumulated that estrogens also have non-genomic actions via the activation of plasma membrane estrogen receptors (Wehling 1997; Falkenstein et al., 2000). Indeed, the cell membrane estrogen receptors have been extensively studied, resulting in the identification of several types of membrane estrogen receptors, such as ER-α, its S-palmitoylated ER variant (Li et al., 2003; Wyckoff et al., 2001), ER-X (Toran-Allerand et al., 2002; Qui et al., 2006), and GPR30 (Thomas et al., 2005; Revankar et al., 2005). Among these, GPR30 may be the most plausible candidate for a membrane estrogen receptor that regulates the various functions induced by estrogens (Funakoshi et al., 2006; Filardo et al., 2007), although the precise cellular localization and functions of GPR30 remain controversial (for review see, Mizukami 2010).

Soybeans have traditionally been consumed as food, especially in East Asian countries. Isoflavones such as daidzein and genistein are soy phytoestrogens and have a weak estrogenic activity due to the fact that their structures are similar to the primary structure of estrogens (Kurzer and Xu, 1997). Recent research attention has been paid to the high dietary intake of isoflavones because of their potentially beneficial effects associated with a reduction in the risk of developing cardiovascular diseases (Arjmandi et al., 1997), osteoporosis (Adlercreutz et al., 1993; Toda et al., 1999), menopausal symptoms (Adlercreutz et al., 1992), and some forms of cancers (Messina et al., 1994). These effects are considered to be mediated by binding to the nuclear estrogen receptors (Kurzer and Xu, 1997; Murkies et al., 1998).

Adrenal medullary cells are derived from the embryonic neural crest and share many properties with sympathetic postganglionic neurons. In cultured bovine adrenal medullary

cells, our previous studies have shown that carbachol, a derivative of acetylcholine, induced $^{22}Na^+$ influx via voltage-dependent Na^+ channels, and then increased $^{45}Ca^{2+}$ influx via voltage-dependent Ca^{2+} channels, a prerequisite for secretion (Wada et al., 1985; Yanagihara et al., 1996) and synthesis (Yanagihara et al., 1987) of catecholamines. Since the mechanism of stimulation of catecholamine synthesis and secretion in adrenal medullary cells are thought to be similar to those of noradrenaline in the sympathetic neurons, adrenal medullary cells have provided a good model for detailed analysis of the actions of cardiovascular drugs, such as α_2-adrenergic agonists (Yanagihara et al., 1987), natriuretic peptides (Yanagihara et al., 1991), carvedilol (Kajiwara et al., 2002) and pimobendan (Toyohira et al., 2005).

In our previous studies, treatment of bovine adrenal medullary cells with environmental estrogenic pollutants such as p-nonylphenol and bisphenol A stimulated catecholamine synthesis and tyrosine hydroxylase activity, probably through plasma membrane estrogen receptors (Yanagihara et al., 2005). Indeed, we demonstrated the occurrence and functional roles of unique estrogen receptors in the plasma membranes isolated from bovine adrenal medullary cells (Yanagihara et al., 2006). 17β-Estradiol stimulated catecholamine synthesis via activation of extracellular signal-regulated kinases (ERKs) through the plasma membrane estrogen receptors.

The present review summarizes the current knowledge of pharmacological effects of daidzein and genistein on catecholamine signaling, such as catecholamine synthesis and secretion in cultured bovine adrenal medullary cells and noradrenaline reuptake by SK-N-SH cells and by COS-7 cells transiently transfected with noradrenaline transporter.

2. Dual effects of daidzein on catecholamine synthesis and secretion

Incubation of bovine adrenal medullary cells with daidzein for 20 min resulted in a small increase in ^{14}C-catecholamine synthesis from [^{14}C]tyrosine (Fig. 1A). The stimulatory effect of daidzein on ^{14}C-catecholamine synthesis was observed to be concentration-dependent (10-1000 nM). Daidzein inhibited the specific binding of [^{3}H]17β-estradiol in a concentration (10-1000 nM)-dependent manner (Fig. 1B) similar to that of ^{14}C-catecholamine synthesis. From these results, it is likely that daidzein stimulates catecholamine synthesis via activation of membrane estrogen receptors in bovine adrenal medullary cells. Previous studies have shown that the serum concentrations of daidzein are around 200-350 nM in Japanese people older than 40 years (Morton et al., 2002) and that the serum levels of daidzein in humans consuming three meals per day, including one meal containing soybeans, can reach a maximum of 4.1 μM (King and Bursill, 1998). It seems that the concentrations of daidzein used in the present study are relevant in people's daily lives because these concentrations partially overlap with those in the plasma of individuals who consume soy products.

Daidzein (1 μM) and acetylcholine (0.3 mM) increased ^{14}C-catecholamine synthesis form [^{14}C]tyrosine by 31% and 245% over the control levels, respectively (Fig. 2A). Concurrent treatment of cells with daidzein and acetylcholine did not enhance but significantly inhibited the stimulatory effect of acetylcholine on ^{14}C-catecholamine synthesis (84% of acetylcholine). To determine which step of catecholamine synthesis was enhanced by daidzein, [^{14}C]DOPA was used as a substrate instead of [^{14}C]tyrosine (Fig. 2B). Neither daidzein nor acetylcholine increased ^{14}C-catecholamine synthesis from [^{14}C]DOPA, suggesting that the increase in catecholamine synthesis induced by daidzein and acetylcholine occurs predominantly upstream of the DOPA decarboxylase step, i.e., tyrosine hydroxylase step. Indeed, incubation of cells with daidzein (1 μM) for 10 min resulted in a significant increase in tyrosine

hydroxylase activity of 27% over the control (Fig. 3B). ICI182,780 (100 nM), an inhibitor of nuclear estrogen receptors when used alone, increased ^{14}C-catecholamine synthesis as well as tyrosine hydroxylase activity, and did not abolish but rather enhanced both stimulatory effects of daidzein (Fig. 3A and B). Furthermore, ICI182,780 enhanced the specific binding of [^{3}H]17β-estradiol to plasma membranes isolated from bovine adrenal medulla (data not presented). This gives rise to the possibility that daidzein and ICI182,780 act synergistically on different sites of the membrane estrogen receptors.

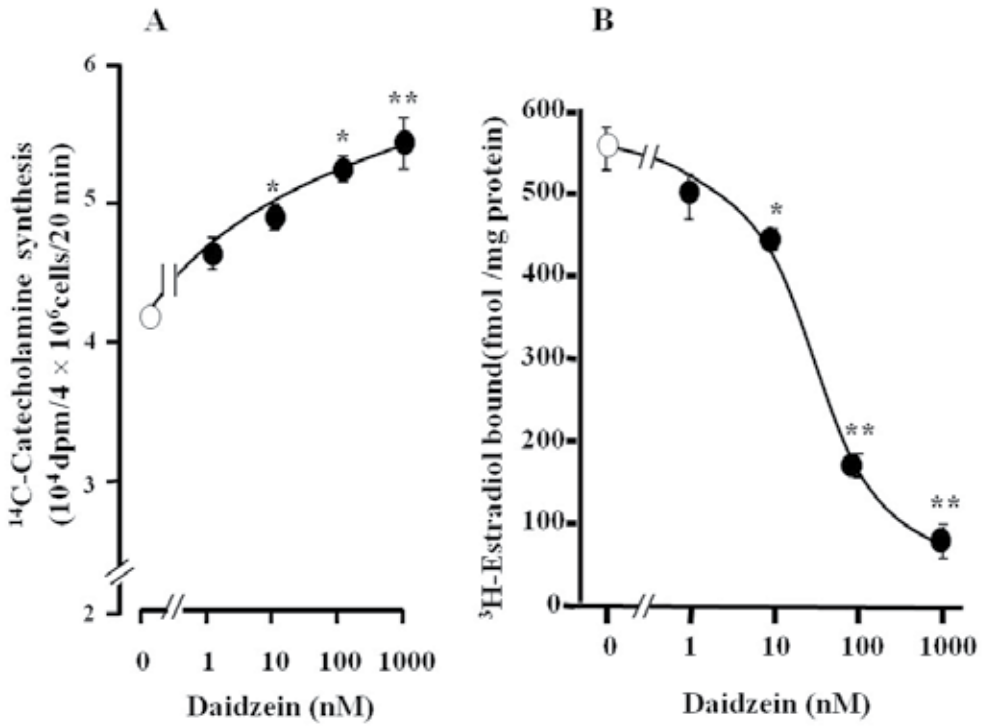

Fig. 1. Concentration–response curve of daidzein for ^{14}C-catecholamine synthesis from [^{14}C]tyrosine in cultured bovine adrenal medullary cells (A) and concentration-inhibition curve for specific binding of [^{3}H] 17β-estradiol to adrenal medullary plasma membranes (B). (A) Cultured bovine adrenal medullary cells (4x10^6 / dish) were incubated with (●) or without (○) various concentrations (1 – 1000 nM) of daidzein at 37 °C for 20 min in 1.0 ml KRP buffer containing L-[U-^{14}C]tyrosine (20 μM, 1 μCi). The ^{14}C-labeled catecholamines formed are measured and shown as the total ^{14}C-catecholamines (adrenaline, noradrenaline and dopamine). Date are expressed as the mean ± SEM of four experiments carried out in duplicate. *p < 0.05 and **p < 0.01, compared with control. (B) Plasma membranes isolated form bovine adrenal medulla were incubated at 4 °C for 30 min with various concentrations of daidzein in the presence of [^{3}H]17β-estradiol (5 nM, 0.1 μCi). Non-specific binding was determined in the presence 1 μM of 17β-estradiol and specific binding was obtained by subtracting non-specific binding from total binding. Values shown are means ± SEM of four separate experiments carried out in triplicate. *p < 0.05 and **p < 0.01, compared with control. Data from Liu et al. (2007) are modified.

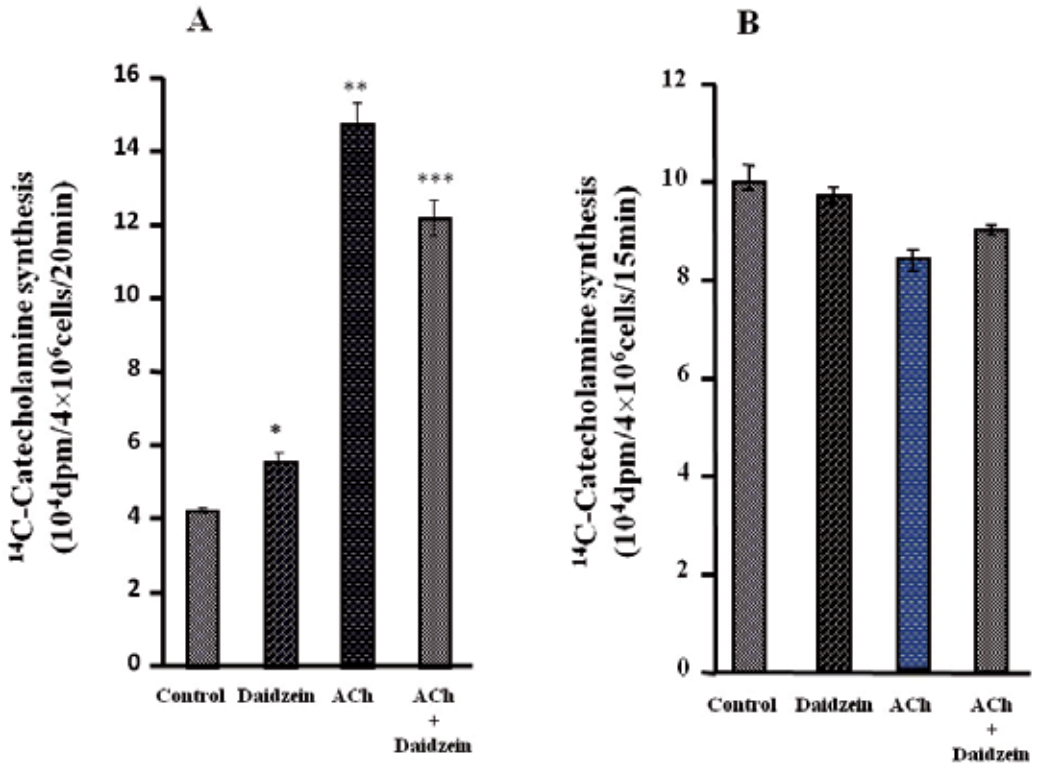

Fig. 2. Effects of daidzein on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine (A) or [^{14}C]DOPA (B). The cells (4 x10^6/dish) were incubated with or without daidzein (1 μM) and acetylcholine (ACh)(0.3 mM) at 37 ˚C for 20 min or 15 min in the presence of L-[U-^{14}C]tyrosine (20 μM, 1 μCi) (A) or [^{14}C]DOPA (20 μM, 0.25 μCi) (B), respectively. The ^{14}C-catecholamines formed were measured. Data are expressed as the mean ± SEM of four experiments carried out in triplicate. *p < 0.05 and **p < 0.01, compared with control; ***p < 0.05, compared with acetylcholine alone. Data from Liu et al. (2007) are modified.

We next studied the effect of daidzein on catecholamine secretion induced by acetylcholine. The catecholamine secretion induced by acetylcholine corresponded to 13.5% of the total catecholamine content in the cells. Daidzein (1, 10, and 100 μM) significantly suppressed catecholamine secretion induced by acetylcholine to 94%, 88%, and 64% of that by acetylcholine alone (Fig. 4), respectively. These findings suggest that daidzein has dual effects on catecholamine synthesis and secretion, i.e., at low concentrations (10-100 nM) daidzein activates tyrosine hydroxylase activity and stimulates catecholamine synthesis, but at high concentrations (≧1 μM) it attenuates catecholamine synthesis and secretion induced by acetylcholine.

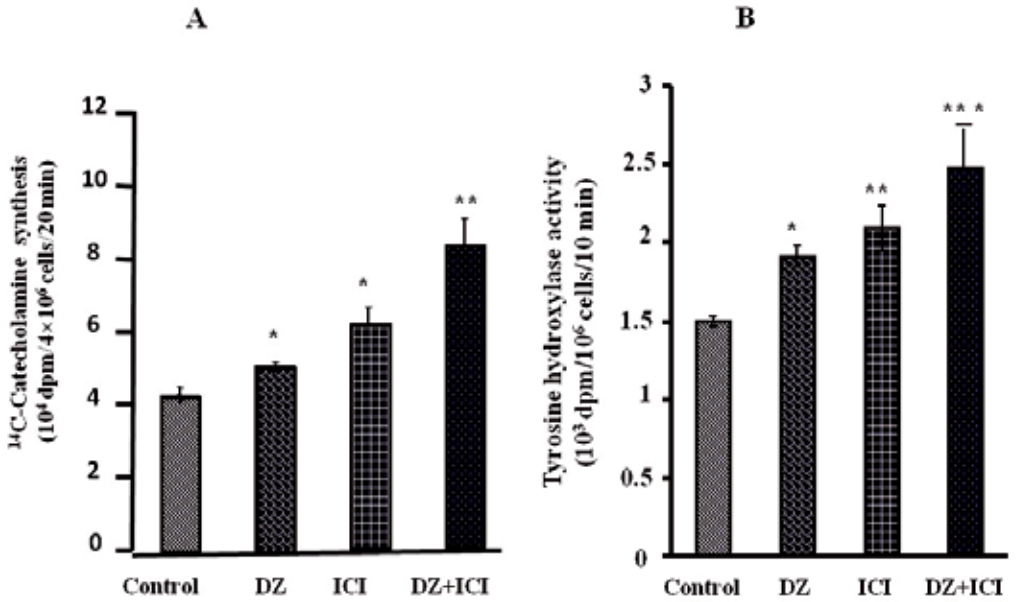

Fig. 3. Effects of daidzein and/or ICI182,780, an inhibitor of classical nuclear estrogen receptors, on ^{14}C-catecholamine synthesis (A) and tyrosine hydroxylase activity (B). (A) The cells ($4x10^6$/dish) were incubated with or without daidzein (DZ) (1 μM) and/or ICI182,780 (ICI) (100 nM) at 37 °C for 20 min in the presence of L-[U-^{14}C]tyrosine. Data are expressed as the means ± SEM of four experiments carried out in triplicate. *$p < 0.05$, compared with control; **$p < 0.05$, compared with daidzein alone. (B) Cells (10^6/well) were preincubated in 250 μl of KRP buffer with or without (1 μM) daidzein and/or ICI182,780 (100 nM) for 10 min and then incubated for another 10 min in the presence of L-[1-^{14}C]tyrosine (18 μM, 0.2 μCi), and tyrosine hydroxylase activity was measured. Data are the mean ± SEM of four separate experiments carried out in triplicate. *$p < 0.05$ and **$p < 0.01$, compared with control; ***$p < 0.05$, compared with daidzein. Data from Liu et al. (2007) are modified.

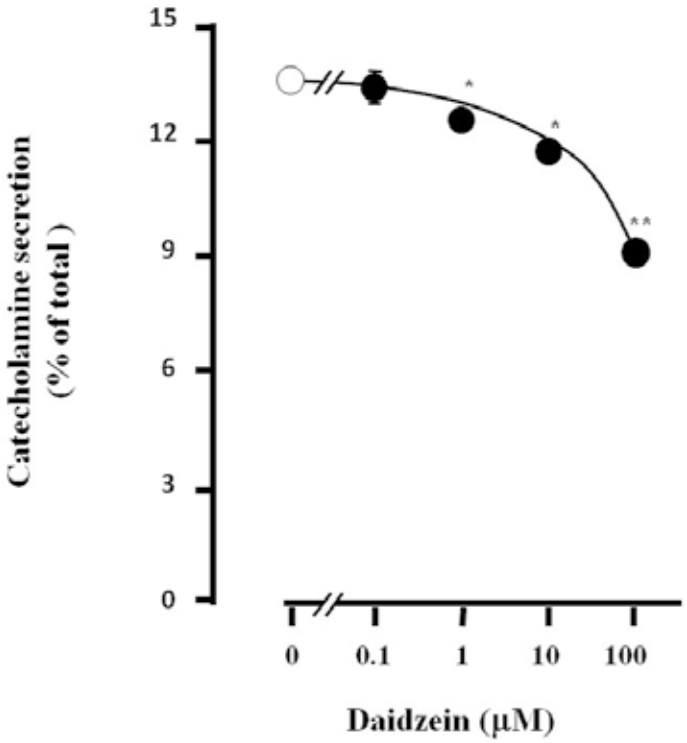

Fig. 4. Effects of daidzein on catecholamine secretion induced by acetylcholine in bovine adrenal medullary cells. Cells ($2x10^6$/dish) were incubated with acetylcholine (0.3 mM) in the presence (●) or absence (○) of various concentrations (0.1-100 μM) of daidzein for 10 min at 37 °C. Catecholamines secreted into the medium were measured, and expressed as percentage of total catecholamines. Data are means ± SEM of four experiments carried out in duplicate. *p <0.05, compared with control (0 μM daidzein). Data from Liu et al. (2007) are modified.

3. Up-regulation of noradrenaline transporter function by genistein

Treatment of SK-N-SH cells (a human neuroblastoma cell line) with genistein, another soy isoflavone, for 20 min stimulated [^{3}H]noradrenaline uptake by the cells in a bell-shaped concentration-dependent manner (0.1-10 μM), whereas neither daidzein nor cumestrol, another phytoestrogen, did so (Fig. 5A). Genistein (0.01-10 μM) also stimulated [^{3}H]noradrenaline uptake by COS-7 cells transiently transfected with noradrenaline transporter (Fig.5B). In Japanese middle-aged women, the dietary intake of genistein was reported to be 111.6 μmol/day/capita (30.1 mg/day/capita), and the median plasma concentration of genistein was 206 nM (Arai et al., 2000). Since Asian individuals generally consume more soy foods than do people in developed Western countries, the mean concentrations of genistein in Japanese and UK men were 493 nM and 33 nM, respectively (Morton et al., 2002). Furthermore, a previous paper (Adlercreutz et al., 1993) reported that the plasma concentration of genistein exceeded 2400 nM in one Japanese man. Therefore, it

seems that the genistein concentrations used in the present study are nutritionally (or pharmacologically) relevant.

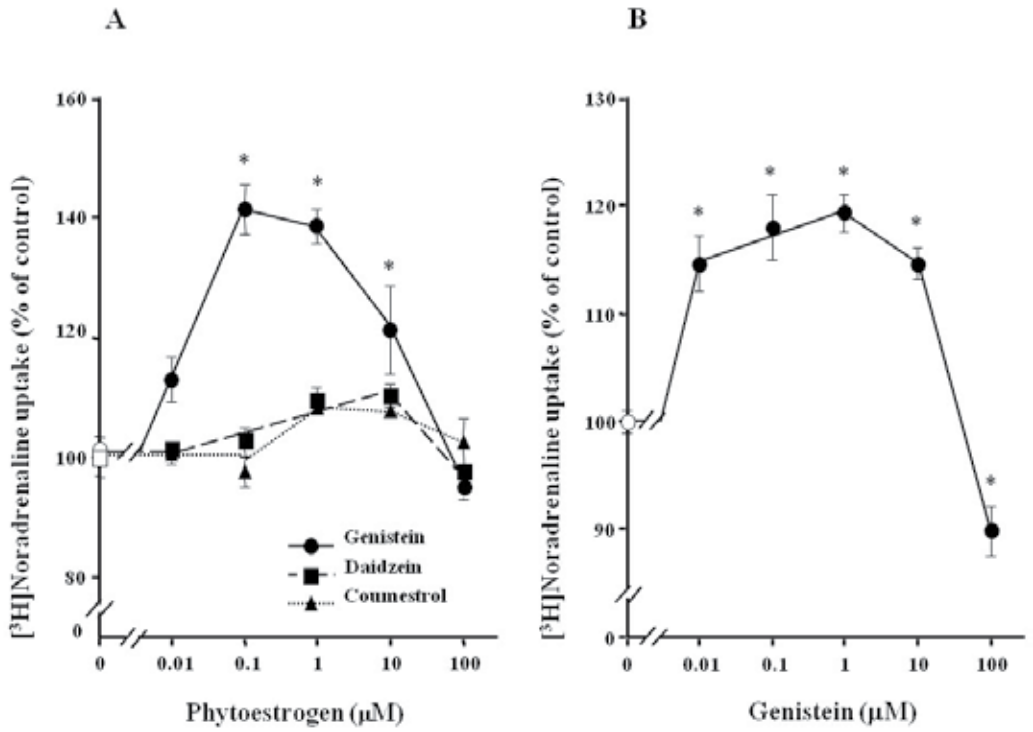

Fig. 5. Effects of various phytoestrogens on [^{3}H]noradrenaline uptake by SK-N-SH cells (A) and COS-7 cells transfected with bovine noradrenaline transporters (B). (A) SK-N-SH cells were pretreated for 20 min with or without various concentrations (0.01 – 100 μM) of genistein (●), daidzein (■), or coumestrol (▲), and then incubated for another 10 min with [^{3}H]noradrenaline (0.1 μM, 0.1 μCi) in the presence or absence of phytoestrogens (0.01 - 100 μM). Results are presented as percentage of control values (62.3 ± 4.2 fmol/10^6 cells/min). Data are means ± SEM from three separate experiments. *p < 0.05, compared with control. (B) The bovine noradrenaline transporter transfected COS-7 cells were pretreated for 20 min with various concentrations of genistein (0.01 – 100 μM), and then the desipramine-sensitive uptake of [^{3}H] noradrenaline by the cells was measured. *p < 0.05, compared with control. Data from Toyohira et al. (2010) are modified.

From Eadie-Hofstee analysis of [^{3}H] noradrenaline uptake, genistein (10 μM) caused a significant increase in the maximal velocity (V_{max}) of noradrenaline transport with little change in the Michealis-Menten constant (K_m) value (Fig. 6A). Scatchard analysis of [^{3}H]nisoxetine binding to COS-7 cells transiently transfected with noradrenaline transporter showed that genistein increases the maximal binding (B_{max}) without any change in the dissociation constant (K_d) (Fig. 6B).

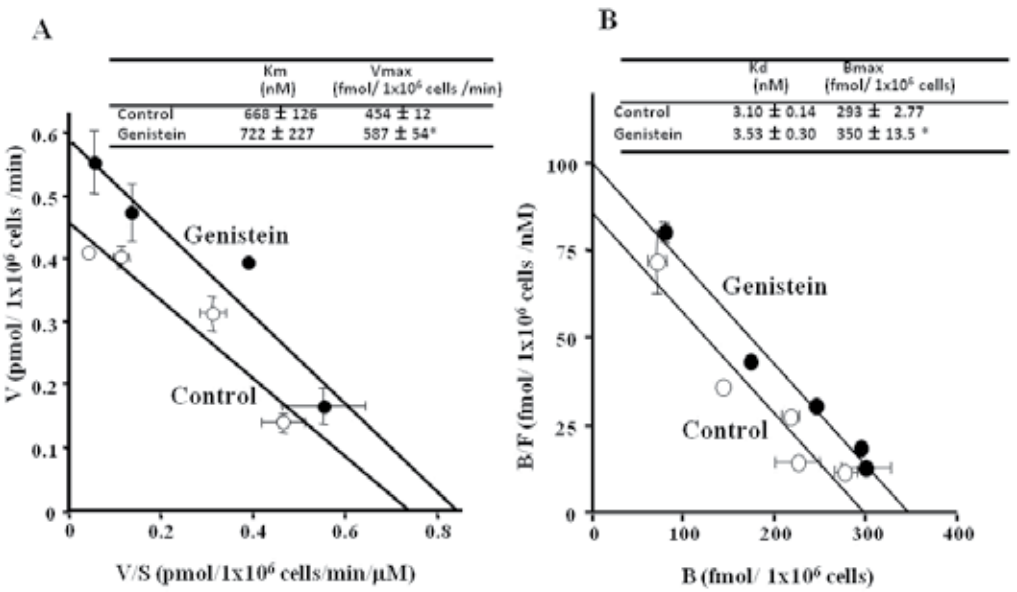

Fig. 6. Eadie-Hofstee plots of [^{3}H] noradrenaline uptake (A) and Scatchard plots of specific [^{3}H]nisoxetine binding (B) in bovine noradrenaline transporter transfected COS-7 cells. (A) Cells were pretreated with or without genistein (10 μM) for 20 min at 37 ˚C. Desipramine-sensitive [^{3}H] noradrenaline uptake was measured with various concentrations (0.1 - 10 μM) of [^{3}H] noradrenaline and analyzed by Eadie-Hofstee method of [^{3}H] noradrenaline uptake. Data are means ± SEM from three separate experiments. Inset: V_{max} and K_m values were calculated by Eadie-Hofstee analysis of the saturation curves in the absence (control) or presence of 10 μM genistein. *p < 0.05, compared with control. (B) The bovine noradrenaline transporter transfected COS-7 cells were incubated with increasing concentrations of [^{3}H]nisoxetine (1 - 24 nM) in the presence or absence of genistein (10 μM) at 4 ˚C for 2 h. Non-specific binding was determined in the presence of 10 μM nisoxetine. The specific binding was 30-40% of the total binding at the K_d concentration of [^{3}H]nisoxetine. The data are plotted by Scatchard plot analysis of the saturation curves of [^{3}H]nisoxetine specific binding in the absence (control) or presence of 10 μM genistein. Data are means ± SEM from three separate experiments. *p < 0.05, compared with control. Data from Toyohira et al. (2010) are modified.

To test the involvement of nuclear estrogen receptors (ER-α and ER-β) in the stimulatory effect of genistein on noradrenaline transport, we used ICI182,780, an inhibitor of both ER-α and ER-β. COS-7 cells transfected with noradrenaline transporters were preincubated with ICI182,780 (100 nM), and then incubated for another 10 min with [^{3}H]noradrenaline in the presence or absence of genistein (100 nM) or 17β-estradiol (100 nM). ICI182,780 by itself stimulated [^{3}H]noradrenaline uptake by the cells (data not presented). ICI182,780 did not suppress but rather enhanced genistein-induced [^{3}H]noradrenaline uptake. Sodium orthovanadate (50 μM), an inhibitor of protein tyrosine phosphatase, significantly inhibited [^{3}H]noradrenaline uptake (data not shown). Although genistein is known to be an inhibitor of tyrosine kinases, daidzein, an inactive analogue of genistein against tyrosine kinase, had little effect on [^{3}H]noradrenaline uptake by SK-N-SH cells (Fig. 5A), suggesting an involvement of tyrosine kinase, but not of membrane estrogen receptors. Furthermore, the

stimulatory effects on [3H]noradrenaline uptake were observed by treatment with tyrophostin 25, an inhibitor of epidermal growth factor receptor tyrosine kinase (Fig. 7A), whereas PP2, an inhibitor of the soluble-type src-family of tyrosine kinases, did not affect it (Fig. 7B). From these findings, it is suggested that genistein increases the activity of noradrenaline transporter, probably through processes involving receptor-type protein tyrosine phosphorylation.

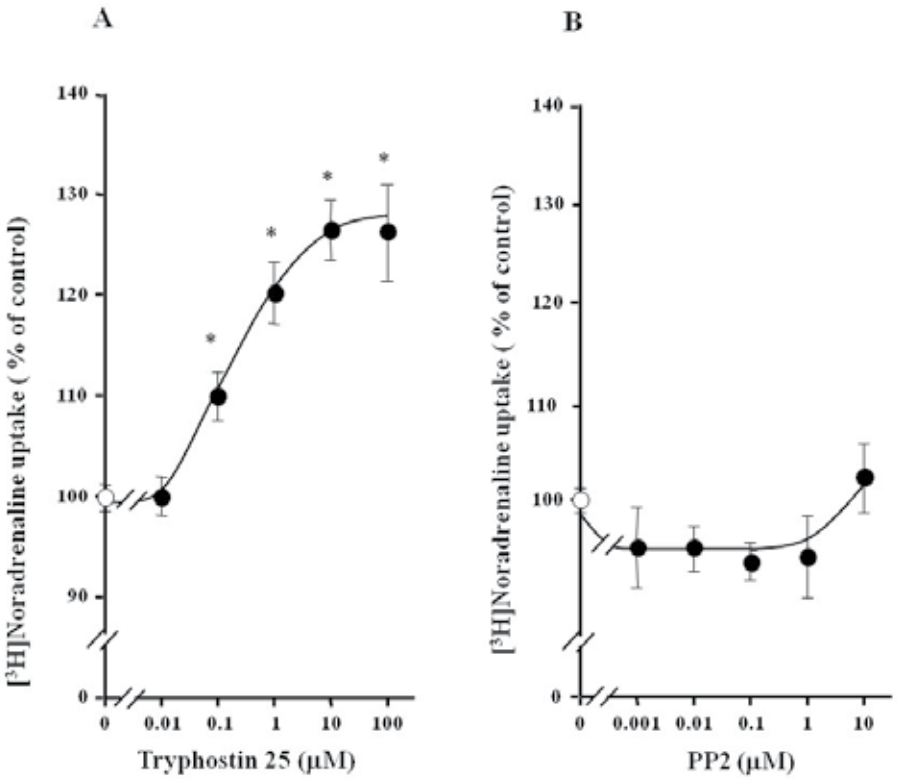

Fig. 7. Effects of tyrphostin 25 (A) or PP2 (B) on [3H] noradrenaline uptake by COS-7 cells transfected with bovine noradrenaline transporters. The bovine noradrenaline transporter transfected COS-7 cells were pretreated for 20 min with tyrphostin 25 (0.01 – 100 μM) (A) or PP2 (0.001 – 10 μM) (B), and then the desipramine-sensitive uptake of [3H] noradrenaline by the cells was measured. Results are presented as percentage of control values. Data are means ± SEM from three separate experiments. *$p < 0.05$, compared with control. Data from Toyohira et al. (2010) are modified.

4. Pharmacological significance of soy isoflavone's effects on catecholamine system

Soy isoflavones (daidzein and genistein) are present at high concentrations as a glycoside in many soybeans and soy foods such as miso, tofu, and soy milk. Several lines of accumulating evidence have indicated that soy isoflavones play a role in the prevention of cardiovascular diseases, reproductive cancers, and menopausal symptoms (Potter et al.,

1998; Cassidy et al., 1999; Upmalis et al., 2000; Ishimi et al., 1999). The cardioprotective ability of these isoflavones has been attributed partially to their ability to lower cholesterol (Wong et al., 1998; Yamakoshi et al., 2000) and cardiovascular disease risk (Lichtenstein 1998). In the present review, daidzein (0.01-1.0 μM) stimulated catecholamine synthesis by 20~30% over the control, suggesting that daidzein at nutritionally relevant concentrations strengthens the catecholamine system in the adrenal medulla and probably in the sympathetic neurons.

On the other hand, daidzein at high concentrations (over 1.0 μM) suppresses catecholamine synthesis and secretion induced by the physiological secretagogue acetylcholine, suggesting that daidzein attenuates the catecholamine synthesis and secretion induced by stress and emotional excitation, thus causing the stimulation of sympathetic nerves and the adrenal medulla. Genistein up-regulates the noradrenaline transporter function. This suggests that genistein also stimulates the termination of neurotransmission by the reuptake of noradrenaline released into the extracellular milieu and suppresses the sympathetic nerve activity. Although catecholamines play a pivotal role in the regulation of normal functions in cardiovascular systems, prolonged stress-induced over-expression of endogenous catecholamines may contribute to the involvement and augmentation of cardiovascular diseases such as heart failure, atherosclerosis, coronary heart disease, and hypertension. Indeed, chronic heart failure is associated with activation of the sympathetic nervous system as manifested by increased circulating catecholamines and increased regional activity of the sympathetic nervous system (Freedman and Lefkowitz, 2004; Westfall and Westfall 2005). Furthermore, it has been shown that up-regulation of adrenal medullary G protein-coupled receptor kinase 2 is a very important mechanism for mediating the sympathetic hyperactivity and circulating catecholamine levels that accompany and aggravate chronic heart failure (Lymperopoulos et al., 2007). The present findings on the effects of daidzein and genistein in catecholamine system may partially explain the cardiovascular protective effects of soy isoflavones.

5. Future perspectives

What are the major pending problems or questions revealed by the present study? While the *in vitro* effects of soy isoflavone have been well clarified using cultured bovine adrenal medullary cells, SK-N-SH cells or COS-7 cells transiently transfected with noradrenaline transporter, the *in vivo* effects are not as clear. Therefore, to confirm the effects of soy isoflavone on catecholamine synthesis, secretion and reuptake, further *in vivo* studies on the effects of administration of daidzein and genistein to animals or humans will be needed in the near future.

Finally, a question arises as to how best to demonstrate the protective effects of soy isoflavone on stress-induced catecholamine synthesis and secretoin? The anti-depressive or anxiolytic effects of soy isoflavone should be examined using laboratory animals under various stress conditions. Analysis with *in vivo* studies will provide more conclusive information and add new pharmacological actions of soy isoflavone on catecholamine signaling.

6. Concluding remarks

Daidzein and genistein are major natural phytoestrogens found in soybeans. In the present review, we have demonstrated that daidzein stimulates catecholamine synthesis at low

concentrations similar to those at which daidzein inhibits the specific binding of [^{3}H]17β-estradiol to the membrane receptors, suggesting that daidzein at low concentrations enhances catecholamine synthesis probably through plasma membrane estrogen receptors (Liu et al., 2007). However, daidzein at high concentrations (1-100 μM) inhibited catecholamine synthesis and secretion induced by acetylcholine, the physiological secretagogue. The latter findings suggest that daidzein at high concentrations suppresses the catecholamine synthesis and secretion induced by stress or emotional excitation that induces the stimulation of the splanchnic nerves and subsequently the adrenal medulla. In addition to daidzein's effects, genistein also increases the activity of the noradrenaline transporter, suggesting an enhancement of termination of noradrenaline transmission at the sympathetic nerve terminals. Although endogenous catecholamines play an important role in the regulation of normal functions in the cardiovascular system, stress-induced over expression of catecholamines may contribute to the involvement and augmentation of cardiovascular disorders The present findings would support the idea that soy isoflavone suppress excessive stress-induced hyperactivity of the sympatho-adrenal system and thereby protect the cardiovascular system (Yanagihara et al., 2008).

7. Funding

This work was supported in part by a grant from Grant-in-Aids (11839030, 20611020, and 20590129) for Scientific Research (C) from the Japan Society for the Promotion of Science, and a grant from the University of Occupational, Environmental Health for Advanced Research and the Smoking Research Foundation.

8. Conflict of interest

The authors have declared no conflict of interest.

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9

Occurrence of Biogenic Amines in Soybean Food Products

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1. Introduction

Biogenic amines (BAs) are known as toxic substances and formed in foods as a result of microbial action during fermentation and storage (Shalaby, 1996; Santos, 1996). BAs could cause diseases with food poisoning symptoms such as stimulating the nerves and blood vessels in man and animals (Joosten, 1988). The most important BAs found in foods are putrescine, cadaverine, β-phenylethylamine, tyramine, spermine, histamine, spermidine, tryptamine and agmatine. BAs exist in fish, meat, egg, cheeses, vegetables, soybean, beer, wine, etc., and their products. BAs are also known as possible precursors of carcinogens, such as N-nitrosamines (Shalaby, 1996; Santos, 1996). They are frequently found in high concentrations in foods and can not be reduced by high-temperature treatment (Shalaby, 1996; Santos, 1996). BAs in food are extensively studied; a lot of information on formation and occurrence of the biogenic amines in foods is given in recent reviews (Davidek & Davidek, 1995; Halasz et al., 1994; Santos, 1996; Stratton et al., 1991; Suzzi & Gardini, 2003). There are various kinds of soy products such as soybean paste, soy sauce, soy milk and soy curd, in which biogenic amines can be analyzed. Major sources of biogenic amines in the soy foods include: fermented/non-fermented foods such as soy sauce, Miso and Tofu. Nutritionally, soybean milk, Tofu and Sufu have the same importance to people of Asia as they prefer the salt-coagulated bean curd, not only because it has the desired texture, but also because it serves as an important source of calcium (Wang & Hesseltine, 1970). BAs are formed in fermented soybean products by microorganisms during fermentation, and high levels of BAs have been reported for soy products (Chin & Koehler, 1983; Mower & Bhagavan, 1989; Nout et al., 1993; Stratton et al., 1991; Yen, 1986). As the microbial spoilage of food may be accompanied by the increased production of decarboxylases, the presence of biogenic amines might serve as a useful indicator of food spoilage. For these reasons, it is important to monitor biogenic amines levels in foods. Soy sauce, a Chinese traditional fermented condiment, is made from soybean and wheat flour. During the manufacturing process of soy sauce, soy sauce is traditionally prepared by growing the koji mold such as *Aspergillus oryzae* (*A. oryzae*) or *Aspergillus sojae* (*A. sojae*) on the raw material containing a mixture of steam-cooked defatted soybean and roasted wheat flour. Soy sauce mash obtained by mixing the finished koji with brine solution is then subjected to various periods of ageing (Whitaker, 1978). During the fermentation of soy sauce, proteins in the raw materials are hydrolyzed into small molecular weight peptides, amino acids and ammonia

by the proteases produced by *A. oryzae* or *A. sojae* (Whitaker, 1978). During the fermentation and ageing, the flavor may develop gradually. Meanwhile, soy sauce contains relatively high amount of free amino acids, which could be a potential sources of biogenic amine formation. There are many factors that affect the production of BAs, such as the ratio of soybean in the raw material, microbiological composition and duration of fermentation (Chin & Koehler, 1983; Nout et al., 1993).

There are various kinds of soy products such as soybean paste, soy sauce, soy milk and soy curd, in which biogenic amines can be analyzed. The most well known biogenic amines are the neurotransmitters such as serotonin, dopamine, noradrenaline and histamine, best known for their role in allergies. Others, which are less well known, include tyramine, tryptamine and β-phenylethylamine. These biogenic amines may act as neurotransmitters,be involved in local immune responses (such as the inflammation produced by histamine release), or regulate the functions of gut. The classic neurotransmitters serotonin dopamine, noradrenaline are all essential to proper brain functioning. Imbalances of these neurotransmitters can lead depression and anxiety. In relation to food intolerances however, we are more concerned with the biogenic amines contained in foods and beverages that can cause local symptoms in the gut includig nausea, diarrhoea and irritable bowel syndrome, as well as triggering symptoms elsewhere in the body, such as migraines, asthma and hives. The chemical structure of biogenic amines can either be:

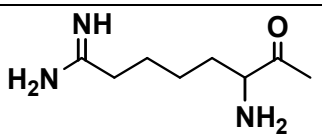
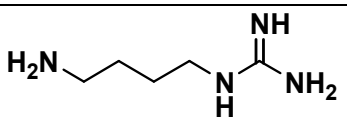
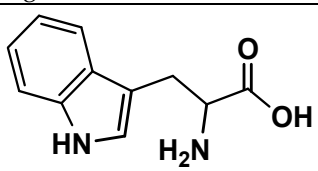
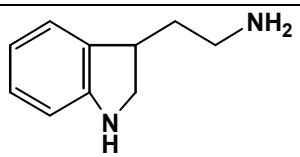
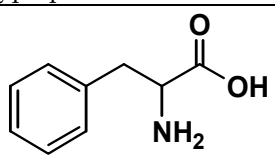
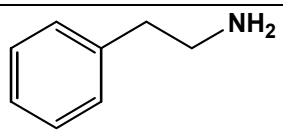
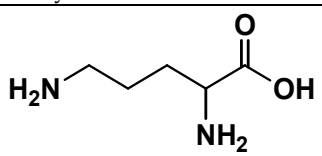
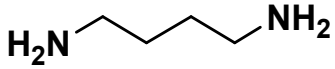
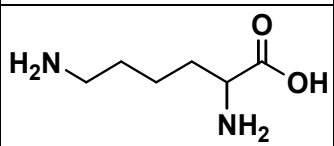
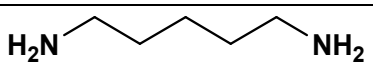
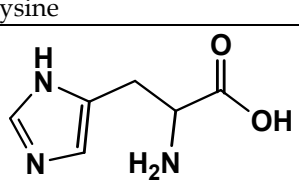
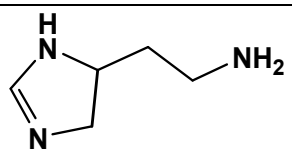
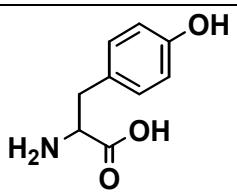
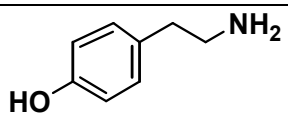
- aliphatic (putrescine, cadaverine, spermine, spermidine)
- aromatic (tyramine, phenylethylamine)
- heterocyclic (histamine, tryptamine) (Santos, 1996)

Amines such as putrescine, spermidine, spermine and also cadaverine are indispensable components of living cells and they are important in the regulation of nucleic acid fraction and protein synthesis and also in the stabilization of membranes (Bardocz, 1995; Maijala et al., 1993; Halasz et al., 1994; Santos, 1996).

2. Mechanism of biogenic amines formation

Amine build-up usually results from decarboxylation of free amino acids by enzymes of bacterial origin. Amino acid decarboxylation takes place by the removal of a carboxyl group to give the corresponding amine. Arginine is easily converted to agmatine, or as a result of bacterial activity can be degraded to ornithine from which putrescine is formed by decarboxylation. Lysine can be converted by bacterial action into cadaverine. Histidine can, under certain conditions, be decarboxylated to histamine. Tyramine, tryptamine and β-phenylethylamine come by the same manner from tyrosine, tryptophan and phenylalanine, respectively. Proteolysis, either autolytic or bacterial, may play a significant role in the release of free amino acids from tissue proteins which offer a substrate for decarboxylases reactions (Shalaby, 1996). The precursors of the main biogenic amines are described in Table 1. Prerequisites for biogenic amine formation by microorganisms are:

1. Availability of free amino acids (Joosten, 1988; Marklinder & Lonner, 1992; Soufleros et al., 1998).
2. Presence of decarboxylase-positive microorganisms (Tiecco et al., 1986; Brink et al., 1990; Huis in't Veld et al., 1990).
3. Conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity (Brink et al., 1990; Santos, 1996).

Compound name	Precusor	Structure	Molecular weight
Agmatine	 Arginine		130.2
Tryptamine	 Tryptophan		160.2
2-Phenylethyl amine	 Phenylalanine		121.2
Putrescine	 Orithine		88.2
Cadaverine	 Lysine		202.2
Histamine	 Histidine		111.0
Tyramine	 Tyrosine		137.3

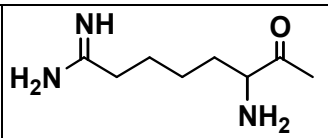
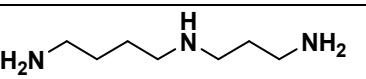
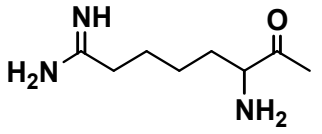
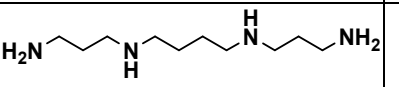
Spermidine	 Arginine		145.3
Spermine	 Arginine		348.1

Table 1. List of some important Biogenic amines and their precoursors

3. Functions of biogenic amines

BAs are sources of nitrogen and precursors for the synthesis of hormones, alkaloides, nucleic acids and proteins (Santos, 1996). They can also influence the processes in the organism such as the regulation of body temperature, intake of nutrition and increase or decrease of blood pressure (Greif et al., 1999). In plants, polyamines such as spermidine and spermine are implicated in a number of physiological processes, such as cell division, flowering, fruit development, response to stress and senescence (Halasz et al., 1994). Polyamines are important for the growth, renovation and metabolism of every organ in the body and essential for maintaining the high metabolic activity of the normal functioning and immunological system of gut (Santos, 1996; Bardocz, 1995). Because of the diversity of the roles of polyamines in cellular metabolism and growth, the requirement for polyamines is particularly high in rapidly growing tissues. Indeed, the importance of putrescine, spermidine and spermine in tumour growth is widely recognized. Inhibition of polyamine biosynthesis in tumour-bearing individuals is one of the major targets of cancer therapy research.

BAs are potential precursors for the formation of carcinogenic N- nitroso compounds (Krizek & Kalac, 1998). The reaction of nitrosating agents with primary amines produces short-lived alkylating species that react with other components in the food matrix to generate products (mainly alcohols) devoid of toxic activity in the relevant contents. The nitrosable secondary amines (agmatine, spermine and spermidine, etc.) can form nitrosamines by reaction with nitrite, while tertiary amines produce a range of labile N-nitroso products (Halasz et al., 1994). In fatty foods, such as bacon, at high temperature and in the presence of water, the carcinogen N-nitrosopyrrolidine can be formed from putrescine or spermidine (Lovaas, 1991). Some BAs such as putrescine, cadaverine and spermidine can act as free radical scavengers. Tyramine has a remarkable antioxidative activity, which increases with its content. Thus, inhibiting effect depends on amino and hydroxy groups (Halasz et al., 1994). Spermine is able to regenerate tocopherol from the tocopheroxyl radical through hydrogenic donor from amino group. The spermine radical next binds lipid or peroxide radicals into a lipid complex (Greif et al., 1999).

Beginning in the early 1990s, a new era dawned in studies of Biogenic amines as neurotransmitter structure, function and regulation, illuminated by the cloning of transporter cDNAs and genes, the development of transporter-specific gene and protein probes, and the characterization of heterologous expression systems suitable for advanced biophysical analyses. It is believed that the brain contains several hundred different types of

chemical messengers (neurotransmitters) that act as communication agents between different brain cells. These chemical messengers are molecular substances that can affect mood, appetite, anxiety, sleep, heart rate, temperature, aggression, fear and many other psychological and physical occurrences. The biogenic amineneurotransmitters dopamine (DA), norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT) are very simple molecules with highly complex actions in the peripheral and central nervous systems ranging from the control of heart rate to the coloring of mood. Pharmacologists have been fascinated by the amines for decades, as the management of amine production, action or inactivation figures prominently in the treatment of autonomic, emotional and cognitive disturbances. The past decade began with an elucidation of the genes responsible for clearance of amines from the synaptic cleft (Povlock & Amara, 1997).
Scientists have identified three major categories of neurotransmitters in the human brain: Some common structures of biogenic amines as a neurotransmitters are shown in Fig.1 & Fig. 2.

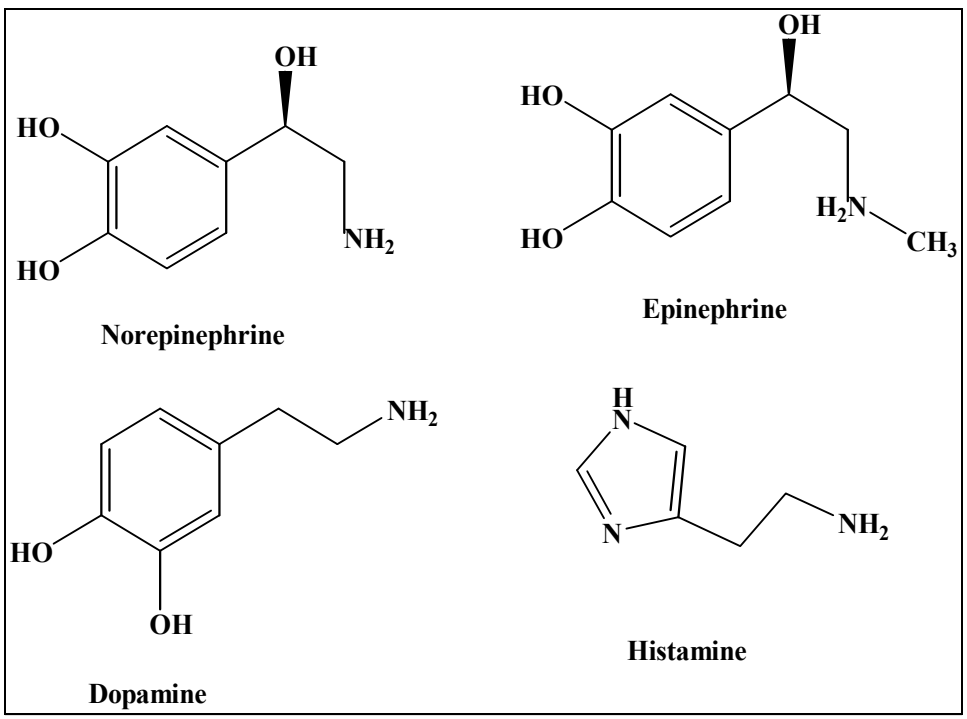


Fig 1. Common biogenic amines as neurotransmitters.

Biogenic amine neurotransmitters have been studied the longest and are probably the best understood in terms of their relationship to psychological disturbances. Some important biogenic amine neurotransmitters are:
Serotonin, is chemical messenger that a role in modulating anxiety, mood, sleep, appetite and sexuality. Serotonin reuptake inhibitors are generally considered first line medication to treat panic disorder.
Norepinephrine, which influences sleep and alertness, is believed to be correlated to fight or flight stress response.

Epinephrine, is usually thought of a stress hormone managed by the adrenal system, but it also acts as a neurotransmitter in the brain.
Dopamine, influences body movement and is also believed to be involved in motivation, reward, reinforcement and adictive behaviours. Many theories of psychosis suggest that dopamine plays a role in psychotic symptoms.
Histamine, is thought to influence arousal, attention and learning. It is also released in response to an allergic reaction. Antihistaine, which are commonly used to treat allergies, have common side effects of sedation, weight gain and low blood pressure.

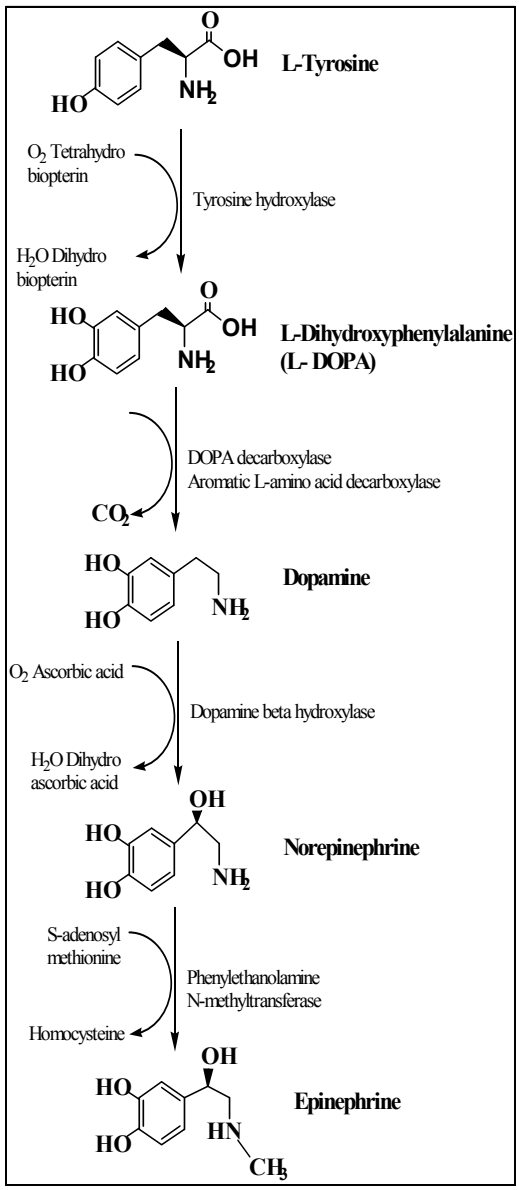


Fig. 2. A synthetic pathway for neurotransmitter biogenic amines

4. Microorganisms producing biogenic amine in soybean food

Microorganisms have a different ability in synthesizing decarboxylases. Most soybean fermented and non-fermented foods are subjected to conditions that enable BAs synthesis. The amount of different amines formed is highly dependent on the nature of the food and the microorganisms present in the food (Brink et al., 1990). BAs are present in a wide range of fermented food products such as fish (Shalaby, 1996), meat (Maijala et al., 1993), dairy (Stratton et al., 1991), soybean products (Chin & Koehler, 1986), wine (Lehtonen et al., 1992) and beer (Dumont et al., 1992), as well as vegetables (Taylor et al., 1978). Soybean paste or Doenjang is a traditional Korean food produced through the fermentation of soybeans by naturally occurring bacteria and fungi, and has been consumed for centuries as a protein rich source and seasoning ingredient in Korea. This paste contains a relatively high concentration of amino acids degraded from soybeans and may be a source for BAs formation. Decarboxylase activity has been described in several microbial groups, including *Bacillus*, *Citrobacter*, *Clostridium*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Photobacterium*, *Lactobacillus*, *Pediococcus* and *Streptococcus* (Rice et al., 1976; Brink et al., 1990; Huis in't Veld et al., 1990). In Miso (Japanese fermented soybean paste), tyrosine decarboxylase bacteria have been identified as *Enterococcus faecium, Lactobacillus bulgaricus* and histamine decarboxylase has been associated with *Lactobacillus* species and *Lactobacillus sanfrancisco* (Ibe et al., 1992). Amine-producing lactic bacteria such as *Lactobacillus breuis, Lactobacillus buchneri, Lactobacillus bulgaricus, Lactobacillus curuatus, Lactobacillus carnis, Lactobacillus dicergens* and *Lactobacillus hilgardii* have been isolated from meat products (Maijala et al., 1993). Moon et al. (2010) isolated two biogenic amine producing bacteria from traditional soybean pastes: one was a histamine producing *Clostridium* strain, and the other was a tyramine producing *Pseudomonas* strain. Moon et al. (2010) reported that *Clostridium* strain, isolated from traditional soybean pastes, was potent histamine producer among the tested cultures. *Clostridium perfringens* grows in protein rich media and can not survive in media that lacks essential amino acid supply (Shimizu et al., 2002). Accordingly, this bacterium is often detected in amino acid rich environment, including protein-fermented foods like Sufu, a traditional Chinese fermented soybean curd (Han et al., 2001). Tsai et al. (2007) identified some histamine producing bacteria belonging to *Lactobacillus* species in Natto products (traditional Japanese fermented soybean food) manufactured in Taiwan. In the case of fermented food and beverages, the introduction of starter cultures can affect the production of biogenic amines either directly or indirectly through interaction between different microbial populations, which are probably very important (Huis int Veld et al., 1990).

5. Occurrence of biogenic amines in soybean food

Virtually, all foods that contain proteins or free amino acids are subjected to conditions, enabling microbial or biochemical activity; biogenic amines can be expected. The total amount of the different amines formed strongly depends on the nature of the food and the microorganisms present (Brink et al., 1990). Biogenic amines are present in a wide range of food products including fermented and non-fermented soybean products (Brink et al., 1990; Halasz et al., 1994; Santos, 1996; Shalaby, 1996; Soufleros et al., 1998). Since several

varieties of molds, yeasts and lactic acid bacteria are involved in the fermentation processes of soybean products where the raw material (soybean) contains considerable amounts of protein, the formation of various amines might be expected during the fermentation (Shalaby, 1996). Several studies have shown that biogenic amines in fermented soybean products are most likely formed by the lactic microflora that remains active during fermentation (Kirschbaum et al., 2000; Stratton et al., 1991). Tyramine and histamine have been found at various levels in fermented products (Stratton et al., 1991). The variability of biogenic amines levels in the commercial fermented soybean products samples had been attributed to the variations in manufacturing processes; variability in the ratio of soybean in the raw material, microbial composition, conditions and duration of fermentation (Shalaby, 1996). The data reported by several authors (Maijala et al., 1995a; Eerola et al., 1998) confirmed the key role played by the raw material quality. However, other variables such as pH, moisture content and NaCl can have an important effect on the production of BAs in soybean food and other food products. In non-fermented foods, the presence of BAs above a certain level is considered as indicative of undesired microbial activity, therefore, the amine level could be used as an indicator of microbial spoilage. However, the presence of biogenic amines in food does not necessary correlate with the growth of spoilage organisms, because they are not all decarboxylase-positive (Santos, 1996). Shalaby (1996) reported that fermented soybean products (Miso) contained high levels of histamine (462 mg/100g), putrescine (1,234 mg/100g), cadverine (634 mg/100 g) and tyramine (3,568 mg/100g). Cho et al. (2006) reported the presence of histamine and tyramine in traditional Korean paste Doenjang at a level of 952.0 mg/kg and 1,430.7 mg/kg. Tyramine was the most abundant BA in different types of soy sauces produced in China (Yongmei et al., 2009). Tsai et al. (2007) tested biogenic amine levels in seven soybean and eleven black bean douchi (traditional chinese fermented soybean product), among which four soybean douchi products had histamine levels greater than 5 mg/100 g while, among the black bean douchi samples, four samples contained histamine at 56.3, 62.1, 80.2 and 80.8 mg/100 g, levels greater than 50 mg/100 g, a hazard action level (Taylor, 1989). However, histamine is not the only compound responsible for scombrotoxicosis (acute onset of gastrointestinal symptoms such as headache, flushing and hypertension after ingesting spoiled fish), since ingestion of pure histamine does not automatically cause toxic symptoms (Bjeldanes et al., 1978). The differences in the contents of biogenic amines between black bean and soybean douchi products could be attributed to the variation of the substrate materials, the microbial composition, conditions and duration of fermentation (Yen, 1986). The toxic effects of histamine are increased in the presence of some other amines, such as putrescine and cadaverine, which inhibit histamine metabolizing enzymes in the small intestine (Arnold & Brown, 1978; Bjeldanes et al., 1978; Lehane & Olley, 2000).

Yen (1986) reported that the average amine contents in 15 samples of commercial Sufu from Taiwan and China were: cadaverine (0.039 mg/g), histamine (0.088 mg/g), β - phenylethylamine (0.063 mg/g), putrescine (0.473 mg/g), tryptamine (0.150 mg/g) and tyramine (0.485 mg/g). Tyramine and putrescine were the major amines found, and these might have a potential harmful effect on human beings if levels are very high. Biogenic amines in different varieties of soybean foods have been analyzed by several other authors as summarized in Table 2.

Food	Tyramine	Tryptamine	Histamine	Putrescine	Cadaverine	Phenylethylamine	Spermine	Spermidine	Reference
Fermented Soy	-	-	4,620	12,340	6,340	-	-	-	Shalaby , 1996
Tempe	4.3	15.6	4.1	116.9	-	-	-	11.6	Saaid et al., 2009
Soy bean sauce	1.0	ND	9.6	1.0	-	-	-	ND	
Salty soy sauce	ND	ND	2.0	ND	-	-	-	ND	
Taucu (salty bean)	ND	ND	0.8	59.0	-	-	-	ND	
Soya bean milk	1.7	20.2	17.5	ND	-	-	-	1.3	
Soy sauce (n mol/ g)	-	-	-	696	-	-	10	82	Nishibori et al., 2007
Miso (Japanese soybean paste) mol/g	-	-	-	296	-	-	5	12	
Korean Doenjang (traditional type)- mg/kg	669.5	105.5	596.4	462.6	23.5	244.7	3.8	15.6	Cho et al., 2006
Korean Doenjang (modern type)- mg/kg	133.0	22.4	83.6	46.4	3.2	6.5	2.4	7.4	
Miso (mg/kg)	48.6	22.6	0.9	19.8	3.0	4.4	2.2	15.7	
Chunngkukjang (mg/kg)	133.8	69.9	10.1	26.4	9.7	22.0	10.7	52.0	
Chunngkukjang powder (mg/kg)	68.1	35.0	1.0	10.2	12.1	17.0	15.5	54.6	
Chunjang (mg/kg)	44.3	16.6	16.8	10.7	3.3	7.0	1.1	6.1	
Soy sauce (traditional tType) mg/kg	241.6	12.1	225.9	376.9	16.1	13.5	6.6	24.5	
Soy sauce (modern type) mg/kg	594.5	36.6	129.8	56.8	6.1	40.8	1.0	6.3	
Kochujang (mg/kg)	3.5	27.2	1.0	2.9	0.5	4.9	1.6	2.5	
Sufu (white) mg/100g	0.80	1.62	0.46	1.51	0.70	ND	1.64	ND	Kung et al., 2007

Sufu (brown)mg/100 g	1.08	ND	2.72	0.24	5.00	ND	ND	ND	
Chinese soy sauce (mg/l)	0-673		0-592		0-550		0-145	0-486	Yongmei et al., 2009
Natto (Japanese) mg/100g	0.12	0.91	3.54	1.71	2.19	ND	0.86	4.50	Tsai et al., 2007
Natto (Taiwan) mg/100g	ND	ND	4.51	0.16	0.05	ND	ND	2.50	

ND: Not detected

Table 2. Biogenic amines in different soybean food products

6. Physiological role of biogenic amines

BAs play a number of crucial roles in the physiology and development of eukaryotic cells (Tabor & Tabor, 1985; Igarashi, 2001). A detail description of their physiological role has been summarized in Table 3. The most active BAs are histamine, putrascine and tyramine. Polyamines such as putrescine, spermine and spermidine also play essential roles in cell growth and differentiation via the regulation of gene expression and the modulation of signal transduction pathways. Histamine is present in many living tissues as a normal constituent of the body and has multiple effects in different mammalian and invertebrate organs (Maintz & Novak, 2007). In humans, it is found in different concentrations in the brain, lungs, stomach, small and large intestines, uterus and the ureter. It is produced and stored predominantly in mast cells, basophiles and neurons. Histamine modulates a variety of functions by interacting with specific receptors on target cells, namely H1, H2 and H3 receptors of the G-protein coupled receptor family. H1 receptors are found in the brain where they are involved in the control of the circadian rhythm, attention and cognition and in peripheral tissues where they mediate vascular and bronchial muscle responses to histamine in allergic processes (Jorgensen et al., 2007). H2 receptors, although widely distributed in body tissues, seem to have a central role only in the regulation of acid secretion. They respond to the presence of histamine, provoking gastric acid secretion and the contraction of intestinal smooth muscle (Ranganchari, 1992). H3 receptors, originally described as presynaptic autoreceptors on brain histaminergic neurons that control histamine synthesis and release, were subsequently recharacterised as heteroreceptors on non-histaminergic neurons in the central and peripheral nervous systems. They have also been found in immune cells and in smooth muscle (Coruzzi et al., 2001; Passani et al., 2007) where they have been associated with immediate and allergic hypersensitivity. When histamine binds with these receptors, they affect the contraction of smooth muscle cells, the dilation of blood vessels and, therefore, an efflux of blood serum is established into the surrounding tissues (including the mucous membranes) and initiating the inflammatory process (Rangachari, 1992). Tyramine and β-phenylethylamine are included in the group of trace amines, a family of endogenous compounds with strong structural similarities to classical monoamine neurotransmitters, although the endogenous levels of these

compounds are at least two orders of magnitude below that of these neurotransmitters. The effects of these low physiological concentrations have been difficult to demonstrate, but it has been suggested that they serve to maintain the neuronal activity of monoamine neurotransmitters within defined physiological limits (Berry, 2007). Tyramine can be converted into octopamine when taken up in sympathetic nerve terminals, where it displaces norepinephrine (NE) from storage vesicles. A portion of this NE diffuses out of the nerve to react with receptors, causing hypertension and other sympathomimetic effects (Berry, 2007). The biological functions of amines are mainly the regulation of gene expression by altering DNA structure and by modulating signal transduction pathways. The optimal functioning of the cell therefore requires the intracellular polyamine content be strictly controlled at the levels of biosynthesis, catabolism, uptake and efflux (Linsalata & Russo, 2008). Small amounts of orally administrated polyamines induce cell growth; larger quantities have no effect or may actually inhibit growth (Deloyer et al., 2001). Amines lies in their physiological functions related to cell membrane stabilization and cell proliferation, since they are involved in DNA, RNA and protein synthesis. Therefore, they are considered important food microcomponents during periods of intensive tissue growth (infant gut maturation, post-operational recovery, etc.), although in some pathological cases (individuals with tumours) the intake of amines should be minimized (Bardocz, 1995).

7. Toxicological effects of biogenic amines

BAs, such as tyramine and β- phenylethylamine, have been proposed as the starters of hypertensive crisis in certain patients and dietary-induced migraine. Another amine, histamine, has been implicated as the causitive agent in several outbreaks of food poisoning. Histamine intake ranged within 8 - 40 mg, 40 - 100 mg and higher than 100 mg may cause slight, intermediate and intensive poisoning, respectively (Parente et al., 2001). Nout (1994) pointed out that the maximum daily intake of histamine and tyramine should be in the range of 50 - 100 mg/kg and 100 - 800 mg/kg, respectively; over 1,080 mg/kg tyramine becomes toxic. Putrescine, spermine, spermidine and cadaverine have no adverse health effect, but they may react with nitrite to form carcinogenic nitrosoamines and also can be proposed as indicators of spoilage (Hernandez-Jover et al., 1997). Tryptamine can induce blood pressure increase, therefore causes hypertension, however there is no regulation on the maximum amount of tryptamine consumption in sausage in some countries (Shalaby, 1996). Food poisoning may occur especially in conjunction with potentiating factors such as monoamine oxidase inhibiting (MAOI) drugs, alcohol, gastrointestinal diseases and other food containing amines. Histaminic intoxication and hypertensive crisis due to interaction between food and MAOI anti-depressants as well as food-induced migraines are the most common reactions associated with the consumption of foods containing large amounts of biogenic amines (Marine-Font et al., 1995). The diamines (putrescine and cadaverine) and the polyamines (spermine and spermidine) favor the intestinal absorption and decrease the catabolism of the above amines, thus, potentiating their toxicity (Bardocz, 1995). Formation of nitrosoamines, which are potential carcinogens, constitutes an additional toxicological risk associated to biogenic amines, especially in meat products that contain nitrite and nitrate salts as curing agents (Scanlan, 1983). Determination of the exact toxicity threshold of biogenic amines in individuals is extremely difficult, since the toxic dose is strongly dependent on the efficiency of the detoxification mechanisms of each individual (Halasz et al., 1994). Normally, during the food intake process in the human gut, low amounts of

biogenic amines are metabolized to physiologically less active degradation products. This detoxification system includes specific enzymes such as diamine oxidase (DAO). However, upon intake of high loads of biogenic amines in foods, the detoxification system is unable to eliminate these biogenic amines sufficiently. Moreover, in case of insufficient DAO activity, caused for example by generic predisposition, gastrointestinal disease or inhibition of DAO activity due to secondary effects of medicines or alcohol, even low amounts of biogenic amines can not be metabolized efficiently (Bodmer et al., 1999).

Some biogenic amines, e.g., histamine and tyramine, are considered as anti-nutritional compounds. For sensitive individuals they represent a health risk, especially when their effects are potentiated by other substances. The intake of foods with high BA loads or the inadequate detoxification of BAs can lead to their entering the systemic circulation, inducing the release of adrenaline and noradrenaline and provoking gastric acid secretion, an increased cardiac output, migraine, tachycardia, increased blood sugar levels and higher blood pressure (Salabhy, 1996). The most serious and studied toxic effects of BA-rich foods have been investigated in patients treated with MAOIs (Stratton et al., 1991; Gardner et al., 1996; Rapaport, 2007). Indeed, the toxic effects of some BAs were first discovered in patients treated with MAOIs who suffered headaches after eating cheese (Blackwell, 1963; Hanington, 1967). Depending on the severity of the symptoms, the effects of BAs are described as a reaction, intolerance, or intoxication or poisoning. Reaction symptoms include nausea, sweating, rashes, slight variations in blood pressure and mild headache. If the amount ingested is too great for efficient detoxification to be performed, or the detoxification system is strongly inhibited, the symptoms become more severe (those of intolerance) with vomiting, diarrhoea, facial flushing, a bright red rash, bronchospasms, tachycardia, oral burning, hypo- or hypertension and migraine. In exceptional cases BA poisoning may occur, involving a hypertensive crisis (blood pressure >180/120 mmHg) that can lead to end-organ damage in the heart or the central nervous system (Blackwell, 1963).

Biogenic amine	Physiological effects	Toxicological effects
Histamine	Neurotransmitter, local hormone, gastric acid secretion, cell growth and differentiation, regulation of circadian rhythm, body temperature, food intake, learning and memory, immune response, allergic reactions	Headaches, sweating, burning nasal secretion, facial flushing, bright red rashes, dizziness, itching rashes, oedema (eyelids), urticaria, difficulty in swallowing, diarrhea, respiratory distress, bronchospasm, increased cardiac output, tachycardia, extrasystoles, blood pressure disorders
Tyramine	Neurotransmitter, peripheral vasoconstriction, increase respiration, elevate blood glucose, release of norepinephrine	Headaches, migraine, neurological disorders, nausea, vomiting, respiratory disorders, hypertension
Putrescine	Regulation of gene expression maturation of intestine, cell growth and differentiation	Increased cardiac output, tachycardia, hypotension, carcinogenic effects

Table 3. Physiological and toxicological effects of biogenic amines

8. Recommended limits of biogenic amines in food

It is very difficult to establish a uniform maximum limit for ingested BAs since their toxic effects depend on the type of amine, the presence of modulating compounds and the efficiency of an individual's detoxification mechanism. Several studies have suggested that the absorption, metabolism and/or potency of one BA might be modified by the presence of another, which might explain why aged cheese is more toxic than its equivalent amount of histamine in aqueous solution (Taylor, 1986). Laboratory studies on the effects of BAs face a number of methodological problems. Most studies have focused on the effect of individual BAs administered intravenously to laboratory animals or healthy volunteers, but these results are difficult to transfer to food intake since the intravenous response is several times higher than that obtained with oral administrations (Simpson & White, 1984). The effects of trace amines are mainly based on clinical observations; no meta-analyses that might confirm their effects are therefore possible (Jansen et al., 2003). Ingestion limits based on case reports may be too high since, usually, only cases of BA poisoning are reported (Taylor, 1985; Rauscher-Gaberng et al., 2009). Although more in-depth studies on the toxic effects of BAs are necessary, some studies have reported minimum toxic levels for some BAs. Wohrl et al. (2004) reported that 75 mg of pure liquid oral histamine, a dose common in normal meals, can provoke immediate as well as delayed symptoms in 50% of healthy females with no history of food intolerance. A concentration of over 125 mg/kg of tyramine in food is considered to be toxic in normal individuals, almost 100 times the concentration considered potentially toxic when ingested in combination with MAOIs (McCabe-Sellers, 1986). Threshold values of 100 mg/kg for tyramine and 30 mg/kg for phenylethylamine have been suggested (Brink et al., 1990). However, since there is always more than one type of BA in food, a maximum total BA level of 750 - 900 mg/kg in food products has been proposed (Brink et al., 1990). Currently, the only BA for which maximum limits have been set in the European Union and the United States of America is histamine. The US Food and Drug Administration (FDA) consider a histamine level of ≥500 mg/kg in food to be a danger to health. This agrees with values cited in histamine intoxication reports in which over 500-1000 mg/kg of food had been ingested (Rauscher-Gaberng et al., 2009). Askar and Treptow (1996) have suggested histamine at a concentration of 500 mg/kg in food to be hazardous for human health. On the other hand, an upper limit of histamine for human consumption has to be 100 mg/kg, 100 - 800 mg/kg of tyramine and 30 mg/kg of phenylethylamine in food products have been reported to be toxic doses in foods (Brink et al., 1990). Total BA levels of 1,000 mg/kg in food are also considered hazardous for human health (Taylor, 1985). An intake of over 40 mg biogenic amines per meal has been considered potentially toxic (Nout, 1994).

9. Factors influencing biogenic amine production in soybean food

Since amines are formed by the enzymatic breakdown of food or by decarboxylase active bacteria, inhibition of such activity and prevention of bacterial growth would be very important for controlling the hazardous amine content of foods. Raw material and various manufacturing conditions influence the production of biogenic amines. Thus, tyramine, putrescine and cadaverine concentration in Tempe were low or high depending on the applied manufacturing process: soaked soybeans, kinds of fermentative microorganisms used and storage temperature (Nout et al., 1993). Biosynthesis of amino acids in fermented soybean paste is an enzymatic process which is catalyzed by synthetases (e.g. glutamine synthetase). Other amino acid metabolizing enzymes have been detected with higher levels,

e.g., aspartate amino transferase and especially histidine decarboxylase during the fermentation process in various food products (Picton et al., 1993). Thus, biogenic amine production in various fermented and non-fermented soybean foods has been related to factors such as variety of raw material, pH, salt concentration, and temperature.

9.1 Effect of pH

The pH is an important factor for fermentation and formation of biogenic amines because amino acid decarboxylase activity remains stronger in an acidic environment (Santos, 1996). Santos (1996) reported that the pH was an important factor influencing decarboxylase activity, and low pH about 3.0 - 6.0 was optimal for bacteria to produce decarboxylase**.** Teodorovi et al. (1994) also reported that amino acid decarboxylase activity was stronger in an acidic environment, being the optimum pH between 4.0 and 5.5. Furthermore, in such acidic environment, bacteria are more strongly encouraged to produce decarboxylase enzymes, as a part of their defence mechanisms against the acidity (Santos, 1996). In addition to this, Kim et al. (2003) reported that low pH of Doenjang samples, about 3.0 - 6.0, was effective for increasing the decarboxylase activity. Koessler et al. (1928) suggested that amine formation by bacteria was a physiological mechanism to counteract an acid environment. Bacterial amino acid decarboxylases usually have acid pH optimum (Gale, 1946). However, amine formation depends on the amount of growth of decarboxylating bacteria (Yoshinaga & Frank, 1982). High production of histamine can be related to inadequate pH decrease in the first day of ripening process (Buncic et al., 1993; Maijala et al., 1993). Also tyramine production by *Carnobacterium divergens* was lower at pH 4.9 than 5.3, associated with a reduced cell yield. This can explain the low tyramine amount found in nordic meat generally characterized by lower pH, which limits bacterial growth, and, consequently, tyrosine decarboxylase activity (Masson et al., 1999).

9.2 Effect of sodium chloride

The variation in the quantity of water and in the salt/water ratio during fermentation and storage of fermented soyproducts has an important role on microbial multiplication. The rate of amines production of a bacterial strain *L. bulgaricus* (now *L. delbrueckii* subsp. *bulgaricus*) was considerably reduced when salt concentration in the medium increased from 0 to 6% (Chander et al., 1989). Chin and Koehler (1986) demonstrated that NaCl concentration ranging from 3.5 to 5.5% could inhibit histamine production. This influence can be attributed to reduced cell yield obtained in the presence of high NaCl concentration and to a progressive disturb of the membrane located microbial decarboxylase enzymes (Sumner et al., 1990). A similar NaCl effect characterized cell yield and BA production in *Enterococcus faecalis* EF37 (Gardini et al., 2001). According to Santos (1996), the presence of sodium chloride activates the tyrosine decarboxylase activity and inhibits histidine decarboxylase activity. At 3.5% content of sodium chloride, the ability of *L. buchneri* to form histamine is partly inhibited, whereas its formation was stopped at the concentration of 5.0% NaCl (Maijala et al., 1995b). Hernandez-Herrero et al. (1999) reported that NaCl contents in the range of 0.5 - 10% had a stimulatory effect on histamine formation for *Staphylococcus capitis* and *Staphylococcus epidermidis*, whereas NaCl level in excess of 20% inhibited their growth and histamine formation.

9.3 Effect of temperature

It is well known that temperature has a marked effect on the formation of BAs in food products. Several authors reported that biogenic amine content depends on temperature

and time (Diaz-Cinco et al., 1992; Halasz et al., 1994). *Carnobacterium divergens* produced more tyramine at 25°C than at 15°C (Masson et al., 1999).Also the temperature has effects on the activity of proteolytic and decarboxylating enzymes and the relationship between the microbial population (Joosten & van Boeckel, 1988 and Maijala et al., 1995b). In addition, the processing temperature also has influence on the formation of biogenic amines in dry sausages as well as on the total amount of amines (Maijala et al., 1995b). Higher temperature can favor proteolytic and decarboxylating reactions, resulting in increased amine concentration after storage. At 15°C, microbial decarboxylases might remain active, even if during storage, most microbial populations have reached the stationary growth or death phase (Bover-Cid et al., 2000). In contrast, during a prolonged meat storage at 4°C before casing, putrescine can be produced due to the action of psychrotrophic pseudomonads (Paulsen & Bauer, 1997). However, lower BA amounts were detected in food products stored at 4°C with respect to those stored at 15°C (Bover-Cid et al., 2000). A better understanding of the mechanisms by which biogenic amines are produced is necessary to prevent their formation. Generally, biogenic amines in foods can be controlled by strict use of good hygiene in both raw material and manufacturing environments with corresponding inhibition of spoiling microorganisms. In case of fermented foods, the use of short fermentation with carefully selected active starter cultures instead of wild fermentations will help to prevent the formation of toxic amines.

10. Analytical methods for the detection of biogenic amines in food

There are two reasons for the determination of amines in foods: the first is their potential toxicity; the second is the possibility of using them as food quality markers. Various methods have been developed for the analysis of BAs in foods such as thin-layer chromatography (TLC), gas chromatography (GC), capillary electrophoretic method (CE) and high performance liquid chromatography (HPLC). Lapa-Guimaraes & Pickova (2004) introduced one dimensional, double development thin-layer chromatographic technique, using the solvent system Chloroform:diethylether:triethylamine (6:4:1) followed by chloroform:triethylamine (6:1) for separation and determination of the dansyl derivatives of BAs. One-dimensional TLC technique was used for the separation of eight biogenic amines. The quantitative determination of biogenic amines has been performed by densitometry at 254 nm (Shalaby, 1996).

Few reports have been published on simultaneous detection of multiple amines. Gradient HPLC with pre- or post-column derivatization is a reproducible and accurate method for the determination of histamine, putrescine, cadaverine and tyramine in fish (Luten et al., 1992). Continuous flow analysis and isocratic HPLC with precolumn derivatization is suitable for the analysis of histamine alone. Good repeatability and reproducibility have been reported with extraction into trichloroacetic acid clean-up by cation exchange and HPLC separation using UV and fluorescence separation for determining putrescine, cadaverine, histamine and tyramine in fish and fish products Feier & Goetsch (1993). A convenient method was described for the analysis of biogenic amines by means of reversed-phase HPLC (Lehtonen et al., 1992).

Various chemical derivatization reagents have also been used for the BAs analysis, for example ninhydrine and o-phthalaldehyde as a postcolumn derivatization reagent, dansyl and benzoyl chloride, fluoresceine and 9-fluorenylmethyl chloroformate with precolumn derivatization (Wei, 1990; Seiler, 1986; Beljaars, 1998). Simplest method for determination of biogenic amines in foods is by chromatography in an amino acid analyser, including the ion-

exchange chromatographic method (Simon-Sarkadi & Holzapfel, 1994). Zhang and Sun (2004) described sensitive capillary zone electrophoresis (CZE) with lamp-induced fluorescence detection method for the simultaneous analysis of histamine and histidine. Kim et al. (2005) developed a method for the determination of biogenic amine in low salt fermented soybean paste by using benzoylchloride as a derivatization agent and amounts of amine were quantified by HPLC analysis. Previously other researchers also reported a similar method for the determination of biogenic amines in Miso and Natto products (Kung et al., 2007; Tsai et al., 2007). Saaid et al. (2009) determined biogenic amines in some Malaysian soybean products such as soybean sauce, tempe, salty soy sauce, taucu and soybean milk. These samples were extracted with 0.1 M HCl and then derivatized with dansyl chloride and finally analyzed by using HPLC. The BAs are determined in derivatized forms as trifluoroacetyl, trimethylsilyl or 2, 4-dinitrophenyl derivatives (Ascar & Treptow, 1986).

Flourometric methods are used owing to fluorescence of BAs at some pH and reaction of BAs with suitable agents to the fluorescence derivatives. Using these methods, histamine can be determined by o-phthaladehyde and tyramine by β-naphthol (Ascar & Treptow, 1986). At suitable conditions amino acid analyzer can be used not only for the determination of BAs as well their representative precursor amino acids (Halasz et al., 1999). Recently due to the commercial availability of enzymes like MAO and putrescine oxidase, several research groups tried to couple the enzymatic reactions with electrochemical sensors in order to obtain simple and reproducible biosensors. In some cases, the BAs have been coupled with oxygen sensors or hydrogen peroxide sensors. The biosensor procedure has advantages, such as low cost, short analysis time and simplicity of use and it can be used outside an organized laboratory. The biosensors show a low detection limit with life-time estimated at one month with a 10 - 30% loss of sensitivity (Casella et al., 2001).

Enzymatic methods including radioimmuno assay and enzyme linked immunosorbent assay system (ELISA) have been applied for the detection of t histamine (Guesdon et al., 1986), with the advantages of rapidity and not requiring expensive instrumentation like HPLC (Stratton et al., 1991). Lange and Wittman (2002) developed an enzyme sensor array methods for the simultaneous detection of biogenic amines (histamine, tyramine and putrescine) in different food samples within the duration of 20 min. Aygun et al. (1999) compared ELISA and HPLC method for the detection of histamine in cheese and found that the ELISA was suitable for the determination of histamine in cheese. Many other authors also reported various analytical detection methods for the determination of biogenic amines in different food samples as summarized in Table 4.

Amines	Food samples	Sample pretreatment	Derivatization	Detection / wavelength	References
Histamine	Natto	Extraction with 6% trichloroacetic acid	Derivatization with dansyl chloride	HPLC, UV-Vis detector/ 254 nm	Tsai et al., 2007
Various amines	Chinese soy sauce	Extraction with 0.4 M-perchloric acid	Derivatization with dansyl chloride	HPLC, Diode-array detector/ 254 nm	Yongmei et al., 2009
Histamine	Sufu	Extraction with 6% trichloroacetic acid	Derivatization with benzoyl chloride	HPLC, UV-Vis detector 254 nm	Kung et al., 2007

Putrescine, Cadaverine, Histamine, Spermidine and Spermine	Fish	Extraction with 5% trichloroacetic acid	Precolumn derivatization with dansylchloride	HPLC, UV detector/ 254 nm	Rosier & Peteghem, 1988
Various amines	Fermented sausages	Extraction with 0.6 M-perchloric acid	Postcolumn derivatization with o-pthaldialdehyde and 3-mercaptopropionic acid	HPLC, Fluorimetric (excitation 390 nm and emission 475 nm)	Straub et al., 1993
Various amines	Doenjang, Miso, Chungkukjang, Soy sauce, Kochujang	Extraction with 0.1 N HCl, 0.4 M perchloric acid and 5% trichloroacetic acid	Derivatization with dansyl chloride	HPLC, UV detector 254 nm	Cho et al., 2006
Various amines	Korean traditional fermented soybean paste	Extraction with 0.4 M-perchloric acid	Derivatization with dansyl chloride	HPLC UV-Vis detector/ 254 nm	Shukla et al., 2010
Various amines	Low salt fermented soybean paste	Extraction with trichloroacetic acid	Derivatization with benzoyl chloride	HPLC, Photodiode array detector/ 225 nm	Kim et al., 2005
Various amines	Leafy vegetables	Extracted with 10% trichloroacetic acid	-	Amino acid analyzer, Colorimetric detection/ 570 nm	Simon-Sarkadi & Holzapfel, 1994
Various amines	Meat products	Extraction with 0.6 M perchloric acid	Derivatization with dansylchloride	UPLC, UV detector/ 225 nm,	Dadakova et al., 2009
Various amines	Alcoholic beverages	-	-	Integrated pulsed amperometric detection (25 UV-Vis detector at 276 nm by Ion exchange chromatography	De Borba & Rohrer, 2007
Various amines	Variety of Food samples	-	-	Enzyme sensor array detection method	Lange & Wittmann, 2002
Various amines	Milk and cheese	Extraction with 0.6 M	Derivatization with o-phthaldialdehyde	HPLC, Fluorimetric (excitation 340 nm	Masson et al., 1996

		trichloroacetic acid		and emission 445 nm)	
Various amines	Alcoholic beverages in Nigeria	-	Derivatization with dansylchloride	HPLC, UV detector 254 nm	Lasekan & Lasekan, 2000
Various amines	Fish products	Extraction with 0.1 M hydrochloric acid	Derivatization with dansylchloride	HPLC, Photodiode array detector/ 254 nm	Park et al., 2010
Various amines	Turkish red wines	-	Derivatization with o-phthaldialdehyde	HPLC, diode array detector/ 200-550 nm	Anli et al., 2004
Various amines	Sucuk (Turkish dry fermented sausage)	Extraction with 0.4 M perchloric acid	Derivatization with dansylchloride	HPLC, diode array detector	Genccelep et al., 2008
Various amines	Dressed fried fish meat product	Extraction with 20% trichloroacetic acid	-	Enzyme based colorimetric method, UV-VIS Spectrophotometer at 505 nm.	Yeh et al., 2006
Histamine	Dressed fried fish meat product	Extraction with deionized water	-	Competitive direct enzyme- linked immunosorbent assay (as descrived by Neogen Corp.), detected at 650 nm.	Yeh et al., 2006
Various amines	Jeotkals, Korean salted and fermented fish products	Extraction with 0.4 M perchloric acid	Derivatization with dansylchloride	HPLC, Photodiode array detector/ 254 nm	Mah et al., 2002

Table 4. Various methods for the detection of biogenic amines in different food samples

11. Conclusions

The biogenic amines represent a group of low molecular mass organic bases occurring in all organisms. Enzymatic decarboxylation of free amino acids and other metabolic processes can lead to the presence of BAs in soybean products. These BAs can also be produced by bacterial decarboxylation of amino acids. Therefore, any fermented soybean foodstuffs produced by fermentation or exposed to microbial contamination during processing or storage may contain BAs. Therefore the concentration of BAs like histamine, tyramine, cadaverine, putrescine and spermidine gives therefore a good indication of the freshness of foods. The determination of biogenic amines in non-fermented or fresh and processed foods is of great interest not only due to their toxicity but also because they can be a useful index of spoilage or ripening. For these reasons, it is important to monitor the levels of BAs in foodstuffs. On the other hand, the same raw material can lead to very different amine levels in final products depending on the presence of decarboxylating microorganisms, either derived from environmental contamination or from starter cultures, and the conditions

supporting the growth activity of amine - producing bacteria. However the quality of raw materials seems to be only one of the many factors affecting amine formation in fermented soybean products. In this perspective, the control of hygiene and storage conditions is essential for the reduction of biogenic amine accumulation.
Analytical determination of biogenic amines (BAs) is not simple because of the complexity of the real matrix to be analyzed. The extraction of amines from real matrices is the most critical in terms of obtaining adequate recoveries for all amines. The most of the analyses include derivatization step. Therefore, estimation of BAs is important not only from the point of view of their toxicity, but also because they can be used as indicators of the degree of freshness or spoilage of food.

12. Acknowledgement

This study was supported by Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea, in 2009.

13. References

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10

Biologically Active Molecules from Soybeans

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1. Introduction

Soybeans (*Glycine max* [L.] Merr.) contain an impressive array of biologically active components. People have been eating soybeans for almost 5,000 years. Unlike most plant foods, soybeans are high in protein. Researchers are interested in both the nutritional value and the potential health benefits of soybeans. Research of the health effects of soyfoods and soybean constituents has been received significant attention to support the health improvements or health risks observed clinically or *in vitro* experiments. This research includes a wide range of areas, such as cancer, coronary heart disease (cardiovascular disease), osteoporosis, cognitive function (memory related), menopausal symptoms, renal function, and many others. This chapter provides up-to-date coverage on biologically active and related organic molecules isolated from soy and soy products. Their biological activities are briefly summarized. Molecules discussed in this chapter are as follows: isoflavones, phytic acid, soy lipids, soy phytoalexins, soyasaponins, lectins, hemagglutinin, soy toxins, and vitamins.

2. Health benefit of soy or soy products

Soy products have been considered as great source of protein for many decades. Japanese, in particular, eat a soy-based diet. Japanese monks eat soy products as their main protein source. They are known to live longer and have lower rates of chronic diseases. Because soybeans contain practically no starch, soybeans are an important part of a diabetic diet. Soybeans are 1) rich in protein (*vide supra*), calcium, and vitamins, and 2) high in mono-saturated fatty acids. In addition, soybeans contain several biologically interesting phytochemicals as minor components, which scientists are interested in understanding their biological functions.

Many studies have shown that American and European males have a ten-fold increase in the risk of prostate cancer development as compared with East Asian countries. The observed difference prevalence of prostate cancers is considered due, in part, to difference in soy consumption. Some studies indicated that isoflavones found in soybeans contribute to the risk reduction of prostate cancer. Although there is a lack of unequivocal evidences that consuming soy as an adult may reduce the risk of breast cancer, several researches have reported that consuming soy products as a teenager may help reduce breast cancer risk as an adult. Some medical research has determined that ingredients of soybeans may help reduce the risk of colon cancer and heart diseases. The dramatic increase in soy products is

due largely to the fact that the US FDA approved soy products as an official cholesterol-lowering food, along with other heart and health benefits due to the evidence that soy product intake is correlated with significant decreases in serum cholesterol, low-density lipoprotein (LDL, bad cholesterol) and triglycerides. Although a significant health benefit has been observed in people with a high soy intake (*vide supra*), some scientists have argued that reported health benefits are poorly supported by the available experimental evidences. It seems to be difficult to support soy product benefit by *in vitro* studies using individually isolated phytochemicals (a wide variety of compounds produced by plants) or crude products prepared from soy. It is important to note that all phytochemicals isolated from soybeans show an array of weak biological activities, thus, normal consumption of foods that contain these phytochemicals should not provide sufficient amounts to elicit a visible physiological response in humans in short-time clinical researches.

3. Chemical composition of soybeans

Remarkably, seeds of soy contain very high levels of protein, carbohydrate conjugates, fatty acids (soybean oil), amino acids, and inorganic materials (minerals). Among these soybean components, protein and fatty acid content account for about 40% and 20%, respectively. The remaining components consist of carbohydrate conjugates, inorganic constituents, and the minor components of biologically interesting small molecules (molecules highlighted below). Thus, soybeans constitute important nutritional components. Soybeans are considered to be a good substituents of protein (essential amino acids), amongst other major vegetables, for animal products. This chapter reviews *secondary* metabolites isolated from soy and soy products that show interesting biological activities.

3.1 Isoflavones

Isoflavones (a subgroup of flavonoids) are known to be highly potent antioxidants (Fig.1). As stated above, the consumption of soy products has many health benefits, including protection against breast cancer, prostate cancer, menopausal symptoms, heart disease and osteoporosis. Many of the health benefits of soy are derived from its isoflavones. Isoflavones are produced *via* a branch of the general phenylpropanoid pathway biosynthesis (begins from phenylalanine) that produces flavonoid compounds in legumes and stored as glucosyl- and malonyl-glucose conjugates (Graham, 1991). The major isoflavones in soybean are genistein, daidzein, and glycitein, comprising about 50, 40, and 10% of total isoflavone profiles, respectively. The chemical structure of isoflavones is similar to that of the primary female sex hormone, estrogen. Because of this similarity in structure, they can interfere with the action of estrogen. Thus, isoflavones are often called "phytoestrogens". The common biological roles of phytoestrogens are to protect plants from stress and to act as part of a plant's defence mechanism. Some scientists postulate that phytoestrogens may have evolved to protect the plants by interfering with the reproductive ability of grazing animals. The estrogen effects of isoflavones are much less effective than estrogen; its effectiveness represents around 1/1000 of estrogen. Isoflavones can also reduce the effect of the estrogen on cells and skin layers when the hormone levels are high, reducing the risk of estrogen linked cancers. There are two other classes (lignans and coumestans) of phytoestrogens that have estrogen-like actions in the human body. Isoflavones have been reported to show estrogenic, antifungal, anti-tumor and anti-mutagenic properties (Rishi, 2002; Dorge and Sheehan, 2002; Coward et al., 1993; Miyazawa, 1999). Isoflavones remain the subject of many scientific studies, as illustrated by the more than 18,000 scientific publications.

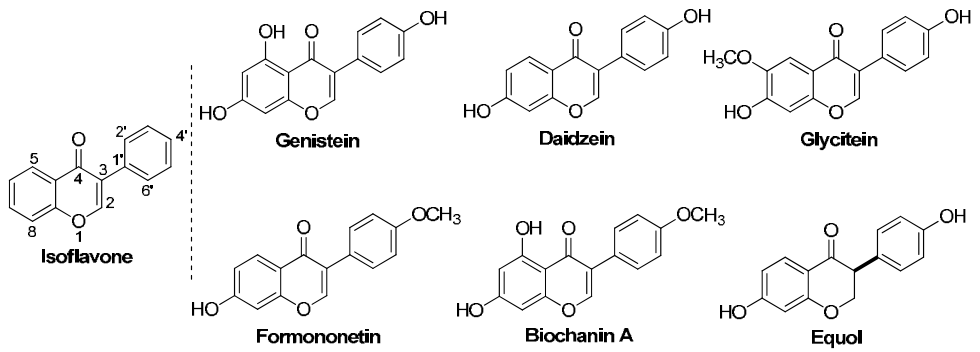


Fig. 1. Structures of soy isoflavones

3.1.1 Genistein

Genistein is found in a number of plants including soybeans, lupin, fava beans, kudzu, psoralea, and coffee. Genistein is the most discussed phytoestrogenic substance, because it is very well represented in soybeans. Genistein influences several targets in living cells. Due to its structural similarity to estrogen (i.e. 17β-estradiol, Fig. 2), genistein can bind to estrogen receptors. Genistein shows much higher affinity toward estrogen receptor β (ERβ) than toward estrogen receptor α (ERα).

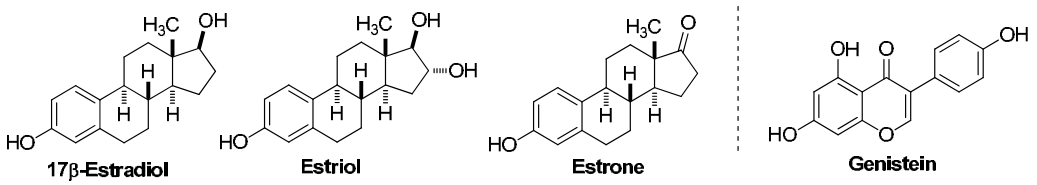


Fig. 2. Structures of estrogen

Estrogen is a key regulator of growth and differentiation in a broad range of tissues, including the reproductive (genital) system, mammary gland, central nervous and skeletal systems. Estrogen is also known to be involved in breast and endometrial cancers. To date, two key conclusions can be highlighted from the significant number of studies on the specific roles of the two receptor subtypes in diverse estrogen target tissues. ERα and ERβ have different transcriptional activities in certain cell-type, which help to explain some of the major differences in their tissue-specific biological actions. Both ERs are widely expressed in different tissue types, however, there are some distinct differences in their expression patters. The ERα is found in the inner membrane of the uterus (endometrium) and breast cancer cells. On the other hand, ERβ is found in kidney, brain, bone, heart, lung, intestinal mucosa, prostate, and endothelial cells. Unwanted effects are generally mediated through ERα. Roles of ERβ have been the subjects of interest in human cancer researches. Recent studies have shown that ERβ is lost in majority of breast tumors and thus *ESR2* gene, encoding ERβ, is suggested to be a possible tumor suppressor gene. Similarly, ERβ overexpression in ovarian cancer cells is suggested to exert antitumoral effects. ERβ is highly expressed in prostate cancer cells, and is the predominant estrogen receptor in the colonic epithelium. Thus, effects of estrogen in these cells or tissues are mediated by ERβ. Therefore, non-steroidal ERβ-antagonist has potential to be a clinically useful drug.

In the 1960's, many researches regarding the physiological effects of genistein were limited to its estrogenic activity. Genistein have also been shown to possess antifungal activities (Weidernbörner, et al. 1989), antiangiogenic effects (blocking formation of new blood vessels), and may block the uncontrolled cell growth associated with cancer, most likely by inhibiting the activity of substances in the body that regulate cell division and cell growth factors. Various studies have found moderate doses of genistein to have inhibitory effects on cancers of the prostate, cervical, brain, breast, and colon. Additionally it has been shown that genistein makes some cells more sensitive to radio-therapy. Genistein has shown a protein tyrosine kinase inhibitory activity. Tyrosine kinases are implicated in almost all cell growth and proliferation signal cascades. Genistatin's inhibition of DNA topoisomerase II also plays an important role in the cytotoxic activity of genistein.

3.1.2 Daidzein

Daidzein is also present a number of plants. Soy foods typically contain more genistein than daidzein. Structurally, daidzein lacks the 5-hroxy group of genistein (Fig. 1). Genistein and daidzein can transfer across the human placenta at environmentally relevant levels and their influence to early puberty in children is unknown. *In vitro* and *in vivo* studies have shown that daidzein stimulates the growth of estrogen-sensitive breast cancer cells. Some epidemiological evidence indicates that soy intake may be more protective when the exposure occurs prior to puberty. More research needs to be conducted on the association between breast cancer risk and daidzein specifically before conclusions can be drawn. Daidzein is metabolized in the colon by bacteria to equol and another isoflavones. Daidzein is available as a dietary supplement.

3.1.3 Glycitein

Glycitein is unique in that it is an isoflavone found in soy with a methoxy group. Methylated isoflavones have been shown to be more bioavailable and biologically stable than non-methylated isoflavones. Glycitein accounts for 5-10% of the total isoflavones in soy food products. Glycitein shows a weak estrogenic activity, comparable to that of the other soy isoflavones.

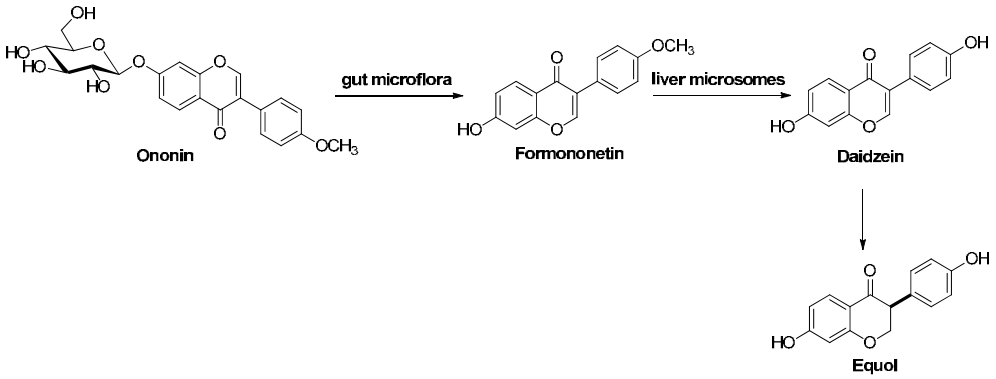
3.1.4 Formononetin

Formononetin is an *O*-methylated isoflavone. It is found in the family Fabaceae and Ranunculaceae (i.e. clovers, soybeans, and cohosh). Formononetin is known to be converted in the rumen (in sheep and cow) into a potent phytoestrogen, equol. Although, *O*-demethylase, catalysing *O*-demethylation, has been attributed to metabolism by gut microflora, incubation of formononetin with human liver microsomes resulted in 4'-*O*-demethylation to yield daidzein (Tolleson, et al. 2002) (Scheme 1).

3.1.5 Biochanin A

Biochanin A is an *O*-methylated isoflavone. Biochanin A can be found in red clover, soy, alfalfa sprouts, peanuts, chickpea and other legumes. Biochanin A-containing supplements are derived from red clover and, in addition to biochanin A, usually contain genistein, daidzein and formononetin. In red clover, biochanin A exists as its glycoside (Fig. 3). However, the glycoside undergoes hydrolysis during extraction to form the aglycone (non-sugar component). Biochanin A has weak estrogenic activities as measured in *in vivo* and *in vitro* assays. In comparison with other isoflavones, biochanin A is expected to have possible

anti-osteoporotic activity. Structurally, biochanin A would be expected to be able to scavenge reactive oxygen species and inhibit lipid peroxidation. *In vitro* and *in vivo* studies using rodents indicated that biochanin A has anticarcinogenic activity.



Scheme 1. Biotransformation of formonetin, daidzein, and equol from ononin.

3.1.6 Equol

Equol has the 3*S* configuration and is produced by bacterial flora in the intestines as a metabolite of daidzein (Scheme 1). However, only about 30-50% of people have intestinal bacteria that produce equol. Equol is a non-steroidal estrogen that acts as an anti-androgen by blocking the hormone dihydrotestosterone. Equol has the ability to bind to ERβ. This may make equol advantageous in estrogen-related cancers, including breast cancer. Equol is unique because it not only has the ability to bind to ERβ, but also acts as an antagonist to androgen actions. Unlike anti-androgen drugs, equol does not bind to androgen receptors, but it binds directly to dihydrotestosterone. This mode of action has prompted studies to determine if men who are equol-producers may have an advantage against prostate cancer.

3.1.7 Isoflavone glycosides

Isoflavones generally exist as aglycones (Fig. 1) and their glycoside forms. Isoflavone glycosides isolated from soybeans are β-glucosidated at C7-position of isoflavone core structure. Soybeans are known to contain daidzein, glycitin, 6″-acetylgenistin, 6″-acetyldaizin (Waltz, 1931; Naim, et al. 1973; Ohta, et al. 1979). Later, malonylglycosides (6"-*O*-malonyldaidzin, 6"-*O*-malonylgenistin, 6"-*O*-malonylglycitin) and succinylglycosides (6"-*O*-succinyldaidzin, 6"-*O*-succinylgenistin, 6"-*O*-succinylglycitin) are found in soybeans or soy products (Wang et al., 1994; Toda, 1999) (Fig. 3). Careful analyses of isoflavones in soybeans revealed that the malonyglycosides are the predominant isoflavones in soybeans. Mass balance of isoflavone glycosides vary depending on manufacturing process of soy products.

As describe above, biological effects of aglycones of isoflavone glycosides found in soybeans have been of great interest in food science, food technology, nutrition and dietary supplements, and disease prevention or treatment. Due to the fact that isoflavones in soybeans are conjugated almost exclusively to sugars, thus, understanding of the mechanism of intestinal absorption of isoflavones in humans is an important subject. Evidence from intestinal perfusion and *in vitro* cell culture studies indicates that isoflavone glycosides are poorly absorbed, yet isoflavones are bioavailable and appear in high

concentrations in plasma, irrespective of whether they are ingested as aglycones or glycoside conjugates. Therefore, it was suggested that hydrolysis of the sugar moiety is an essential prerequisite for bioavailability of soy isoflavones (Setchell et al. 2002).

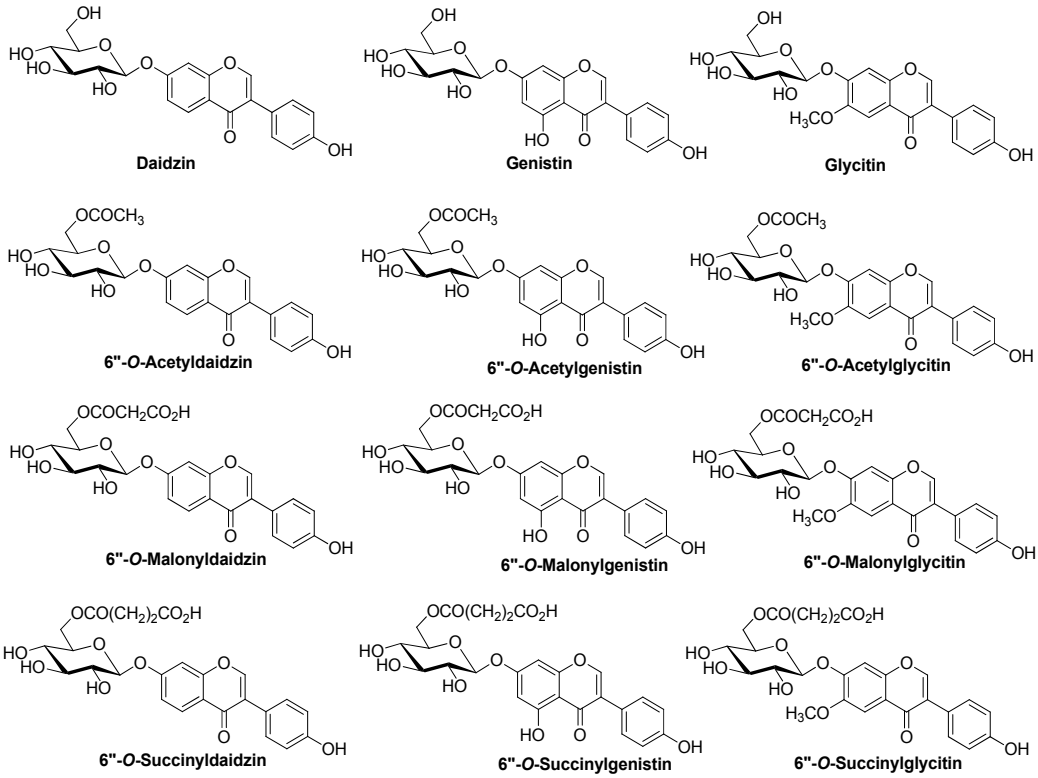


Fig. 3. Structures of soy isoflavone glycosides

3.1.8 Controversial effect of soy isoflavones

Recently, a lot of articles regarding the negative aspects of soy have been published. However, several controversy reports about the adverse effects are always not clear. This may be due to the lack of understanding of metabolism and bioavailability of isoflavones in soy products. Some studies concluded that the bioavailability and pharmacokinetics of isoflavones are significantly influenced by type of soy products. Examples of adverse effects of isoflavones are that genistein 1) increased the rate of proliferation of estrogen-dependent breast cancer *in vitro* when not co-treated with an estrogen antagonist, 2) decreased efficiency of tamoxifen and letrozole, drugs commonly used in breast cancer therapy, and 3) inhibited immune response towards cancer cells due in part to the reduction of thyroid function. In some analyses of current concerns regarding the estrogen-like effects of isoflavones in the breast cancers on the clinical trial data and recent evidence regarding estrogen therapy use in postmenopausal women, Messinia and Wood concluded that there is little clinical evidence to suggest that isoflavones will increase breast cancer risk in healthy women or worsen the prognosis of breast cancer patients. They also pointed out that the clinical trials often involved small numbers of subjects, and there is no evidence that

isoflavone intake increases breast tissue density in pre- or postmenopausal women or increases breast cell proliferation in postmenopausal women with or without a history of breast cancer. The Israeli health ministry has recommended only moderate consumption of soy products because of reported adverse effects of isoflavones (*vide supra*). On the other hand, the British Dietary Association concluded that evidence suggesting isoflavones reduce the symptoms of menopause is inconsistent. Although more clinical researches should be performed to definitively alleviate above concerns, the existing data should provide some degree of assurance that isoflavone exposure at levels consistent with a large amount of soy product intake does not result in adverse effects on breast tissue (Messina et al, 2008).

3.2 Phytic acid

Phytic acid [hexakisphosphate (IP6)) or phytate] is present in the brans and hulls of most grains, beans, nuts, and seeds. Rich sources of phytic acid are wheat bran and flaxseed. Phytic acid is inositol hexaphosphate, and thus it is highly charged, which provided chelative (or binding) properties. Phytic acid binds to minerals and metals. Phytate is not digestible to humans or nonruminant animals. The chelated forms of phytic acid with Zn, Ca, and Mg make them impermeable molecules through cell membranes. Phytic acid blocks the body's uptake of essential minerals such as magnesium, calcium, iron and especially zinc. On the other hand, phytic acid is known to be an antioxidant as well as helpful in eradication of heavy metals and other toxic cation species from the body.

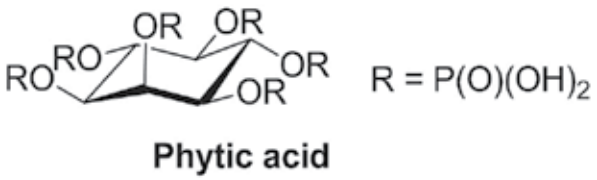


Fig. 4.

3.3 Soybean lipids

Lipids are broadly defined as hydrophobic or amphiphilic molecules. Lipids include fatty acids, sterols, lipid-soluble vitamins (vitamins A, D, E and K), glycerolipids, phospholipids, glycolipids, and sphingoglycolipid. Soybeans contain 82% of triacylglycerol, 13% of phospholipids, about 1% of sterols, and 4% of unsaturated and saturated fatty acids in a total lipid extracted with chloroform-methanol (2/1). Phospholipid composition in a soybean lipid extract is phosphatidylcholine (42%), phosphatidylethanolamine (30%), phosphatidylinositol and phosphatidylserine (20%), lysophosphatidylcholine (1%), sphingomyeline (0.6%), phosphatidic acid and others, respectively (Takagi et al. 1985).

Lipids remain an important research subject because of associations between consumption of lipids and the incidence of some chronic conditions including coronary artery disease, diabetes, cancer and obesity. Dietary lipids (or fats) serve multiple purposes. The importance of antioxidant ability of unsaturated fatty acids including β-carotene in the prevention of cardiovascular disease as well as many cancers is being increasingly recognised. Although saturated fatty acids are generally considered cholesterolemic, it is now evident that the effect of some fatty acids on blood lipids and lipoproteins suggest that the major dietary fats containing in some food products (i.e. soybeans or palm oils) do not

raise plasma total fatty acids and LDL cholesterol levels. In recent times, adverse health concerns from the consumption of trans fatty acids arising from hydrogenation of oils and fats have been the subject of much discussion and controversy.

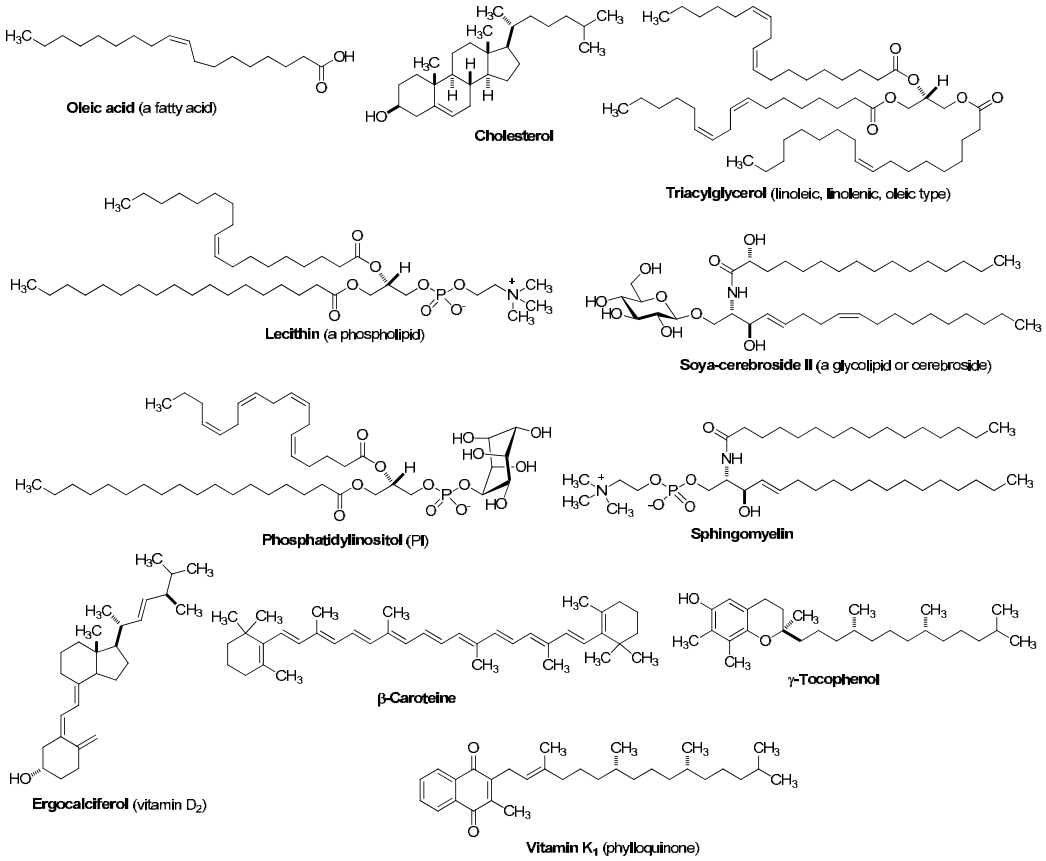


Fig. 5. Structures of representative lipids isolated from soybeans

3.3.1 Fatty acids and soybean oil

Soybean oil is rich in polyunsaturated fatty acids, including the two essential fatty acids, linoleic and linolenic, that are not produced in the human body. Linoleic and linolenic acids aid the body's absorption of vital nutrients and are required for human health.

In many applications, the higher saturate oils have been replaced with partially hydrogenated vegetable oils. Partially hydrogenated oils make the oil more stable and more resistant to air oxidation. Saturated fatty acids are more difficult to digest than unsaturated fatty acids and are seldom used for food product industry applications. Nature makes most mono- and polyunsaturated fatty acids in the *cis* form. However, during the partial hydrogenation process, the *cis* geometry of unsaturated fatty acids is partially isomerized to the *trans* form. Numerous research and epidemiological studies have been conducted to determine the impact of *trans*-fatty acids on cholesterol levels and coronary heart disease. The study by Troisi, et al. suggested a correlation between increased consumption of *trans* fatty acids and an increase in LDL (bad) cholesterol, which increases lipoprotein level and is

an independent risk factor for the development of coronary heart disease, and decrease in HDL (good) cholesterol. This could represent an increased risk of heart attacks by *trans*-fatty acid intake.

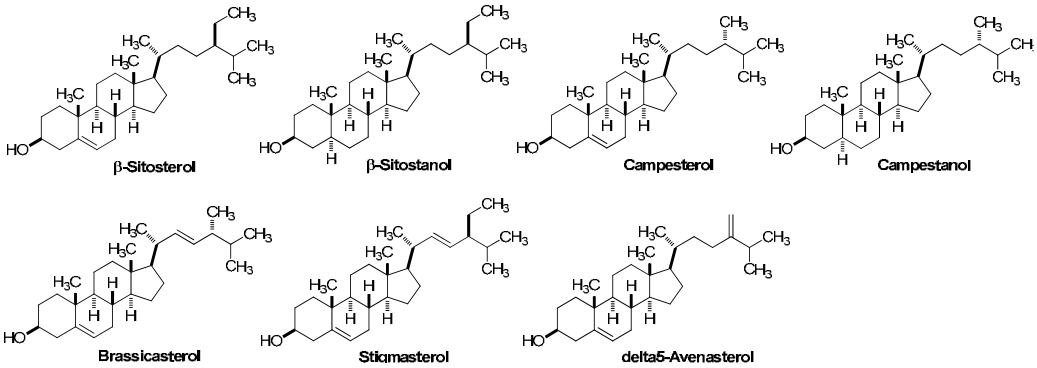


Fig. 6. Structures of plant sterols from soybeans

3.3.2 Soy sterol

Soybeans contain plant sterols, β-sitosterol, β-sitostarol, campesterol, campestanol, brassicasterol, stigmasterol, and Δ5-avenasterol, and cholesterol (Fig. 6). Plant sterols are natural dietary components, and known to have serum cholesterol-lowering properties. The lowing of serum cholesterol by plant sterols is believed to be the result of an inhibition of cholesterol absorption in small intestine. Several studies suggested that unsaturated or saturated plant sterols showed different effects on cholesterol absorption and sterol excretion (Normén et al. 2007).

3.3.3 Glycerolipids

Glycerolipids are composed of mono-, di- and tri-substituted glycerols. In these compounds, the three hydroxy groups of glycerol are esterified (triacylglycerols) or one of hydroxy group forms the ether linkage. Subclasses of glycerolipids are represented by glycosyl glycerols and glycerophospholipids. Glycerophospholipids are subdivided into distinct classes which are characterized by the presence of one or more sugar or phosphate residues (i.e. phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl serine).

Soybeans contain a wide variety of triacylglyceroles, however LC-MS analyses revealed that fatty acids incorporated in soy triacylglycerols are stearic acid, palmitic acid, oleic acid, linoleic acid, and linolenic acid (Neff et al. 1995). Fatty acids in soybeans are considered to be stored as triacylglycerides.

Lecithin can easily be extracted from soybeans (or egg yolk) and soy lecithin is an additive found in many everyday foods (Fig. 5). It has low solubility in water. Due to its amphipathic characteristic, lecithin phospholipids can form either liposomes, bilayer sheets, micelles, or lamellar structures in aqueous solution. In cooking, it is sometimes used as an emulsifier and to prevent sticking (for example, in non-stick cooking spray) and or as a stabilizer in various food applications. Lecithin has been a popular supplement because it's high choline (*N,N,N*-trimethylethanol) content. Choline is an essential nutrient that has benefit for heart health and brain development, as choline deficiency plays a role in liver disease,

atherosclerosis, and possibly neurological disorders. It is particularly important for pregnant women to get enough choline, since low choline intake may raise the rate of neural tube defects in infants, and may affect their child's memory.
Phosphatidylinositol (PI) is classified as a glycerophospholipid that contains a glycerol backbone, two non-polar fatty acid tails, a phosphate group substituted with an inositol (*myo*-D-inositol in animals) polar head group. The most common fatty acids of PIs are stearic acid in the SN_1 position and arachidonic acid in the SN_2 position. Phosphatidylinositols play important roles in lipid signaling, cell signaling and membrane trafficking. The inositol ring can be phosphorylated by a variety of kinases.

3.3.4 Cerebroside

Sphingolipids are structural components of eukaryotic cell membranes. A large number of recent reports have indicated that sphingolipid are involved in a number of important regulatory processes in cell development. Cerebrosides (monoglycosylceramide) is the common name for a group of glycosphingolipids.
Soya-cerebroside (Fig. 5) is a glucosylceramide isolated from soybeans, exhibited a Ca^{2+}-binding activity. The basic structure of soya-cerebroside II including the absolute stereochemistries of (2*R*)-hydroxy fatty acids are identical to one of the neural glucosylceramide. However, the main long-chain base (sphingosine moiety) is C_{18}-4,8-diunsaturated (E/Z). Biological functions of the cerebrosides in soybeans have not been thoroughly studied. Recently, a soya-cerebroside was reported to exhibit moderate tyrosinase inhibitory activity, and applied for making skin-care cosmetics for removal of (black) freckles.

3.3.5 Sphingomyelin

Sphingomyelin is a type of sphingolipid found in animal cell membranes, especially in the membranous myelin sheath that surrounds some nerve cell axons. It consists of phosphorylcholine and ceramide (Fig.5). In humans, sphingomyelin represents ~85% of all sphingolipids. On the other hand, only 0.6% of sphingomyelin was found in a total phospholipid isolated from soybeans (Takagi, et al. 1985). The accumulation of sphingomyelin (i.e. Niemann-Pick Disease) in brain causes irreversible neurological damage. Sphingomyelin in food products is not bioavailable, and thus the accumulation of sphingomyelin in human body is not considered possible by sphingomyelin containing food intake.

3.3.6 Vitamin K

Vitamin K is a lipid-soluble essential vitamin that is stable to air but susceptible to air under sunlight. The "K" is derived from the German word “koagulation”. Natural forms of vitamin K, vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone), exist in the human liver and other tissues at very low concentrations; vitamin K_1 concentrates in the liver while vitamin K_2 is well distributed to other tissues (Fig.6). Vitamin K_1 is derived from dietary intake and vitamin K_2 is produced by intestinal bacteria. Thus, vitamin K is not listed among the essential vitamins. Human get most of our dietary vitamin K in the form of phylloquinone (biosynthesized by plants). In prokaryotes, especially in Gram-positive bacteria, vitamin K_2 will transfer two electrons in a process of aerobic or anaerobic respiration (electron transport systems). Respiration occurs in the cell membrane of prokaryotic cells. Electron donors will ,with the help of another enzyme, transfer two electrons to vitamin K_2. Vitamin K_2, with the help of another enzyme, will in turn transfer these two electrons to an electron acceptor.

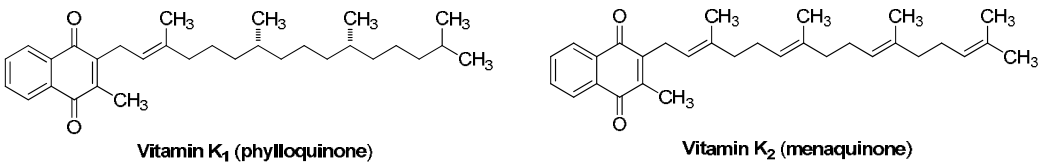


Fig. 6. Structures of phylloquinone and menaquinone

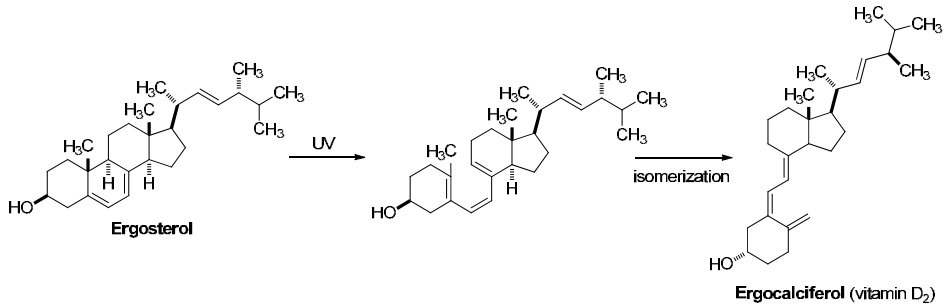
One tablespoon of soybean oil contains about 25 μg of vitamin K_1 (about 47 μg of vitamin K_1 in 100g of soybeans). Green leafy vegetables and some vegetable oils are major contributors of dietary vitamin K. Nattō, a fermented Japanese soybean product, contains large amounts (approximately 870 μg per 100 grams of nattō) of vitamin K_2. Vitamin K_2 is known to be more effective than vitamin K_1 with respect to osteroclastogenesis, hypocholesterolemic effects, and ability to slow atherosclerotic progression. To date, no adverse effects have been reported for higher levels of vitamin K intake from food and/or supplements, there are no documented toxicity symptoms for vitamin K.

3.3.7 Carotene

In the human diet, carotenoids have been shown to have antioxidant activity which may help to prevent certain kinds of cancers, arthritis and atherosclerosis. β-Carotene is a precursor of vitamin A (retinal) which is biosynthesized *via* the action of β-carotene 15,15'-monooxygenase. There are nearly 600 carotenoids in nature. In humans, four carotenoids (β-carotene, α-carotene, γ-carotene, and β-cryptoxanthin) have vitamin A activity, and they can be converted to retinal. Mature soybean seeds contain about 1 μg of carotenoids. However, concentrations of carotenoids are increased several fold during germination. Due to versatility of soybeans in use, genetic modification of soybeans is a popular subject. Genetically engineered soybean can now produce β-carotene 1,400-fold over non-trasngenic soybeans.

3.3.8 Vitamin E

Vitamin E is a lipid-soluble compound and a family of eight related compounds that includes both tocopherols and tocotrienols. α-Tocopherol is most abundant in foods and also dominates in vitamin E supplements; the other leading types are β-, γ-, and δ-tocopherol. α-Tocopherol has become synonymous with vitamin E, and vitamin E is one of the most popular supplements. Vitamin E has antioxidant activities that stop the production of reactive oxygen species formed when unsaturated fatty acids undergo oxidations. Soybeans and corns contain γ-tocopherol (about 12.0 mg of γ-tocopherol in 100g of total soy lipids) (Fig. 5), while α-tocopherol is found in olive oil. *In vitro* experiments, γ-tocopherol killed animal cells at high concentrations, but α-tocopherol did not show cytotoxicities at the same concentrations. Interestingly, although people in the United State tend to use corn and soybean oil for cooking, most abundant in the body is α-tocopherol. Other than antioxidant activities, α-tocopherol is reported to inhibit protein kinase C activity, which is involved in cell proliferation and differentiation. Vitamin E inhibits platelet aggregation and enhances vasodilation. Vitamin E enrichment of endothelial cells downregulates the expression of cell adhesion molecules, thus decreasing the adhesion of blood cell components to the endothelium.



Scheme 2. Synthesis of ergocalciferol from ergosterol (vitamin D_2)

3.3.9 Vitamin D_2 (Ergocalciferol)

There are only a few food sources (fish, liver, and egg yolk) of vitamin D. These are many fortified foods (i.e. milk, soy drinks, orange juice and margarine) that contain vitamin D. Ergosterol, called provitamin D_2, is found in ergot, yeast, and other fungi. It is converted to vitamin D_2 (ergocalciferol) upon irradiation by ultraviolet (UV) light or electronic bombardment (Scheme 2), whereas, vitamin D_3 (cholecalciferol) is normally synthesized in the human skin from 7-dehydrocholesterol. Vitamins D_2 and D_3 are about equal in activity in all mammals (some literatures described that vitamin D_3 is slightly less bioactive). Deficiency of vitamin D can result in rickets (a softening of bones) in children and osteomalacia in adults. The relationship between ergosterol content in soybeans or soybean oils and soybean fungi was studied. For an example, in the studies of soybeans inoculated with spores of *Aspergillus ruber*, ergosterol concentrations in seeds increased with time of storage (Dhingra, et al. 1998).

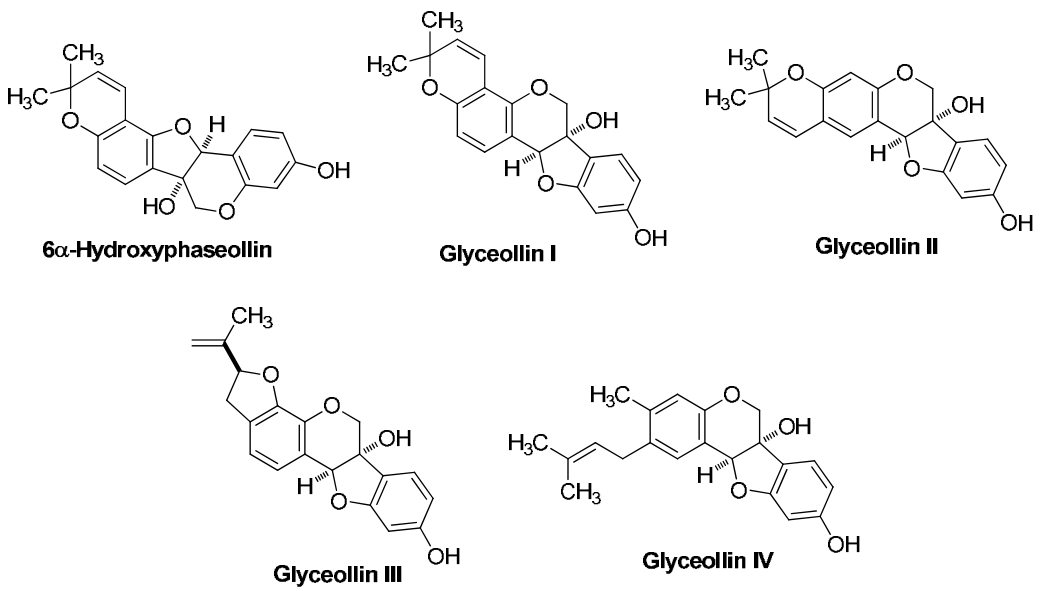
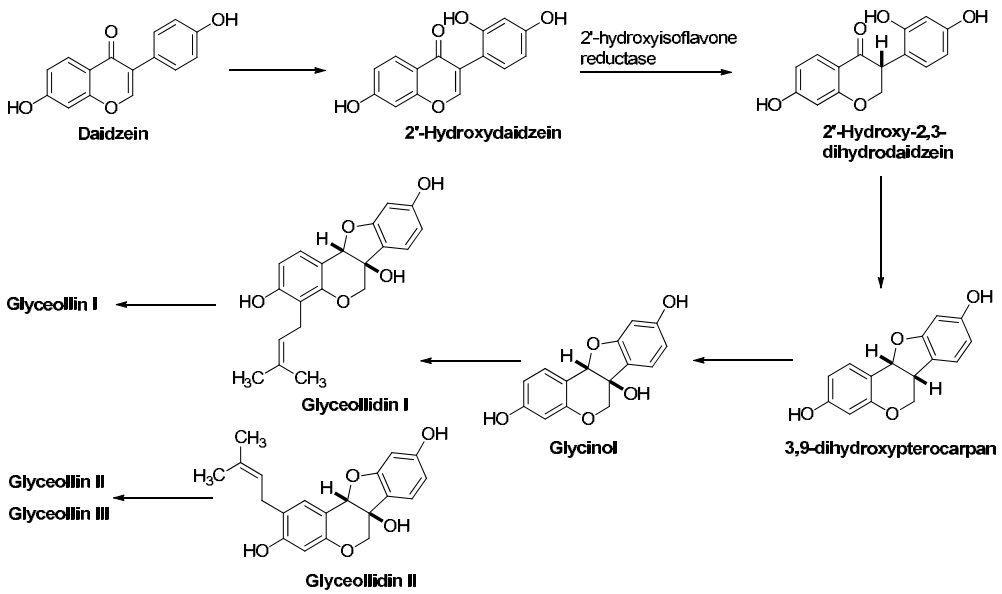


Fig. 7. Representative structures of soy phytoalexins

3.4 Soy phytoalexins

Phytoalexins are antimicrobial substances synthesized by plants that accumulate rapidly at areas of pathogen infection. They are, in general, broad spectrum inhibitors and structurally diverse molecules have been isolated from different plant species. Phytoalexins are known to inhibit bacterial or fungus cell wall biosynthesis, or delay maturation, or disrupt metabolism. To date, several soy phytoalexins have been reported. 6a-Hydroxyphaseollin was the first structurally defined phytoalexin isolated from fungal infected soybeans. Several other hydroxypterocarpan (benzopyrano-furanobenzenes) derivatives are biosynthesized as phytoalexins by soybean tissues on treatment with a variety of biotic or abiotic agents. Glyceollins are a family of pterocarpan found in the Fabaceae family including activated soy. They are biosynthesized from the isoflavone, daidzein (Fig. 1) *via* 2'-hydroxyisoflavone reductase as illustrated in Scheme 3. The concentration of phytoalexins in soybeans is very low since the compounds are only produced by soy as a defence mechanism from disease or infection. For an example, the accumulation of glyceollins in soybean cotyledon tissue was observed using four species of *Aspergillus*; 955 µg/g of glyceollins could be isolated from soybean cotyledon tissue inoculated with *A. sojae*. Representative phytoalexins identified from fungal infected soybeans are summarized in Fig. 7. Besides their antifungal or antibacterial activities, glyceollins have recently been demonstrated to be novel antiestrogens that bind to the estrogen receptor (ER) and inhibit estrogen-induced tumor progression (Zimmermann et al. 2010). Therefore, glyceollins may represent an important component of a phytoalexin-enriched food diet in terms of chemoprevention as well as a novel therapeutic agent for hormone-dependent tumors.

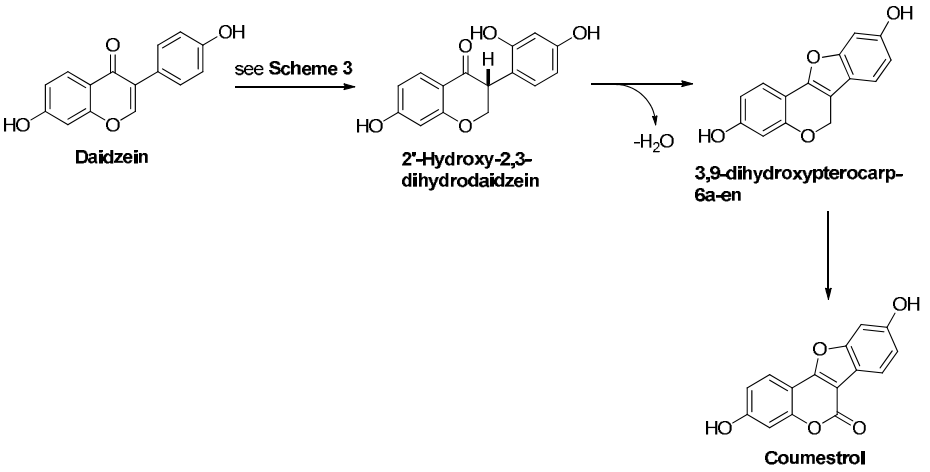


Scheme 3. Biosynthesis of glyceollins

3.5 Coumestrol

Coumestrol is classically categorized as phytoestrogens because this molecule binds to the estrogen receptor (ER). Coumesterol is originally isolated from alfalfa (Bikoff et al. 1957), but later soybeans and clover contain the highest concentrations of this molecule. Biosynthesis

of coumestrol in soy is proposed based on the feeding experiments of labeled precursors to a coumestrol producing bacteria (Berlin et al. 1972). As illustrated in Scheme 4, the isoflavone, daidzein is reduced to form 2′-hydroxy-2,3-dihydrodaidzein which undergoes intramolecular condensation to yield 3,9-dihydroxypterocarp-6a-en. Biological oxidation of the dihydroxypterocarpen furnishes coumestrol.



Scheme 4. Biosynthesis of coumestrol

Coumestrol has less estrogen activities than estrogen and therefore may reduce the risk of developing breast or prostate cancer in humans by preventing estradiol binding to estrogen receptor (ER). Coumestrol was reported to inhibit the enzymes involved in the biosynthesis of steroid hormone (aromatase and hydroxysteroid dehydrogenase), and inhibition of these enzymes results in the modulation of hormone production.

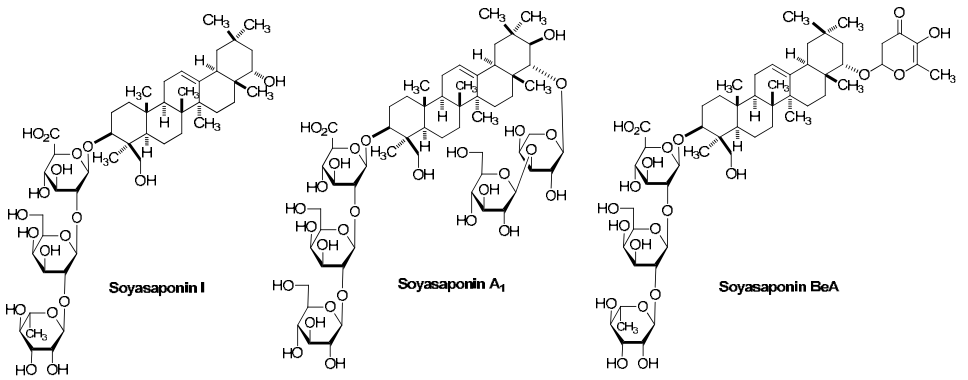


Fig. 8. Representative structures of soyasaponins

3.6 Soyasaponins

Saponins are amphipathic glycosides grouped phenomenologically by the formation of soap-like froth when shaken in aqueous solutions. Structurally, saponins contain one or more glycoside moieties combined with a lipophilic triterpene derivative. Many health

benefits of soybeans are believed to be attributed to their Saponins. Many soy products contain high levels of saponins. Raw soybeans contain between 2 and 5 g saponins per 100 g. Soy saponins are divided in two groups; group A saponins have and undesirable astringent taste, and group B saponins have the health promoting properties.
The blood cholesterol-lowering properties of dietary saponins are of particular interest in human nutrition. Saponins bind cholesterol and bile acids in the gut. Cancer cells have more cholesterol-type compounds in their membranes than normal cells. Soy saponins can bind cholesterol *in vitro*, and thus interfere with cell growth and division. Soy saponins also showed antifungal activities probably due to interference with cholesterol in fungus. Saponins cannot permeate the intestinal wall, but showed effectiveness in binding to cholesterol and making it unavailable for re-absorption within the small and large intestine. Apart from their important role in binding ability to cholesterol, soy saponins showed adjuvant effects for vaccines. An *in vitro* study demonstrated that soy saponins exhibited potent antiviral effects on the HIV virus. To date, over 26 soyasaponins have been isolated from soybeans and their gross structures were determined *via* high-magnetic field NMR or X-ray crystallography. Representative soyasaponins are illustrated in Fig. 8.

3.7 Lectins and hemagglutinins

Lectins are plant derived proteins which are capable of binding to carbohydrate moieties of complex glycoconjugates but do not possess immunoglobulin nature. They typically agglutinate certain animal cells and/or precipitate glycoconjugates. Many members of the lectinic protein family agglutinate red blood cells. This particular nature of lectinic proteins is classified into hemagglutinin. Lectins are stable proteins that do not degrade easily. For examples, some lectins are resistant to stomach acid and digestive enzymes. Unfermented soy products contain high levels of lectins/hemagglutinins. Hemagglutinin renders red blood cells unable to absorb oxygen. However, the soybean fermentation process deactivates soya hemagglutinins, and thus the amounts of lectins present in soybeans have not been considered to be as potentially toxic components. On the other hand, some dried bean products may still contain a large amount of active lectins. These lectins are believed to trigger allergic reactions or toxic reactions in a person's body. Person's lectin sensitivity is largely due to 1) genetics, 2) a failure of mucosal immunity (secretory IgA), and 3) bacterial or viral infections that damage human cells, making human body susceeptable to lectin antibody/antigen reactions.

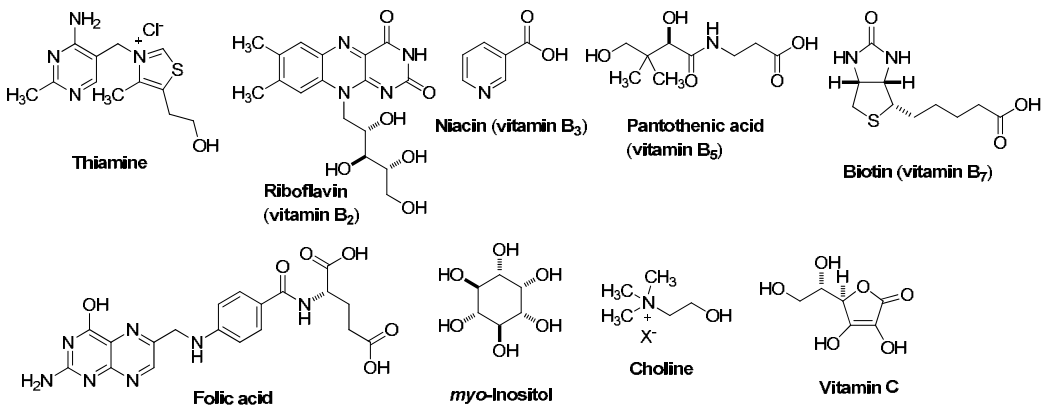


Fig. 9. Water soluble vitamins isolated from soy

3.8 Soy toxins

Soy contains several naturally occurring compounds that are toxic to humans and animals. The best known of the soy toxins is the trypsin (a serine protease found in the digestive system) inhibitors. *In vivo* studies using rat, high levels of exposure to trypsin inhibitors isolated from raw soy flour cause pancreatic cancer whereas moderate levels cause the rat pancreas to be more susceptible to cancer-causing agents. However, the US FDA concluded that low levels of soy protease inhibitors pose no threat to human health. Recently, a metalloprotein possessing toxicity to mice (LD_{50} 7-8 mg/kg mouse upon intraperitoneal injection) was identified. This protein has a size of 21 kDa and was named soyatoxin. Some other biological properties of soyatoxin include hemagglutination and trypsin inhibitory activity.

3.9 Water-soluble vitamins

Soybeans are not considered to be very rich sources of any particular vitamin, but they contain a wide variety of vitamins and do contribute to an overall nutritional well-being. Lipid-soluble vitamins (vitamin K, E, D and carotene) in soybeans are discussed above. The water-soluble vitamins in soybeans are thiamine, riboflavin, niacin, pantothenic acid, biotin, folic acid, inositol, choline, and vitamin C. Vitamin B_6 was also reported to contain in soybeans. Thus, soy includes essential vitamins except for vitamin B_{12} and E. However, soy contains a vitamin E precursor, carotene. Numerous studies have been conducted over the past decades on the relative distribution and concentrations of these vitamins in different portion of soy. The cotyledons contain notably greater amount of all water soluble soy vitamins than those in the hypocotyl. The quantity of all vitamins except thiamine increases through germination (Wai et al., 1947).

4. Conclusion

This chapter summarizes structures of biological active molecules isolated from soy, and their biological activities are briefly reviewed. Since the discovery of soybeans as rich source of protein and oil in 1904, a numerous number of experimental data have been accumulated on chemistry and biochemistry of phytochemicals isolated from soy and soy products. To date, a wide variety of organic compounds have been characterized from soy. The interest of structure elucidation studies of bioactive molecules in soy was to obtain insight into correlation between the reported health benefits, which are associated with soy intake, and soy phytochemicals. Thus, many structural studies on soy have not aimed to discover novel molecules, albeit these efforts resulted in discovery of complex soyasaponins (Fig. 8.). The consumption of soy products has many health benefits, including protection against breast cancer, prostate cancer, menopausal symptoms, heart disease and osteoporosis. These health benefits of soy are believed to be due in part to phytoestrogenic activity of isoflavones, which are stored as glucosyl- and malonyl-glucose conjugates in soybeans. Isoflavon-glucosyl conjugates show very poor oral bioavailability. Recently, the negative aspects of soy have been reported (3.1.8). However, the controversy reports about the adverse effects of soy are not clear. This may be due to the lack of understanding of metabolism and bioavailability of isoflavone-glucosyl conjugates in soy. Some researchers concluded that there is little clinical evidence to suggest that isoflavones will cause the adverse effects (e.g. breast cancer risk in healthy women). It is important to note that all phytochemicals isolated from soy show an array of weak biological activities, and thus, normal consumption of

foods that contain these phytochemicals should not provide sufficient amounts to elicit a visible physiological response in humans in short-time clinical researches.
Unfermented soy products contain high levels of lectins/hemagglutinins, and very low level of soy toxins (protease inhibitors). Most of these proteins will be deactivated through food processing. Remarkably, seeds of soy contain very high levels of protein, carbohydrate conjugates, oil, and minerals. On the other hand, remaining components discussed in this chapter can be isolated minute quantities from soybeans, however, a wide range of health benefit of soy phytochemicals (lipids, phytoestrogens, soy sterols, vitamins, soyasaponins) contributes to the overall nutritional well-being of humans.

5. Acknowledgment

I would like to thank Emeritus Professor Isao Kitagawa for valuable discussions for this document.

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11

From Soybean Phytosterols to Steroid Hormones

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1. Introduction

Phytosterols, which are structurally and physiologically similar to cholesterol, are a large group of steroidal triterpenes. They are essential to maintaining normal function in plant cell membranes. In recent years, some of their beneficial effects on human health have come to light. Phytosterols can lower intestinal cholesterol absorption, thereby reducing serum levels of low-density lipoproteins and the risk of atherosclerosis. The pharmaceutical industry has a long history of converting phytosterols to therapeutic steroid hormones by microbial transformation. One commercially available phytosterol product is a phytosterol mixture extracted from soybean oil deodorizer distillate. Soybean phytosterols usually include four sterols: β-sitosterol, stigmasterol, campesterol, and brassicasterol, all of which make good raw materials for the production of steroid hormones because of their typical A-ring molecular structure with a 3β-hydroxyl group and a 5,6-double bond (Fig. 1). Two kinds of steroid hormone intermediates can be produced from soybean phytosterols through microbial transformation. The first of these are the C19-steroids, which include androsta-4-ene-3,17-dione (AD), androsta-1,4-dien-3,17-dione (ADD), 9α-hydroxy-androstra-4-ene-3,17-dione, and testosterone; the second are the C22-steroids, such as 20-carboxy-pregna-l,4-dien-3-one and 20-hydroxymethylpregna-1,4-dien-3-one (Fig. 2). C19-steroids are the products of complete side chain cleavage. They can be used as precursors to almost all kinds of steroid hormones, including sex hormones, anabolic steroids, and even adrenocortical hormones. C22-steroids are the products of truncated side chain. These make good precursors to adrenocortical hormones. The chemical conversion of sapogenins to steroids is a well-established alternative to microbial transformation of phytosterols to steroids. This method has many shortcomings, however, such as higher costs, more steps, low yield, the waste of land resources, and the destruction of wild plant resources. In light of this, microbial conversion of soybean phytosterols to steroids shows great value in the synthesis of steroid hormones.

Here, we summarize our knowledge of the occurrence of phytosterols in soybeans and the technology that can be used to transform them from phytosterols to steroids through the regulation and modification of microbial catabolism. Based on analysis of the metabolic mechanisms of phytosterols and the bottlenecks inherent in the microbial transformation process, we will also discuss areas for development and improvement.

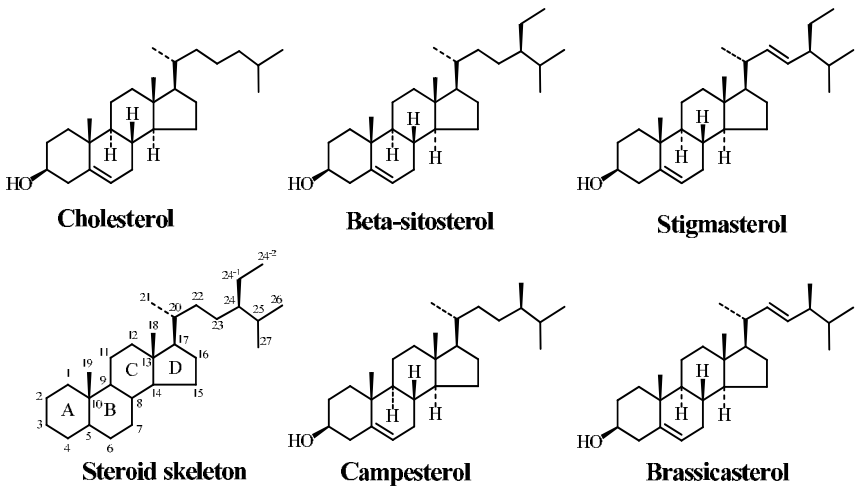

Fig. 1. Cholesterol and soybean phytosterols based on steroid skeleton

2. Soybean phytosterols

2.1 Soybean phytosterols and human health

Phytosterols are a group of steroid alcohols that occur naturally in plants. They are generally isolated during the process of producing vegetable oils, especially soybean oil. Soybean oil deodorizer distillate (SODD) is one main wastes of the soybean-processing process. It is rich in phytosterols and has become the main source of commercial phytosterols (Ramamurthi & McCurdy, 1993; Hirota, *et al.*, 2003, Benites, *et al.*, 2009). β-sitosterol, stigmasterol, campesterol, and brassicasterol are the four major types of soybean phytosterols soybean. They differ in the double bond at C22 and the substituents at C24 (Fig. 1). Just like cholesterol in animals, phytosterols regulate of the fluidity of plant cell membranes and feature in cellular differentiation and proliferation (Benveniste, 1986, Piironen, *et al.*, 2000).

Soybeans are a well-known health food, rich in quality protein, fatty acids, and other healthy components, including phytosterols. Because most people enjoy taking in high-fat, high-energy foods that raise the serum cholesterol level out of healthy range, dyslipidemia, together with its accompanied syndromes, hyperlipidemia, adiposis, and cardiovascular disease, are now threatening the health of citizens of the developed and developing world (Kes Niemi & Miettinen, 1987; Genest, *et al.*, 2003). In recent years, many studies have indicated that phytosterols may be beneficial as food additives because they can lower the absorption of cholesterol in intestines by 10% to 15% (Ostlund, *et al.*, 2003; St-Onge, *et al.*, 2003). The FDA has stated that daily intake of moderate amounts of phytosterols can reduce the risk of heart disease. The long-term intake of phytosterol-rich foods could efficiently diminish plasma cholesterol and atherosclerotic risk. For patients who cannot tolerate cholesterol-lowering drugs, it may be advisable to adopt a phytosterol-rich diet as a substitute (Ostlund, *et al.*, 2003).

2.2 Phytosterol biosynthesis

Like cholesterol, phytosterols are biosynthetically derived from squalene. However, the synthesis of phytosterols involves a relatively complicated mechanism. In plants, the

anabolism of phytosterols can be divided into two parts with squalene being the critical point (Fig. 3) (Piironen, *et al.*, 2000).

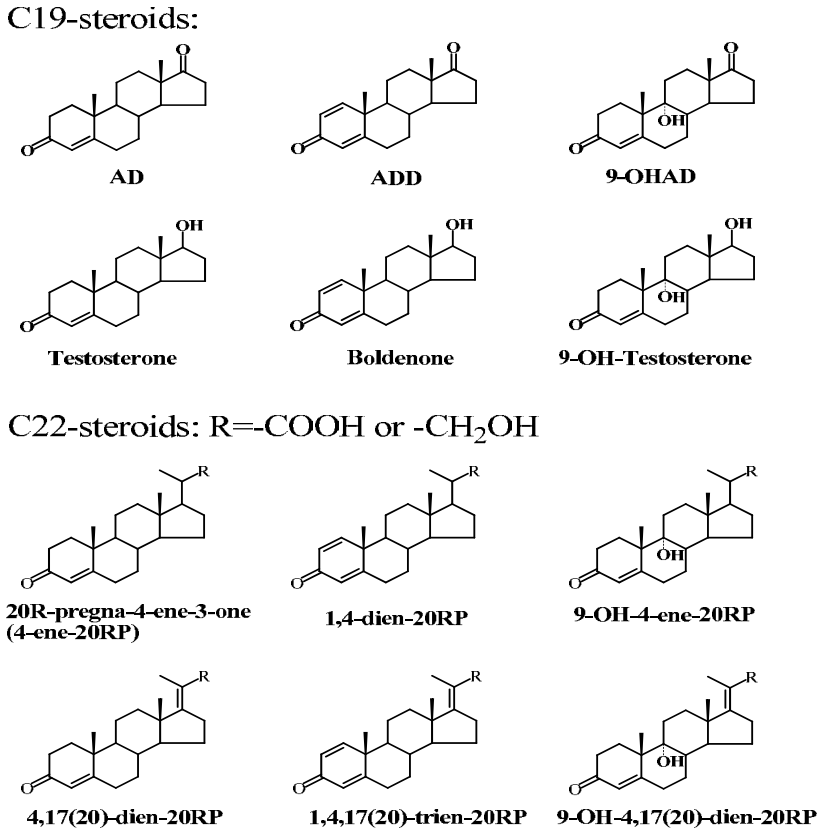

Fig. 2. Steroids derived from soybean phytosterols by microbial transformation

The pre-squalene pathway is a nucleus-accumulating process derived from isoprenoid, using isopentenyl pyrophosphate (IPP) as the fundamental building block. It is carried out by the mevalonic acid pathway (Guo, *et al.*, 1995). It has been determined that the series of enzymes involved in the pre-squalene pathway from acetyl coenzyme A to squalene, via the formation of 3-hydroxymethyl-3-glutaryl coenzyme A (HMG) are mevolonate, isopentenyl pyrophosphate (IPP), and farnesyl pyrophosphate (FPP). Within this process, 3-hydroxymethyl-3-glutaryl coenzyme A reductase (HMGR) is one predominant but controversial factor believed to determine the capability of phytosterol synthesis (Bach, 1986; Goldstein & Brown, 1990; Stermer, *et al.*, 1994). Squalene synthesis was reported to be involved in competition with sesquiterpene, which is regulated by sesquiterpene cyclase (SC) (Vogeli & Chappell, 1988). The activation of sesterpene synthesis would correspondingly result in the suppression of sterol accumulation. Consequently, the inhibition of SC should be regarded as an important control mechanism for enhancing sterol formation.

The post-squalene pathway mainly refers to the modification of branch chain on steroidal compounds. Conversion of cycloartenol to β-sitosterol and stigmasterol involves two

methylation steps, whereas conversion to campesterol and brassicasterol involves one. There are two different families of sterol methyl transferases (SMT 1 and SMT 2) known to be responsible for biosynthesis of the 24-methyl and 24-ethyl sterols, respectively (Nes & Venkatramesh, 1999; Bouvier-Navé, *et al.*, 1998). SMT has been found to be associated with the Δ5-sterol production. Most importantly, it has the highest degree of sterol specificity f any compound involved in the pathway. In other words, the ultimate concentration ratio of phytosterol components, campesterol/β-sitosterol, primarily depends on SMT activity.

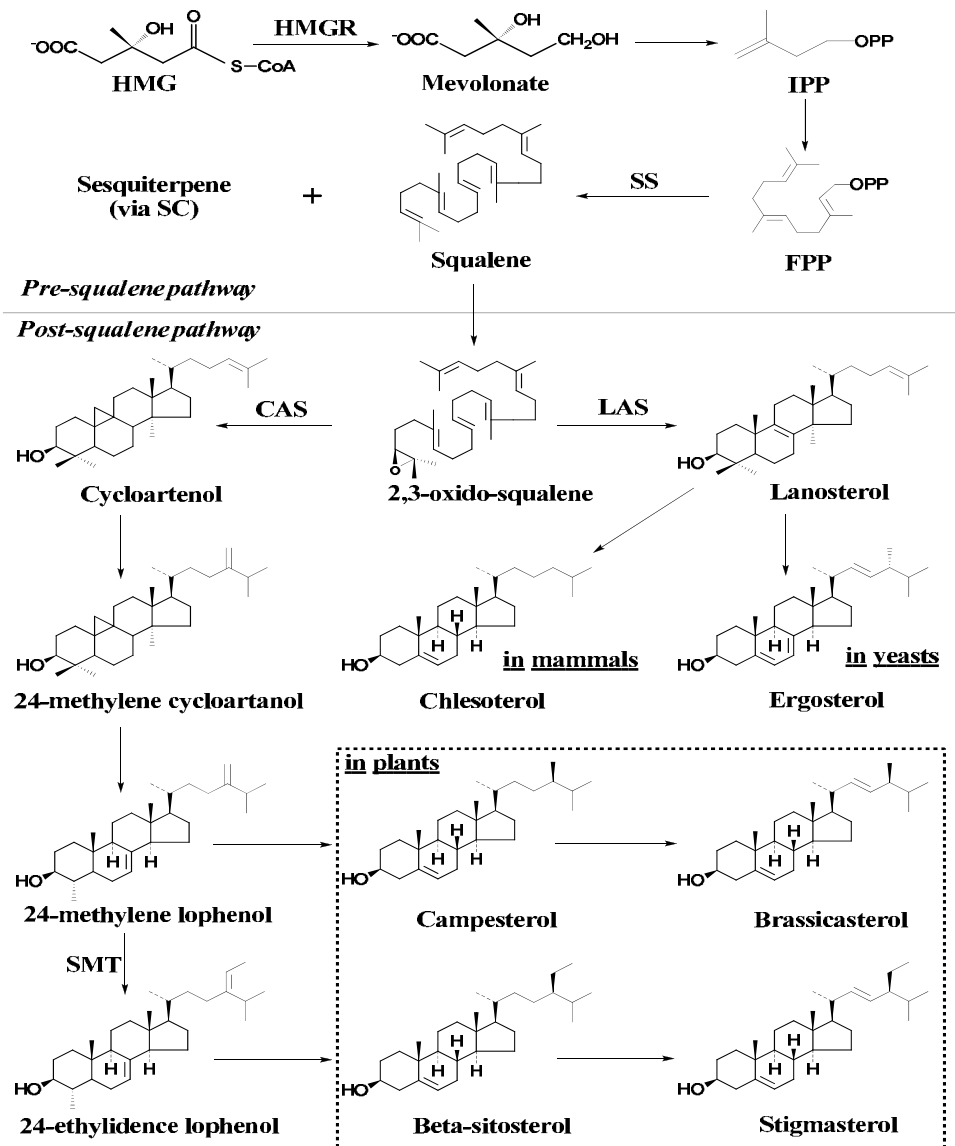

Fig. 3. Sterol synthesis in plants, yeasts and mammals.

3. Conversion of soybean phytosterols to steroid hormones

3.1 Steroid hormones

Steroids are a class of endocrine hormones secreted by the sexual organs and the adrenal cortex in animals. Sterols are closely related to the regulation of physiological effects and the development of reproductive structures, bone, and brain. Many synthetic steroid hormones have been developed and used as pharmaceuticals to cure diseases resulting from the secretion defects of endocrine steroid hormones. Steroid hormones can be grouped into three categories according to their physiological functions: corticosteroids, sex steroids, and anabolic steroids. Corticosteroids can be further divided into two kinds: glucocorticoids and mineralocorticoids. Glucocorticoids play a critical role in regulating many aspects of metabolism and immune function. They also exhibit favorable anti-inflammatory potencies. Mineralocorticoids help maintain blood volume and control renal excretion of electrolytes (Craigie, *et al.*, 2009). Common corticosteroids include C21-steroids, such as hydrocortisone and prednisone. Sex steroids are a type of sex hormone. They include androgens, estrogens, and progestagens, which affect the sex differences and support reproduction (Callewaert, *et al.*, 2010). Anabolic steroids are similar to androgens, interacting with androgen receptors to increase muscle and bone synthesis (Kicman, 2008). Both sex and anabolic steroids have C19 structures. Aside from their endocrine roles, some steroids also perform antiviral, anticancer, cholesterol management, cardiovascular, and neuroprotective functions (Wang, *et al.*, 2007; Garcia-Segura & Balthazart, 2009).

Usually, steroid hormones are synthesized by semi-synthesis using natural steroids as starting materials. Sapogenins and phytosterols are two basic kinds of starting materials commonly used for the production of steroids. These form two main technical routes in the pharmaceutical industry (Fernandes, *et al.*, 2003; Malaviya & Gomes, 2008). One is the chemical transformation of sapogenins such as diosgenin, hecogenin and solasodine to C21 steroids such as 16-dexosy-prognenolone. The other is the microbial transformation of phytosterols to C19 steroids or C22 steroids (Fig. 2). Compared to the chemical transformation of sapogenins, the microbial transformation of phytosterols has the advantage of a simple and environmental friendly process, a stable supply, and low costs.

3.2 Microbial transformation of phytosterols to valuable steroids

Early in the 1980s, the putative metabolic pathway for synthesizing sterols in microorganisms had already been proposed according to the identification of metabolic intermediates (Fig. 4). So far, the cleavage process of sterol nucleus has been extensively studied, and many of the steps in its multi-step reactions have been identified. However side chain cleavage has not been well studied until now. In 2007, van der Geize disclosed the gene clusters involved in sterol catabolism from *Rhodococcus* sp. RHA1 and *Mycobacterium* species, signifying a new starting point for the biotransformation of phytosterols (van der Geize, *et al.*, 2007).

As shown in Figure 4, the biotransformation of phytosterols is initiated by the oxidation of 3-hydroxyl-5-ene moiety of sterols to 4-ene-3-one moiety. This is carried out by cholesterol oxidases (Cho) (Aparicio & Martin, 2008; Doukyu, 2009). Then the transformation process bifurcates into two independent routes: steroid nucleus decomposition and side chain cleavage (Szentirmai, 1990).

3-ketosteroid-9α-hydroxylase (Ksh) and 3-ketosteroid-1-dehydrogenase (KstD) are the enzymes responsible for the decomposition of the steroid nucleus, which acts independently

and successively on rings A and B to form 1,4-dien-9α-hydroxy-steroids, structurally unstable chemicals that spontaneously evoke the opening of ring B (van der Geize, *et al.*, 2001, 2002a). Maintaining an intact steroid nucleus is key to producing valuable steroids from the catabolism of phytosterols. Kshs and KstDs have attracted much attention for their special roles in initiating cleavage of the steroid nucleus. One enzyme must be inactivated to block the decomposition of the steroid nucleus for the development of steroid-producing strains (van der Geize, *et al.*, 2000, 2002a; Petrusma, *et al.*, 2009). As shown in Figures 2 and 4, the inactivation of Kshs, KstDs, or both can result in the production of C19 steroids such as AD, ADD, and 9α-OHAD. In theory, the production of AD can be achieved via the inactivation of both Kshs and KstDs in a microbial cell, and the accumulation of ADD or 9α-OHAD can be achieved by disrupting the Ksh or KstD genes. Traditional mutagenesis-screening techniques have long been employed as a practical means of producing C19-steroid-producing strains (Donova, *et al.*, 2005c; Huang, *et al.*, 2006; Gulla, *et al.*, 2010). Nevertheless, C19 steroids such as AD, ADD, and testosterone are known to coexist in products (Donova, *et al.*, 2005c; Huang, *et al.*, 2006). This is one of the more significant deficiencies between the strains selected from random mutations; the subtle differences in structure between C19 steroids make them difficult to separate in industrial applications. This seriously increases production costs and limits the application of these mutant strains. In addition, mutant strains have other deficiencies, including residual decomposition of the steroid nucleus and reduced transformation efficiency, such as we observed in our study. These deficiencies can be attributed to the complex metabolic processes of the sterols and multi-isoenzymes of KstD and Ksh within microorganisms. These make it difficult to target and block all of the KstD and Ksh genes without interfering with other important genes. Genetic engineering can be considered a viable technique toward facilitating the development of ideal steroid-producing strains. However, genetic engineering was limited by the genetically undefined mechanisms of sterol catabolism until 2000 and 2002, when van der Geize *et al*. first revealed the KstD and Ksh genes responsible for the catabolism of sterols in *Rhodococcus erythropolis* SQ1 (van der Geize, *et al*., 2000, 2002a). This team went on to describe the isoezymes of KstD and Ksh (van der Geize, *et al.*, 2002b, 2008). Notably, in 2007, they revealed the existence of a gene cluster encoding sterol catabolism in *Rhodococcus* and *Mycobacterium* species (van der Geize, *et al*., 2007). The research of van der Geize and co-workers facilitated the development of ideal sterol-transformation strains for the synthesis of valuable C19-steroids. Subsequently, targeted disruption or augmentation of KstDs and Kshs was successfully used in the development of C19 steroid-producing strains (van der Geize, *et al*., 2000; Wei, *et al.*, 2010).

Cleavage of the sterol C17-side chain has been well depicted as similar to the fatty acid β-oxidation and the genes likely to be responsible for this process have been shown to be part of the gene cluster responsible for sterol catabolism (Szentirmai, 1990; Van der Geize, *et al.*, 2007). However, the genetic definitions of these processes have yet to be outlined. Using mutagenesis-screening techniques, the C17-side chain cleavage can be blocked to produce products with truncated side chains, such as C22-steroids, which are valuable candidate intermediates for the synthesis of steroid hormones (Fig. 2) (Szentirmai, 1990). C22-steroid-producing strains are rarer than C19 strains, partly because of the complex enzyme systems likely to be involved in side chain cleavage.

In the terms of practical applications, both C19-steroids and C22-steroids can be readily used to produce higher value-added steroid hormones. The C19-steroids can be grouped by pharmacological activity into anabolic and androgen steroids. These steroids are already

used as the main precursors for all kinds of sex and anabolic hormones, largely because their structures are already similar to those of the desired final products (Malaviya & Gomes, 2008). The C19-steroids can also be used as precursors for the synthesis of C21 corticosteroids (Andrews, *et al.*, 1996). C19-steroids are highly important precursors of steroid hormones and can be used to synthesize almost all of the steroid hormones used in clinical settings. C22-steroids show their advantage in the synthesis of C21 corticosteroids, which can be achieved by simple oxidative decarboxylation (Toröa & Ambrus, 1990).

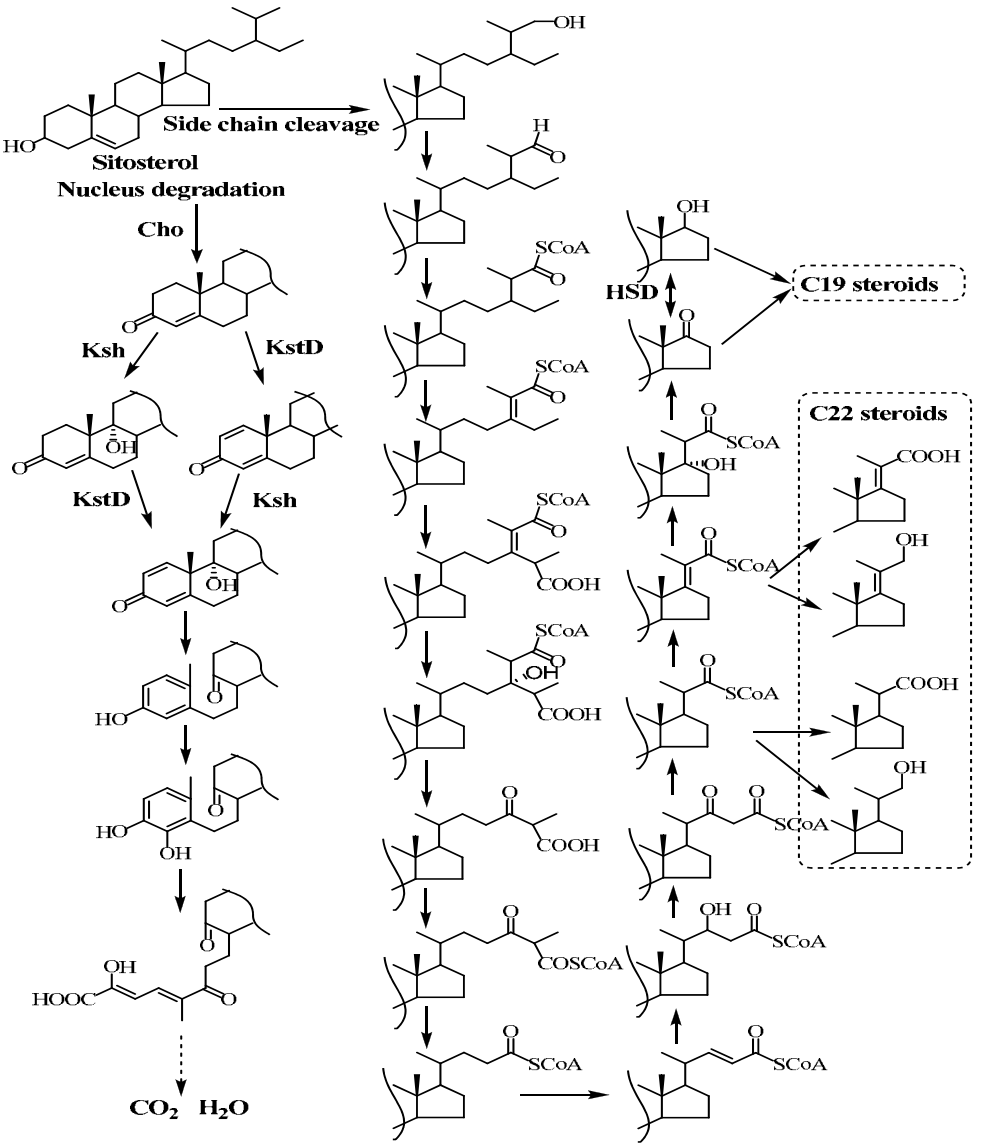

Fig. 4. Catabolic pathway of phytosterols (with β-sitosterol as substrate). (HSD represents the enzyme 17β-hydroxy steroid dehydrogenase.) (Kieslich, 1985; Szentirmai, 1990; van der Geize, *et al.*, 2007)

4. Inherent problems and solutions in strain improvement

The microbial metabolism of sterols has already been shown to be a promising means of preparing valuable steroids. During the second half of the twentieth century, many microbial strains were developed and tailored to synthesize many kinds of C19 and C22 steroids using phytosterols as substrates (Szentirmai, 1990). So far, however, the production of steroids from phytosterols by microbial transformation is still not widely used in the pharmaceutical industry. This may be because of two inherent problems in the phytosterol biotransformation process: (1) The low water solubility of phytosterols can lead to poor bioavailability. (2) The final products are toxic to microbial cells. Many technological strategies have been proposed to overcome these problems. These have been reviewed in detail by Malaviya and Fernandes (Fernandes, *et al.*, 2003; Malaviya & Gomes, 2008). Here, we mainly focus on how to develop robust organisms that can withstand their own products.

4.1 Low water solubility and adaptive mechanisms

Most steroids have solubility below 0.1 mM. Phytosterols are more hydrophobic, their water solubility usually being no more than 1 μM. The poor solubility of phytosterols seriously retards their bioavailability because of inadequate mass transfer (Goetschel & Bar, 1992; Phase & Patil, 1994; Malaviya & Gomes, 2008). Many efforts have been carried out to improve the dispersity and dissolubility of phytosterols in reaction media. Among these, surfactant-facilitated emulsification, favored for its practicality and effectiveness, is a common means of enhancing the bioavailability of phytosterols (Fernandes, *et al.*, 2003; Malaviya & Gomes, 2008). There are other strategies, but many of these seem inadequate in practice. For example, organic solvent facilitated dissolution significantly raises the complexity of the process and production costs and also results in environmental pressures. It is also not very productive.

The mechanism of sterol uptake by microbial cells has already been demonstrated: during the transformation process, microbial cells adhere to the surfaces of sterol particles, forming stable agglomerates. Sterol uptake takes place via the direct contact between the cells and the sterol particles (Atrat, *et al.*, 1991; Goetschel & Bar, 1992; Fernandes, *et al.*, 2003). This is an adaptive mechanism that allows microorganisms to use minimally water-soluble hydrocarbons as carbon sources. Like phytosterols, many polycyclic aromatic hydrocarbons (PAH), such as naphthalene and anthracene, also exhibit extremely low water solubility and are also poorly bioavailable (Johnsen & Karlson, 2004; Harms, *et al.*, 2010a, 2010b). Many microorganisms have been shown to use these hydrophobic hydrocarbons as carbon sources through similar evolutionary adaptations (Miyata, *et al.*, 2004; Heipieper, *et al.*, 2010; Parales & Ditty, 2010; Harms, *et al.*, 2010a). Generally, the microorganisms adapted to use hydrophobic hydrocarbons have the following physiological properties: (1) lipophilic cell walls and adaptive changes in the surface properties allowing direct adhesion to the hydrophobic substrates, (2) high affinity uptake systems such as active transporters and membrane-associated enzymes for initial degradation, (3) the ability to excrete biosurfactants or bioemulsifiers in order to increase the bioavailability of hydrophobic hydrocarbons (Wick, *et al.*, 2002; Heipieper, *et al.*, 2010; Miyata, *et al.*, 2004; Parales & Ditty, 2010; Johnsen & Karlson, 2004; Perfumo, *et al.*, 2010). Several studies regarding adaptive responses to PAH have been carried out with Gram-positive bacteria belonging to the mycolate-containing bacteria families, including *Mycobacterium*, *Rhodococcus*,

Corynebacterium, and *Nocardia*. Interestingly, mycolate-containing actinobacteria are also used in the transformation of phytosterols (Heipieper, *et al.*, 2010; Kim, *et al.*, 2010). Although these adaptive properties have not been well studied, some studies have shown that the similar adaptive responses play key roles in the transformation process of phytosterols (Atrat, *et al.*, 1991; Rajkhowa, *et al.*, 2000; Fernandes, *et al.*, 2003).

4.1.1 Cell envelope and adaptive changes affecting bioavailability

The mycolate-containing actinobacteria are well known as hydrophobic organisms. They have complex and extremely lipophilic cell envelopes containing large amounts of long-chained mycolic acids specific to this group of bacteria. Mycolic acids may reach up to 60% of the dry mass of the cell walls of some actinobacteria and typically range in size from 30–90 carbon atoms. These are often modified in structure by alkylation and hydroxylation and cross-linked to polysaccharide components, forming an exceptionally thick and rigid envelope. This envelope is of particular interest with respect to adaptations allowing the microorganism to degrade a broad range of hydrophobic solid compounds (Korycka-Machala, *et al.*, 2001; Heipieper, *et al.*, 2010; Kim, *et al.*, 2010). Many researches have also found that bacterial hydrophobic envelopes play a critical role in enhancing the uptake capacity of phytosterols (Atrat, *et al.*, 1991; Rajkhowa, *et al.*, 2000; Fernandes, *et al.*, 2003). Mycolate-containing bacteria are able to change their physiochemical surface properties by modifying the composition of their envelopes (Wick, *et al.*, 2002; Falkinham, 2009; Heipieper, *et al.*, 2010). In the case of mycobacteria, anthracene-grown cells respond to the low bioavailability of their hydrophobic substrate by modifying their cell envelopes via the synthesis of mycolic acids with longer alkyl chains. This significantly increases the hydrophobicity of the outer membrane, leading to up to 70-fold stronger adhesion to hydrophobic surfaces than glucose-grown cells (Wick, *et al.*, 2002). Although the adaptive changes of the cell envelope relevant to phytosterol-transforming activity are not well understood, cell wall lipid content has been proved to more than double in the presence of sterols. High lipid content and long-chain fatty acids in the envelope of sterol-grown cells is believed to contribute to the high adherence activity of these cells to sterols (Rajkhowa, *et al.*, 2000). It is conceivable that enhancing the hydrophobicity of the cell envelope by strain development based on natural adaptive mechanisms may be an effective means of overcoming the problem of low bioavailability.

It has been noted that the superficial envelope of mycolate-containing actinobacteria does not always seem to be advantageous with regard to phytosterol bioavailability. Some researchers have shown that the cell envelope might be a barrier to permeability, which is disadvantageous for phytosterol uptake and biotransformation. This can be improved by compounds such as vancomycin, glycine, lecithin, protamine, polymixin B nonapeptide, bacitracin, and ethambutol (Fernandes, *et al.*, 2003; Malaviya & Gomes, 2008). Vancomycin, glycine, and bacitracin are effective reagents, affecting the peptidoglycan layer of the cell membrane. Ethambutol is can disrupt the biosynthesis of arabinogalactan in the cell envelope. Lecithin and protamine have been shown to alter the fatty acid profile of the cell envelope. Structurally speaking, in the envelope of mycolate-containing actinobacteria, mycolic acids attach to the 5'-hydroxyl groups of D-arabinose residues of arabinogalactan and form a mycolyl-arabinogalactan-peptidoglycan complex, becoming barriers to permeability for exogenous chemicals (Draper, 1998). The chemicals listed above have been found to affect the outer part (mycolic acid layer) of the cell envelope. This may motivate

research into enhancing the bioavailability and conversion productivity of phytosterols through strain improvement focusing on a structurally changed envelope.

4.1.2 Affinity uptake of sterols

In 1991, Atrat *et al*. described a model of flexible multi-component mesophase (FMCM) on the cellular uptake of sterol substrates via direct contact between cells and the substrate particles based on observation by freeze-fracture electron microscopy. In this model, they assumed that a FMCM exists between cells and sterol particles and that this FMCM mediates sterol uptake, forming a sharp concentration gradient to drive transport. At the same time, they considered that there should be channels composed of sterol-binding or steroid-transforming proteins connecting the cytoplasm with the cell surface to reinforce the transport of sterols across the thick cell walls (Atrat, *et al*., 1991).

In 2007 and 2008, van der Geize, Mohn, and their colleagues disclosed and substantiated the idea that there is a steroid uptake system in the gene cluster encoding sterol catabolism (van der Geize, *et al*., 2007; Mohn, *et al*., 2008). The steroid uptake system is a kind of mce4 ("mammalian cell entry") locus encoded by an operon including 10 genes conserved in diverse mycolate-containing actinobacteria. This locus is composed of two transmembrane permease (supAB) genes and eight mce4 protein (mce4ABCDEFHI) genes (Mohn, *et al*., 2008). The 10 genes have been shown to encode components of complex ATP-binding cassette ABC transporters. The deletion and inhibition of these genes has been shown to result in an absence of sterol uptake. Additionally, transcriptional analyses have indicated that the operon was up-regulated 4.0-fold during growth on cholesterol, relative to growth on pyruvate. This strongly supports the idea that these 10 genes are specifically involved in sterol uptake. Ultimately, Mohn speculated that the proteins encoded by the mce4 loci might form a complex of ABC uptake transporters in the membrane of mycolate-containing bacteria to mediate the movement of the sterol substrates across the thick, hydrophobic cell envelope (Mohn, *et al*., 2008). This research on the biological function of the mce4 system provides us with new insight into the mechanism of active transport of phytosterols in the microbial transformation process.

Atrat also speculated that steroid-transforming proteins, especially extracellular enzymes, such as cholesterol oxidase (3β-hydoxysteroid dehydrogenase), might make up part of the FMCM structure and stimulate sterols transport (Atrat, *et al*., 1991). Cholesterol oxidases are extracellular flavoenyzmes that catalyze the oxidation and isomerization of cholesterol to cholest-4-en-3-one (cholestenone or ketocholesterol), which is in charge of the first compulsory step in bacterial sterol catabolic pathways that transform sterols into sterones (Kreit & Sampson, 2009). These enzymes often occur in secreted and cell-surface-associated forms. This is closely related to their role in oxidizing exogenous sterols. Cholesterol oxidases are in a class of interfacial enzymes that catalyze reactions with membrane-soluble substrates. An enzyme is catalytically active at the contact membrane interface of cholesterol or other sterols (Sampson & Kwak, 2008). In a recent study, we identified two cholesterol oxidases, ChoM1 and ChoM2, from a gene cluster encoding sterol degradation in a *Mycobacterium* strain. ChoM1, a class I cholesterol oxidase, does not occur as a secreted form, while ChoM2, a class II cholesterol oxidase, can be secreted into the medium. The roles of these two ChoMs in the utilization of phytosterols have been demonstrated by genetic inactivation, which showed that both ChoM1 and ChoM2 are necessary for the *Mycobacterium* strain to use phytosterols as carbon sources. Notably, the deletion of ChoM2 led to the *Mycobacterium* strain failing to survive on a medium with phytosterols as the sole

carbon source. Both enzymes could enhance transformation efficiency by augmenting their activity in an ADD-producing strain. We also found *Mycobacterium* strains to make use of sterones to a greater degree than sterols. Cholesterol and phytosterols are indispensable to cellular membranes in animals and plants, respectively. They can exist in the plasma and mimic membranes. They function in altering the properties of lipids in membranes to maintain membrane integrity and fluidity. However, many studies have indicated that cholestenone dose not have any effect to change the properties of membrane lipids and is not stable in the membrane (Bacia, *et al.*, 2005; Beattie, *et al.*, 2005; Lintker, *et al.*, 2009). The differences in intra-membrane behavior between sterols and sterones and the physiological functions of cholesterol oxidases imply that cholesterol oxidases may be part of the sterol transport process. Although further investigation is still needed to confirm their specific roles on cellular sterol uptake, there is little doubt that the augmentation of cholesterol oxidases will improve phytosterol-transforming strains.

4.1.3 Excretion of biosurfactants and bioemulsifiers and bioaccessibility and bioavailability of phytosterols

The bioaccessibility of hydrophobic hydrocarbons is considered an important part of their bioavailability. It depends on the contact surface area between microbial cells and substrates (Harms, *et al.*, 2010b). The smaller the particle size, the greater this area will be. Experimental observation has indicated that the most favorable arrangement to sterol absorption is substrate particles that are very similar in size to the intended microbial cells (Atrat, *et al.*, 1991). Although the size of the phytosterol particles can be affected by mechanical attrition, the hydrophobic phytosterols tend to self-aggregate in water to form agglomerates. This seriously limits their bioaccessibility and bioavailability. In most cases, surfactant-facilitated emulsification is used to inhibit the aggregation of phytosterols and keep the phytosterol particles homodisperse in the reaction medium. However, synthetic surfactants and emulsifiers are generally toxic to bacteria. They can solubilize cell membranes and induce enzymatic disorders, leading to necrosis and cell lysis (Li & Chen, 2009). In recent years, biosurfactants and bioemulsifiers have emerged as alternatives to synthetic surfactants and emulsifiers. They have attracted attention for their biocompatibility with cells. In natural environments, many bacteria secrete biosurfactants or bioemulsifiers to render minimally water-soluble carbon sources accessible (Li & Chen, 2009, Perfumo, *et al.*, 2010). Mycolate-containing actinobacteria such as *Rhodococcus*, *Corynebacterium*, *Mycobacterium*, and *Nocarida* have a catabolic capacity for phytosterols and have been shown to have the ability to produce biosurfactants (Perfumo, *et al.*, 2010). From this, it can be concluded that the selection and development of organisms with a robust ability to produce biosurfactants or bioemulsifiers may be an alternative to synthetic surfactants.

4.2 Toxic effects of steroid products on microorganisms

The cellular toxicity of steroids to microbial cells is one of the major factors limiting the productivity of conversion from phytosterols to steroid products. Some steroid products, such as AD and ADD, even at low concentrations, are believed to inhibit cell growth and respiration and suppress the enzyme activity in the sterol degradation pathway, causing low product yield (Zeillinger & Spona, 1986; Perez, *et al.*, 2003; Donova, 2007). Many attempts to overcome this problem are being investigated by segregating steroid products

from the reaction media through *in situ* product recovery and by developing mutants with improved tolerances to the steroid products (Perez, *et al.*, 2003; Malaviya & Gomes, 2008).

4.2.1 *In situ* product recovery

In situ product recovery is a common and effective means of reducing the negative effects of toxic products on microbial cells. Some special adsorbents, such as amberlite XAD-7 resin for AD and ADD, are often added to the reaction media to increase the yield of AD and ADD by absorbing steroid products (Huang, *et al.*, 2006; Malaviya & Gomes, 2008). Positive results have also been obtained by using polymers as reservoirs, reducing the concentration of steroid products in the reaction medium. For example, poly dimethyl siloxane and cyclodextrins have been used to recover ADD and AD from the reaction medium. This can improve transformation productivity. In addition, aqueous or organic-aqueous two-phase systems are also clean and effective tools for *in situ* product recovery. In two-phase systems, the water-immiscible organic phase acts as reservoir for toxic products. The significantly higher solubility of sterols in the organic phase allows the system to instantaneously recover toxic steroid products during biotransformation. For a more detailed discussion of *in situ* steroid recovery, we refer to the review by Malaviya and Gomes (2008).

4.2.2 Development of mutants with increased resistance to steroid products

The developing mutants with improved tolerance to toxic products may be a more effective means of solving the problem of toxic products than product sequestration. Perez *et al.* demonstrated that high concentrations of androstanes could be used to isolate tolerant mutants and increase androstane yield. In that case, colonies growing at least 1 mg/ml ADD in culture medium after nitrosoguanidine mutagenesis showed improved ADD yield, going from 18.81 mg/ml to 38.99 mg/ml (Perez, *et al.*, 2003). So far, the actual nature of the toxicity of steroids to microbial cells has not been elucidated. Similarly, the mechanisms by which microbial cells become resistant remain unknown. Even so, robust tolerance to toxic steroid products is one of the most important characteristics of improved phytosterol-transforming strains.

4.2.3 Eflux transporters for steroid products

In natural environments, tolerance to toxins is important for survival. In the case of phytosterol-transforming mycobacteria, there are two common mechanisms involved in resistance to toxicity: the rigid envelope acts as a barrier to extracellular toxins and the active multidrug efflux pumps remove intracellular toxins (Nikaido, 1994; De Rossi, *et al.*, 2006). In the phytosterol transformation process, most of the reactions occur intracellularly. Therefore, it is reasonable to conclude that multidrug efflux pumps play a role in resistance to toxic steroid products. Genes encoding drug efflux transporters have been identified in several mycobacterial species, including *M. fortuitum*, *M. tuberculosis*, and *M. smegmatis* (Ainsa, *et al.*, 1998; Li, *et al.*, 2004). These genes, which exist in the form of multi-copy plasmids or replicates in the genome, encode efflux proteins that transport tetracycline, fluoroquinolones, and other compounds outwards. Because steroid compounds resemble tetracycline and fluoroquinolones, it is reasonable that some non-specific efflux transporters may transport steroids outside the cell. These efflux transporters should be investigated for applicability to the development of phytosterol-transforming organisms resistant to toxic products.

4.3 Development of ideal organisms

Strain performance plays a central role in the commercial development of microbial conversion systems. In essence, the abovementioned problems can be attributed to deficient microbial strains. The fundamental solution is to develop robust, efficient, highly productive microbial strains. Random mutagenesis is the conventional breeding approach and has been the main means of developing custom phytosterol-transforming strains (Egorova, *et al.*, 2002; Donova, *et al.*, 2005a; Sripalakit, *et al.*, 2006). However, strains surviving multiple rounds of mutation are genetically undefined and vulnerable to further changes. Directed genetic modification may be a more feasible option for subsequent isolation of mutant strains with desired functions. Rational metabolic engineering provides a more effective and well-defined alternative to strain development than random mutagenesis. Strategies for developing the desired organisms by metabolic engineering can be summarized as follows: (1) inhibition of B-ring cleavage to maintain the steroidal nucleus by inactivating the key enzymes KstDs and Kshs; (2) overexpression of genes encoding rate-limiting reactions and blockage of downstream and branched reactions to increase the accumulation of desired product and reduce the production of unwanted by-products; (3) enhancement of cellular uptake of phytosterols by increasing the activity of active transporters and/or the permeability of the cell wall; (4) enhancement of cellular tolerance to toxic products and efflux capacity of toxic products.

Ideal microorganisms should have the following characteristics:

(1) Lack of pathogenicity, ease of culture

Many organisms that can transform phytosterols into valuable steroids have been isolated. These include mycobacteria, which seem perform the best. This is why most of the microbial strains used in industrial and academic research are members of the genus *Mycobacterium* (Atrat, *et al.*, 1991; Fernandes, *et al.*, 2003; Perez, *et al.*, 2006). However, mycobacteria have two serious deficiencies. First, many *Mycobacterium* species are known to be stubborn pathogens. These include *M. tuberculosis* and *M. leprae*. Many other *Mycobacterium* species, although generally non-pathogenic, have been shown to be opportunistic pathogens (Malaviya & Gomes, 2008; Cassidy, *et al.*, 2009; van Ingen, *et al.*, 2009). For example *M. fortuitum* can cause local cutaneous disease, osteomyelitis, and joint infections (Wallace, *et al.*, 1983). The other problem is that mycobacteria are difficult to culture because of their complicated physiological states, rod-coccus growth cycle, slow growth rate, and special nutritional requirements (Falkinham, 2009; Kim, *et al.*, 2010). In addition, the process by which mycobacteria convert phytosterols to steroid hormones is complicated and can last more than a week. This reduces its economic benefit. For these reasons, it may be best to select safer and more rapidly growing organisms, such as those in the genera *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Brevibcterium*, *Rhodoccocus*, *Norcardia*, and others.

(2) Ease of modification

Unfortunately, genetic manipulation techniques suitable to most of the organisms with proven capacity for phytosterols conversion lags behind those that work on other bacterial species. This makes it difficult to improve these organisms by means of metabolic engineering. For example, it is difficult to perform genetic recombination on mycobacteria because of the high rate of illegitimate recombination and the lack of universal tools among *Mycobacterium* species (Kim, *et al.*, 2010). This has hindered the progress of strain improvement. In addition, phytosterol metabolism in microbes is a complex process. To

build an ideal strain, many metabolic reactions and physiological properties will need to be modified and regulated. An ideal phytosterol-transforming organism, therefore, must be easy to be modified, preferably by relatively simple genetic recombination techniques.

(3) Single-product production

Most phytosterol-transforming strains can transform phytosterols into many structurally similar products, such as the C19 steroids AD, ADD, and their derivatives, at the same time (Egorova, *et al.*, 2002; Donova, *et al.*, 2005a, 2005b, 2005c; Molchanova, *et al.*, 2007). Because the differences in the structures of these products are very subtle, it is difficult to separate and purify them. This significantly complicates the production process and increases production costs (Molchanova, *et al.*, 2007). An ideal microbial strain, therefore, must be able to transform phytosterols into only a single desired product.

(4) Powerful phytosterol uptake capacity

The extreme hydrophobicity of phytosterols seriously limits their bioavailability. As mentioned above, most of the strategies that enhance phytosterol uptake increase the complexity of the production process and add to its costs. However, many microorganisms already have numerous physiological adaptations that allow them to take up highly hydrophobic compounds, such as phytosterols. These adaptations can be further improved to enhance the organisms' phytosterol uptake capacity. The following strategies should be attempted: i) Increase the direct contact between microbial cells and phytosterol particles by changing the physiochemical properties of the cell envelope. ii) Enhance the active transport capacity for phytosterols by augmenting the activity of mce4 transporters. iii) Overexpress cholesterol oxidases. iv) Increase the bioaccessibility of phytosterols by reinforceing the excretion of biosurfactants and/or bioemulsifiers.

(5) Strong tolerance to toxic products

Although several methods of sequestering toxic steroids from the reaction media have been tested, the development of mutants with improved tolerance to steroid products may be a more effective way to bypass the toxic effects of steroid products on microbial cells (Molchanova, *et al.*, 2007; Perez, *et al.*, 2003; Malaviya & Gomes, 2008). An ideal organism should have physiological functions that reduce or eliminate the harmful effects of toxic products. Such organisms may be developed using strategies that allow the selection of evolutionary mutants by continuous, prolonged exposure to high concentrations of toxic products and augment efflux transporters.

5. The future of phytosterol production

Phytosterol catabolism in microbes is extremely intricate. It involves not only dozens of specific biocatalytic reactions but also cellular properties catering to phytosterol metabolism, such as phytosterol uptake systems and resistance to toxic products. Currently, only the genes directly encoding sterol catabolism were found as a cluster of 51 genes in *Rhodococci* and 80 to 82 genes in mycobacteria (van der Geize, *et al.*, 2007). Most of which have not been well characterized in function until now. The most urgent task is to clearly characterize and elucidate the mechanisms of phytosterol catabolism and the cellular properties related to phytosterol uptake, tolerance to toxic products, and global regulations.

Along with gradual in-depth research on the mechanisms by which phytosterols are transformed into steroid hormones, metabolic engineering may be useful to the

development ideal microbial "factories" for the production of desired steroid hormones from phytosterols. To meet the basic standards depicted in section 4.3, strain improvement by metabolic engineering should include three steps:

The first step is to rationally modify the catabolic phytosterol pathway toward formation of the target product, avoiding cleavage of the steroid nucleus and formation of any byproducts. The cleavage of the steroid nucleus can be prevented by disrupting the key enzymes in steroid B-ring cleavage, KstDs and Kshs (Fig. 2). The accumulation of byproducts can be eliminated by blocking side reactions and amplifying rate-limiting reactions. To produce ADD, for example, Kshs should be blocked and KstDs should be activated (Fig. 5). Several similar projects have already been successful in *Rhodococcus* species and *Mycobacterium* (van der Geize, *et al.*, 2007; Wei, *et al.*, 2010).

The second step should focus on economically feasible productivity. The strains developed in the first stage may be limited in efficiency. To increase their economic benefit, they should be reconstructed to avoid limitations in productivity. The phytosterol uptake capacity of the organisms should be enhanced by augmenting sterol transporters such as Mce proteins, and reducing the barrier effect of the cell envelope (Mohn, *et al.*, 2008; Korycka-Machala, *et al.*, 2005; Hoffmann, *et al.*, 2008). The tolerance of the microbial cells to toxic products should be improved. This may be achieved by enhancing the cells' ability to expel active products by augmenting or rationally designing specific efflux pumps or by reducing the sensibility of the strain to the toxic products. Negative regulators and feedback inhibitors limiting the phytosterol metabolism may also be of use and should be examined and deregulated. For example two TetR-type transcriptional regulators control sterol utilization in mycobacteria (Kendall, *et al.*, 2010).

The third step is to comprehensively reprogram phytosterol-transforming organisms based on systems biology. In brief, the metabolic flux from phytosterols to steroid products should be comprehensively investigated, modified, and optimized based on industrial requirements. Approaches to this include transcriptomics, proteomics, metabolomics, and computational modeling.

Microorganisms with good phytosterol conversion capacities may be not good candidates for modification by metabolic engineering. This is partly because of the complexity of physiological process of reprogramming the cell. Recently, metabolic engineering has become capable of creating novel, finely controlled metabolic and regulatory circuits that can produce desired products effectively. Reconstructing the transformation pathway from phytosterols to steroid homones in a heterologous host organism with suitable physiological trains, then, may an alternative to developing engineered organisms. In 2003, Szczebara and co-workers described a process that rerouted ergosterol biosynthesis in *Saccharomyces cerevisiae* to compatible brassicasterol and campesterol, two ingredients in the four soy phytosterols (Fig. 1) and then extended the pathway to produce hydrocortisone by mimicking hydrocortisone biosynthesis seen in the mammalian adrenocortex. In other words, an artificial, fully self-sufficient biosynthetic pathway was successfully built in a microbial host, suggesting that the production of steroid hormones may also take place in this simple, environmentally friendly, low-cost manner (Szczebara, *et al.*, 2003). This kind of revolutionary approach to the production of steroid hormones may be the future of the steroid pharmaceutical industry.

The integrated reconstruction of the phytosterol transformation system in microbes may become a reality in the near future, most likely in one of two ways.

The first way would be to rebuild the phytosterol transformation system in robust hosts that would be superior to existing phytosterol-transforming organisms in tolerance to high

concentrations of steroid hormones, phytosterol uptake capacity, simplicity of gene manipulation, and cell cultivation. This would need to be founded on a comprehensive understanding of the processes by which phytosterols are converted to products, including the degradation of C17-side chains. In order to facilitate the assembly and control of this system, the process of design and reconstruction should based on synthetic biology, standardizing the gene parts required, designing controllable and efficient gene circuits, developing functional modules, and integrating and optimizing the rebuilt system in the selected host. Therefore, a novel engineered organism with ideal phytosterol-transforming properties may be developed (Fig. 5A).

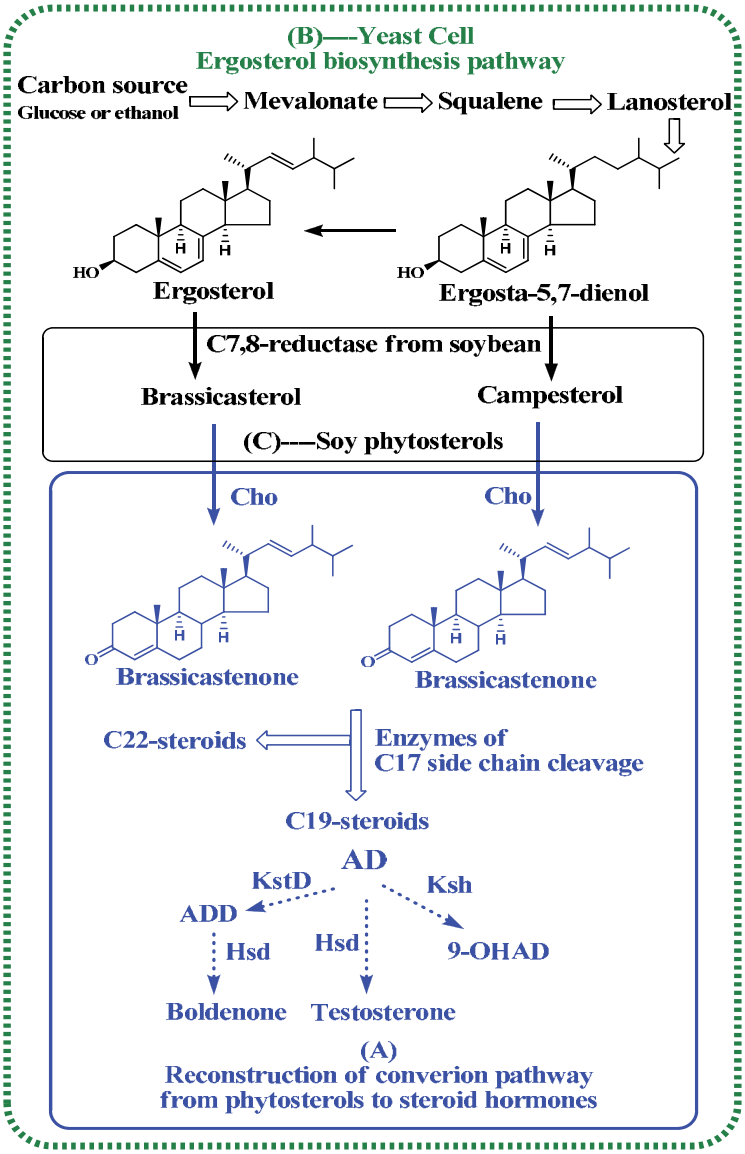

Fig. 5. Transformation pathway of phytosterols in *S. cerevisiae*.

The second way would be to learn from the works of Szczebara and Dumas and reconstitute the phytosterol conversion system in yeast (Szczebara, *et al*., 2003; Dumas, *et al*., 2006). Yeast is a proven producer of endogenous sterols, such as ergosterol and ergosta-5,7-dienol. Ergosterol and ergosta-5,7-dienol are similar to the precursors of phytosterols, and they can be converted to brassicasterol and campesterol by C7-reductases from plants (Hartmann, 2003; Szczebara, *et al*., 2003). Brassicasterol and campesterol are two components of the four main phytosterols found in soybeans (Fig. 1). Therefore, the endogenous sterol biosynthesis seen in yeast may be rerouted to produce soybean phytosterols by the introduction of C7-reductase from a plant, such as the soybean (Fig. 4C). In this way, the conversion pathway of soybean phytosterols to C19 steroids or C22 steroids can be reassembled (Fig. 4A) and then linked to the natural ergosterol biosynthetic pathway in yeast. This would result in steroid hormones in self-sufficient in yeast fed on a simple carbon source, such as glucose or ethanol (Fig. 4). The benefits of this method are that the many reactions involved in producing C19 or C22-steroids from phytosterols would be integrated in a single fermentation step, avoiding the need for the production or addition of phytosterols. Such a simple production process would revolutionize the synthesis of steroid hormones in industry and significantly lower production costs and environmental impact. Nevertheless, there are also some drawbacks to this technique. One important issue is the inherent difficulties of the yeast system to adapt to the exogenous biocoversion process, such as the toxicity of the steroids to the yeast organism. Much more additional research must be performed to improve the physiological properties of yeast before it can become an industrially advantageous means of producing steroid products.

6. Aknowlegements

The work was supported by the National Basic Research Program of China (2009CB724703) and the Fundamental Research Funds for the Central Universities and China Postdoctoral Science Foundation (20100470757).

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12

Genistein Derivatization - From a Dietary Supplement to a Pharmaceutical Agent

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1. Introduction

Many soy supplements in the market focus on estrogen and antioxidant properties of isoflavonoid, genistein. Due to the structural similarity to estrogen and binding to estrogen receptor, genistein is often referred to as a phyto-hormone or a dietary estrogen from soy. Target applications of isoflavone-enriched whole soy concentrates include natural hormonal replacement therapy and women's health products (Knight & Eden, 1996; Messina, 1995). However, beneficial effect of genistein on human health extends to the prevention of cancer, cardiovascular events, diabetes, and incidence of inflammatory diseases and management of some metabolic diseases (Birt et al., 2001, Kurzer, 2002; Rimbach et al., 2008; Węgrzyn et al., 2010).

The view of genistein as a safe and healthy food supplement is not entirely clear, however. Although most studies show no risk of high consumption of soy-based products for human growth, development or reproduction, there are some data urging caution in genistein overdose. Genistein is implicated as a possible cause of infertility and liver disease in some animal species. Consumption of genistein-rich food and supplements during pregnancy have been suggested to increase the risk of infant leukemias (Hengstler et al., 2002). In addition, experimental data showing stimulatory effect of genistein on proliferation of some breast cancer cells lines raise the problem of safety of genistein supplementation in women with diagnosed breast cancer (Lavigne et al., 2008).

Despite the controversies about safety and benefits of soy food supplementation, the pleiotropic activity of genistein, resulting from its interaction with numerous molecular targets, along with the possibility of chemical derivatization of a molecule place genistein among leading compounds for drug development.

This chapter deals with two main issues: (1) describes the main molecular targets affected by genistein and indicates alterations in signaling pathways, revealed by global gene profiling analyses, and (2) delineates the relevant examples of genistein derivatives synthesized with aim to obtain compounds exhibiting improved pharmacological activity, increased affinity

to molecular targets or altered mode of action as compared to the parent drug. The clinical utility of genistein-based pharmaceuticals is also discussed.

2. Molecular targets affected by genistein

Genistein is known for its pleiotropic effects, mediated by alteration of the activity of key enzymes involved in cell signaling, and by changes of the expression of the genes involved in various physiological processes. To the large group of genistein targets new proteins discovered with use of potent computational methods have joined recently. The interplay between different signaling pathways is extensively studied; however, exact molecular mechanisms have not been clearly defined yet.

2.1 Binding of genistein to different proteins

The proteins affected directly by genistein belong to many families. Among them are nuclear receptors, tyrosine-specific protein kinases, topoisomerases, ABC transporters and transport proteins present in the bloodstream (Tab. 1).

Nuclear receptors are members of a large family of transcription factors activated by binding of a ligand and regulating gene expression underlying a plethora of physiological and pathological states. Genistein binds to and activates estrogen receptors, peroxisome-proliferators activated receptors, liver X receptors and estrogen related receptors.

It was recognized very early that the chemical structure of genistein bears strong similarity to 17β-estradiol, and that genisten binds to estrogen receptors (ER) and to sex hormone-binding globulins (Klinge, 2000; Kuiper et al., 1998; Kurzer, 2002). A functional interaction of genistein with estrogen receptors, leading to stimulation of ER responsive genes was confirmed in experiments in vitro (Birt et al., 2001; Kostelac et al., 2003). There are two isoforms of estrogen receptor, ERα and ERβ, differentially expressed in tissues and exhibiting different biological functions. The hydrophobic ligand-binding cavity displays 53% of similarity between two isoforms (Manas et al., 2004). This pocket contains 12 α-helices and two β-sheets, connected by several short straight fragments. Inside a ligand binding cavity the ligand molecule is oriented with hydrogen bonding at the ends and hydrophobic van der Waals contacts along the body of the hormone. In the ERα functionally important polar amino acids in the binding pocket include: Glu-353, Arg-394, and His-524 (Tanenbaum et al., 1998). These aminoacids interact with 7 and 4′ OH groups of genistein. Several other amino acid residues create hydrophobic bonds essential for stabilizing the nonpolar elements of the ligand ring structure. Genistein is regarded as an agonist of ERα. Although ligand binding domain sequence and structure in ERβ is very similar to ERα and stabilization of a ligand occurs due to analogous hydrogen bonding with the side chains of Glu-305, Arg-346 and His-475 (Fig. 1), genistein binds to this form with higher affinity than to ERα (Manas et al., 2004). The position of the ligand in both receptor types is similar, but the volume of a cavity is smaller in ERβ, and genistein is bound more tightly in the cavity. Despite the higher affinity to ERβ than ERα, genistein is only partial agonist of ERβ. The explanation may be derived from the different conformations of a ligand-bound state of ERα and ERβ. Hormone-dependent activation of a receptor is a result of the movement of helix 12 from one position to another. The positional variability of this substructure appears to be a critical attribute of the ER receptors in their variable response to different ligands (Pike et al., 1999).

Recently it was shown that proteins constituting the family of estrogen related receptors (ERRs) are also able to bind genistein (Suetsugi et al., 2003). Although they display similarity to estrogen receptors, they do not bind estrogens. ERRs are referred to as orphan receptors, because no endogenous ligands required for their activity have been identified so far. The ligand-binding pockets of ERRs are very small (about half size of ERα) and partially filled with the aromatic bulky side chains of aminoacids forming the cavity, what is supposed to mimick the ligand-bound state inducing constitutive activity of the receptor. Surprisingly, genistein is a strong agonist of those receptors, augmenting their basal activity. Docking results showed that genistein, due to the small size of the cavity, is tightly packed and deeply docked into the ligand-binding site (Suetsugi et al., 2003) (Fig. 2).

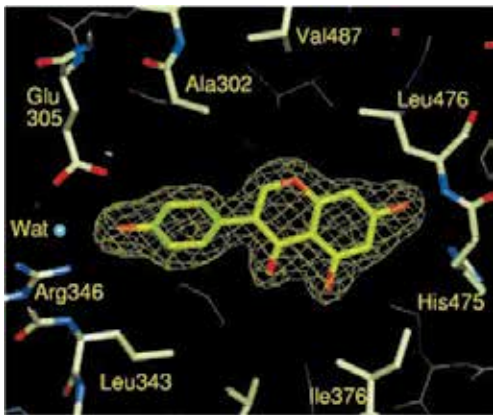

Fig. 1. The ligand-binding cavity in the genistein bound human estrogen receptor β. 4'OH group of genistein forms hydrogen bonds with the Glu-305, the Arg-346 and a water molecule; 7OH group forms hydrogen bond with His-475. Reprinted by permission from Macmillan Publishers Ltd: Pike et al. 1999, EMBO Journal Vol.18, No.17, 4608-4618, Copyright 1999.

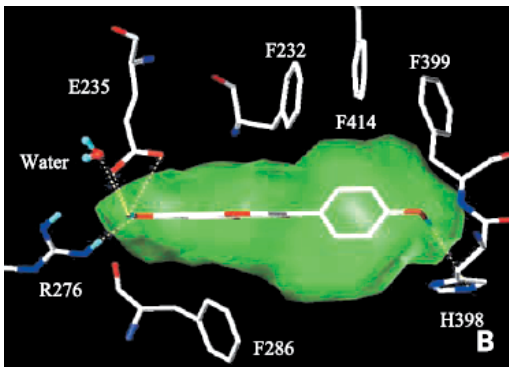

Fig. 2. The binding pocket of genistein-bound ERRα. 7OH group of genistein forms hydrogen bonds with the glutamate in helix 3, the arginine in helix 5, and a water molecule; 4'OH group forms hydrogen bond with H398. Adapted and reprinted by permission from American Association for Cancer Research, Suetsugi et al. 2003, Molecular Cancer Research Vol.1, No.13, 981-991.

Other members of nuclear receptor family activated by genistein are peroxisome proliferators-activated receptor γ (PPARγ) (Dang et al., 2003) and PPARα (Kim et al., 2004; Kim et al., 2005). The main role of PPARγ is to control the genes involved in adipocyte differentiation and lipid storage. The structure of ligand binding domain in the PPARγ is similar to both PPARα and PPARγ, and consists of 12 helices arranged in an antiparallel helix sandwich, and additional 3-stranded antiparallel β sheet (Cronet et al., 2001). Docking studies, supported by the functional assay, characterized genistein as a full PPARγ agonist (Salam et al., 2008). Subsequent studies shown, that agonistic action of genistein on PPARγ stimulates adipogenesis (Relic et al., 2009). It is worth to keep in mind that genistein concurrently activates two different transcriptional factors, ERs and PPARγ, which have opposite effects on osteogenesis or adipogenesis. Thus, genistein as an agonist of both receptors may affect the balance between activated ERs and PPARγ, which determines the final effects on osteogenesis and adipogenesis.

Very weak binding of genistein to androgen receptor (AR) was reported by Bectic et al. (2004). However, the inhibition of specific androgen binding was less than 25% at 1000-fold higher concentration of genistein (1 μM) in a radioligand-binding assay. Moreover, this weak binding did not influenced AR transcriptional activity measured by a reporter gene assay in PC-3 and DU 145 cells. The authors concluded, that genistein influence the expression of AR dependent genes indirectly, by down-regulation of AR.

Another group of enzymes binding genistein are proteins, which share in their structure the consensus sequence for ATP binding, such as tyrosine kinesis, topoisomerases, ABC transporters, and ion channels. Although genistein does not resemble ATP, its binding is competitive to ATP.

Historically, the first proteins affected by genistein were protein tyrosine kinases (PTK) (Akiyama et al., 1987). Suppression of PTKs is thought to occur due to genistein binding with a common, highly conservative sequence at, or near to, the ATP-binding domain (Markovits et al., 1989; Akiyama et al., 1987). Genistein is a competitive inhibitor of ATP in a number of tyrosine kinases that utilize the G-X-G-X-X-G consensus for ATP binding (Akiyama et al., 1987).

To another group of proteins affected by genistein belong hexose transporters. The 12 transmembrane α-helical domains of the monomeric GLUT protein form a central water-filled pore, facilitating glucose transport. Vera et al. (1996) found that genistein inhibited transport of substrates by the GLUT1, blocking its ATP-binding domains. Similar mechanism of glucose uptake inhibition by genistein occurs in GLUT4 (Bazuine et al., 2005). Exact binding mode of genistein in GLUT1 is not known. It is hypothesized, that genistein, binding to the cytoplasmic surface of GLUT1, uses the sequences of close homologies to the sequences in the ligand-binding cavity of estrogen receptors (Afzal et al., 2002) (Fig. 3). These domains are in close vicinity or partially overlap the ATP-binding sites, so binding of estrogen, tamoxifen and genistein may competitively inhibit ATP binding.

The inhibition of topoisomerases by genistein is thought to be mediated by its binding to N-terminal ATP-binding motif. Topoisomerase II maintains the integrity of the cleaved DNA by forming covalent bonds with each newly created 5'-phosphate termini of the cleaved DNA segment. This transfer of phosphodiester bonds from DNA to topoisomerase II is similar to the autophosphorylation reaction of tyrosine kinases, where the enzyme forms a bond between its tyrosine and the phosphate group of ATP. Eucaryotic topoisomerase IIα has two consensus ATP-binding motifs. Only Walker A site (residues 161-166 in human topoisomerase IIα) is utilized for ATP binding in the eukaryotic type II enzyme. The Walker B site (residues 472-477), located near the region of the enzyme, in close proximity to the site

of DNA cleavage and ligation, has no known function for topoisomerase activity, but its consensus sequence (G-X-G-X-X-G), is the same, as the ATP-binding consensus sequence in a number of tyrosine kinases (Markowitz et al., 1989). Probably, this non-functional consensus site, common with protein kinases is the sequence of topoisomerase IIα, which binds genistein (Bandele &Osheroff 2007).

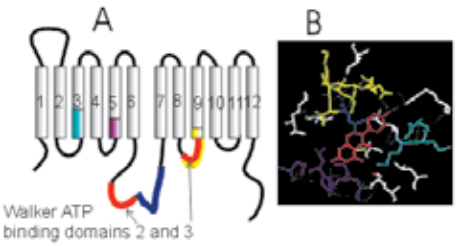

Fig. 3. Structure of GLUT1. A. Two-dimensional model of GLUT1 with Walker ATP-binding domains (red), and homologies to ERβ (turquoise, violet, blue and yellow). B. Close view of H bonding interactions of genistein (dark red) with His and Arg of GLUT1. Colours on A and B match each other. Reproduced with permission, from Afzal et al. 2002, Biochemical Journal Vol. 365, No.3, 1707-1719, © the Biochemical Society.

Cystic fibrosis transmembrane conductance regulator (CFTR) belongs to ABC transporter-class ion channels, and transports chloride through epithelial cells membranes. Potentiation of Cl^{2-} ion efflux through CFTR by genistein is thought to result from interaction of the isoflavonoid with one of two ATP-binding sites in the regulatory nucleotide-binding domains (NBD) of a protein. In CFTR, the membrane spanning domains form a pathway for passive anion flow that is gated by cycles of ATP binding and hydrolysis (Hwang & Sheppard 2009) (Fig. 4). It is established that ATP hydrolysis at ATP-binding site 2 is responsible for a rapid closure of the gate opened by ATP (Chen & Hwang, 2008; Gadsby et al., 2006). Competitive binding of genistein to the second ATP-binding site disables channel gating (Wang et al., 1998), what means that potentiation of CFTR Cl- current is a result of the channel locking by genistein in a stable open state. Exact mode of genistein binding to CFTR is unknown, however docking studies indicate several putative binding sites in the protein (Huang et al, 2009) (Fig. 4B).

The next group of proteins binding genistein are blood proteins. Binding of genistein to human sex hormone-binding proteins (hSHBG) is reversible and competitive for both [3H]testosterone and [3H]17b-estradiol (Dechaud et al., 1999). Genisten binding to hSHBG may influence its bioavailability to cell tissues, and displace endogenous sex steroid hormones from hSHBG binding sites.

Human serum albumin (HSA) with high affinity binding sites is a major transporter for delivering several endogenous compounds and drugs *in vivo*. Structural analysis showed that genistein binds to HSA *via* polypeptide polar groups (Mandeville et al., 2009). Binding of genistein to albumins is reversible, rapid and the concentration of unbound isoflavone is in an equilibrium state.

It is worth to note, that hemoglobin is able to bind genistein, what makes this most abundant protein of blood, important for genistein transportation, distribution and storage (Yuan et al., 2008).

Transthyretin (TTR) is a tetrameric β-sheet-rich transporter protein involved to some extent in the transport of thyroid hormone, thyroxine. TTR binds genistein via the thyroxine (T4)

binding sites. TTR has two identical funnel-shaped thyroxine-binding sites located at the dimer–dimer interface (Fig. 5). Binding of thyroxine stabilizes native, tetrameric state, whereas dissociation of the hormone promotes dissociation of the protein to monomers, and their abnormal aggregation, observed in human amyloid diseases. Since only 1% of TTR is bound to thyroxine, small molecules, such as genistein may help to stabilize the tetrameric structure. Genistein is known to potently inhibit TTR amyloid fibril formation (Green et al., 2005). In TTR, the mode of genistein binding is sequential, with negative cooperativity observed. Binding of the first genistein molecule to TTR generates allosteric adjustment of conformation in the second genistein binding site. The most stable conformation of TTR protein is observed when two molecules of genistein are bound to the tetramer (Trivella et al., 2010).

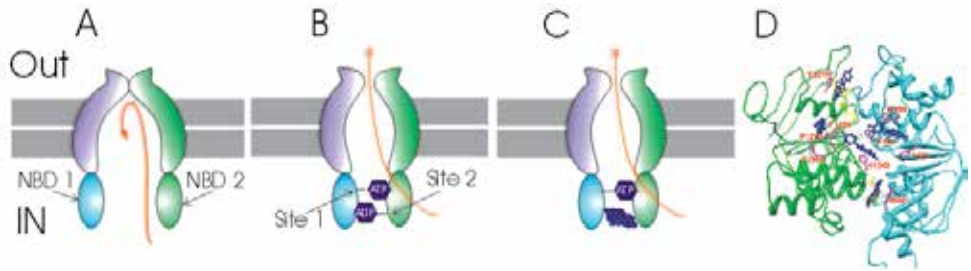

Fig. 4. The simplified model of CFTR Cl^- channel opening. A. closed state, B. open state, C. stable open state, forced by genistein (dark blue molecule), which blocks ATP-binding site 2. IN and OUT denote the intra- and extracellular sides of the membrane, respectively. NBD1 – turquoise, NBD2 – green, site 1 and site 2 – ATP-binding sites. D. A stereo view of five putative binding sites of genistein to human CFTR. Ball-and-stick molecules of genistein are shown in blue, and the ATP in yellow. Residues are important for interacting with genistein are in magenta. A and B - Reproduced with permission from John Wiley and Sons, Hwang & Sheppard 2009, Journal of Physiology, Vol.587, No.10, 2151–2161, D - Reprinted from Journal of Molecular Graphics and Modelling, Vol.27, No. 7, Huang et al. 2009, Pages 822-828, Copyright (2009), with permission from Elsevier.

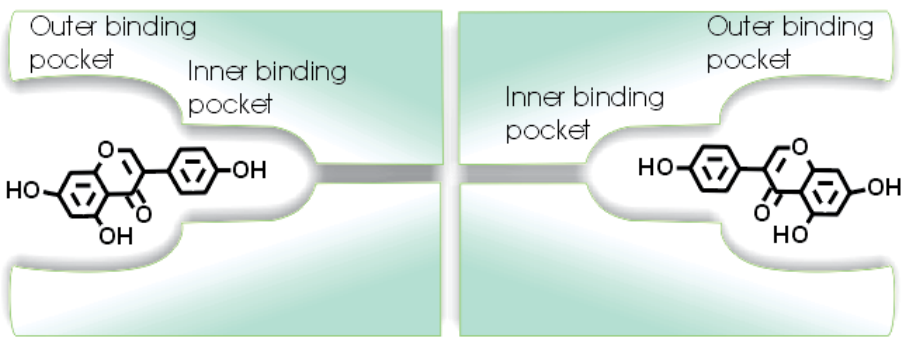

Fig. 5. Structure of transthyretin. A. Schematic representation of the tetrameric structure of TTR depicting the two thyroxine-binding sites with two molecules of genistein stabilizing the tetramer. Modified from Green et al., 2005, with permission. Copyright (2005) National Academy of Sciences, U.S.A.

There are also many intracellular proteins which functions are affected upon genistein binding listed in the Tab. 1., which does not fit any group described above, such as ornithyne decarboxylase, aromatase CYP19 or tubulin. Cytoskeleton protein, tubulin binds genistein at ANS-binding site, what leads to depolymerization of microtubules (Mukherjee et al., 2010).

Classess of proteins binding genistein	Proteins	Type of influence on a protein activity	Binding affinity (Ki) or 50 % inhibitory concentration (IC50)	Source
Estrogen receptors	Erα	Agonist	Ki=370 nM IC_{50}=1.97-14 μM	Matsuda et al., 2001, Güngör et al., 2006, Kostelac et al., 2003
	ERβ	Partial agonist	Ki=24nM; IC_{50}=1.3-395 nM	Pike et al., 1999, Matsuda et al., 2001, Chesworth et al., 2005; Mewshaw et al., 2007
Estrogen related receptors	ERRα	Agonist	IC_{50}=2.4 μM	Suetsugi et al., 2003
	ERRβ	Agonist	IC_{5}0>10 μM	Suetsugi et al., 2003
	ERRγ	Agonist	IC_{50}>10 μM	Suetsugi et al., 2003
Peroxisome proliferator activator receptor	PPARγ	Agonist	Ki=5.7 μM IC_{50}=16.7 μM	Dang et al., 2003, Salam et al., 2008
Androgen receptor	AR	No transcription activation	Ki>>1 μM	Bektic et al., 2004
Liver X receptor	LXR-α	Agonist	IC_{50}=31 μM	Dodo et al., 2008
	LXR-β	Agonist	IC_{50}=22 μM	Dodo et al., 2008
Tyrosine kinases	EGFR	Inhibitor	IC_{50}=22 μM	Akiyama et al., 1987
	Pp60 v-src	Inhibitor	IC_{50}=26 μM	Akiyama et al., 1987
	Pp110 gag-fes	Inhibitor	IC_{50}=24 μM	Akiyama et al., 1987
	MEK4	Inhibitor	IC_{50}=0.4 μM	Xu et al., 2009
	ABL	Inhibitor	IC_{50}=10 μM	Traxler et al., 1995
	Protein kinase C	Inhibitor	IC_{50}=15 μM	Traxler et al., 1995
	Tyrosine Kinase Syk	Inhibitor	IC_{50}=39 μM	Xie et al, 2009
FGF receptor 1	FGF1	Inhibitor	IC_{50}<25 μM	Rao, 1997
Ion channels	CFTR	Potentiator	Ki=1.8 μM	Melani et al., 2010

Classess of proteins binding genistein	Proteins	Type of influence on a protein activity	Binding affinity (Ki) or 50 % inhibitory concentration (IC50)	Source
Glucose transporters	GLUT1	Inhibitor	Ki=4-12 μM	Afzal et al., 2002; Vera et al. 1996
	GLUT4	Inhibitor	IC_{50}=20 μM	Bazuine et al., 2005
MDR proteins	ABC G2	Substrate	n.a.	Imai et al., 2004; Zhang et al., 2004; Perez et al., 2009
Topoisomerase I	Topo I	Inhibitor	n.a.	Okura et al., 1988
Topoisomerase II	Topo II α	Inhibitor	IC_{50}<30 μM	Bandele & Osheroff, 2007
	Topo II β	Inhibitor	IC_{50}=30 μM	Constantinou et al., 1995; Okura et al., 1988; Markovitz et al., 1989
	Bacterial gyrase	Inhibitor	IC_{50}>200 μM	Bernard et al., 1997
	Bacterial topo IV	Inhibitor	IC_{50}=93 μM	Bernard et al., 1997
Adenosine receptors	A1	Inhibitor	IC_{50}=2.6 μM	Schulte & Fredholm, 2002
	A2A	Inhibitor	IC_{50}=15.3 μM	Schulte & Fredholm, 2002
Aromatase	Cyp 19	Inhibitor	Ki=100 μM	Paoletta et al., 2008
Tubulin	Tubulin	Polymeriza-tion inhibitor	Ki=20 μM IC_{50}>87 μM	Mukherjee et al., 2010
Ornithine decarboxylase	ODC	Inhibitor	>10 μM	Fang & Cassida, 1999
Sex hormone-binding globulin	hSHBG	Competitive binding to testosterone and estradiol	Ka=1.7x10^5 M^{-1} (Testosterone) Ka=6.3 x10^5 M^{-1} (estradiol)	Dechaud et al.,1999
Albumins	HAS		K=2.4×10^4 M^{-1}	Mandeville et al., 2009 ; Bian et al. 2004
Haemoglobin			n.a.	Yuan et al., 2008
Transthyretin	TTR	Competitor of T4	Ki=70nM	Radovic et al., 2006
		Stabilisator of a tetramer	K_{d1}=40 nM, K_{d2}=1.4 μM	Green et al., 2005

Table 1. Proteins binding genistein and the type of influence on their activity

New efficient computer-assisted methods facilitate rapid identification of protein targets of potential drugs (Chen et al., 2003). INVDOCK is one of *in silico* techniques specifically applied to identification targets of medicinal plants ingredients and synthetic chemicals. It is based on ligand–protein inverse docking of a tested ligand to known ligand-binding pockets of the proteins from a 3D structural database. Many proteins indicated by the aid of INVDOCK as potential targets of genistein were previously confirmed or implicated by experiments. This approach allowed also indication of multiple new targets with therapeutic implications, able to bind genistein. Among them are: thymidylate synthetase, purine nucleoside phosphorylase, cyclophilin A, farnesyltransferase, guanylyl cyclase, carbonic anhydrase (cancer treatment), DNA polymerase, (cancer and Herpes viral infection), inosine dehydrogenase, purine nucleoside phosphorylase (Malaria), dihydrofolate reductase (leprosy), phospholipase A2 (inflammation), carbonic anhydrase I (hypertension and glaucoma), protein kinase C (cardiovascular disease).
From many studies on the oral bioavailability of genistein it is clear that *in vivo* plasma concentrations of genistein is 0.1–8 μM at a dose of 16 mg/kg of body weight (Bloedon et al., 2002; Setchell et al., 2001). Tab. 1. shows that the concentration of genistein, inhibiting or stimulating several potential targets, is much higher than its concentration in plasma, thus its physiological relevance is disputable. However, it might be possible, that under chronic exposure, even weak effects of genistein on molecular targets influence the overall physiological status.

2.2 Signalling pathways influenced by genistein in microarrays profiling

Specific changes in gene expression profiles brought about by the genistein treatment can occur either due to direct influence of the flavonoid on activity of transcription factors or to indirect compensatory homeostatic mechanisms. A global analysis of gene expression in response to a plejotropic compound, such as genistein, in models of different organs under physiologic and disease conditions appears to be essential for understanding the molecular mechanisms of genistein action and seems to be advantageous approach in comparison to "gene-by-gene" studies. In this chapter we summarize the most important observations derived from transcryptomic, microarray-based studies aimed to identify gene sets influenced by genistein in normal and tumor tissues.
In general, microarray data published so far supports existing hypotheses on the mechanisms of action of genistein, both beneficial for human health (i.e. reduction of cancer risk, amelioration of postmenopausal syndrome and decrease of bone resorption in postmenopausal women, lipid metabolism, cardiovascular homeostasis) (Kim et al., 2005; Lee et al., 2007; Pie et al, 2006; Rice et al., 2007) and unfavorable (as estrogen-dependent cancer cell proliferation and adverse effects on the reproductive organs or involution of thymus and auto-immune disorders) (Lee et al., 2007; Naciff et al., 2002; Selvaraj et al., 2005). Those homeostasis-maintaining or homeostasis-affecting effects are often observed at low concentrations of genistein, essentially not exceeding 5 μM.
Functional categorization of genes affected by genistein on transcriptional level repeatedly indicates genes involved in cell growth, cell cycle, apoptosis, cell signals transduction, angiogenesis, tumor cell invasion and metastasis, cholesterol synthesis and lipid metabolism (Kim et al. 2005; Li et al., 2004; Li & Sarkar, 2002; Niculescu et al., 2007; Pie et al., 2006; Rice et al., 2007; Selvaraj et al., 2005). Among the genes regulated by genistein is a group of genes with common mechanism of regulation by nuclear receptors: estrogen receptors (Selvaraj et al., 2005) and androgen receptor (Rice et al., 2007).

The papers comparing the expression profiles of cells treated with estradiol and genistein show a considerable overlap in the genes not only in reproductive organs, but also in other tissues influenced by estrogens. Among non-reproductive organs in which strong changes of expression profile occurred in estrogen receptor-dependent manner after treatment with genistein are bones (Pie et al., 2006), liver and adipose tissue (Kim et al., 2005), thymus (Selvaraj et al., 2005), lymphocytes (Niculescu et al., 2007), brain (Lyou et al, 2002), endothelial cells (Rimbach et al., 2008), cardiovascular system and muscles (Velders et al., 2010). It has to be noted that the expression profile influenced by high genistein uptake may not depend on genistein directly, but on the ability of individuals to metabolize genistein to equol (Niculescu et al., 2007).

The findings that dietary isoflavones may play a beneficial role in lipid and carbohydrate metabolism prompted the group of Kim et al. (2005) to perform cDNA microarray-based analysis in mice fed with high-fat diet (HFD) and supplemented with genistein. Mice fed the high-fat diet had abnormal lipid profiles, significantly greater body weight, and visceral fat accumulation and all these effects had been significantly reduced by genistein supplementation. Of much importance was also the observation that the expression of 84 genes affected by the high fat diet was normalized by genistein. The expression of genes encoding enzymes of cholesterol biosynthesis, which decreased by at least 50% in the high fat diet fed mice, returned to normal level as a result of genistein supplementation.

Pie et al. (2006) in their cDNA microarray study shown changes in the expression levels of bone metabolism-related genes, including those encoding calciotropic receptor, cytokines, growth factors and bone matrix proteins by genistein in ovariectomized mice. The study demonstrated that genistein prevented bone loss caused by estrogen deficiency without substantially affecting the uterus.

Many studies have correlated the soy-rich diet with a decreased risk of developing hormone-dependent cancers, including breast and prostate cancer. The result of a study carried on HCC1395 cells line derived from an early-stage primary breast cancer showed that genistein dose-dependently decreased cell viability and inhibited the invasion potential (Lee et al., 2007). The gene expression profile revealed upregulation of some genes which inhibit invasion and metastasis and downregulation of genes promoting these processes, indicating that genistein-induced alternations of gene expression involving metastasis may be exploited for setting up chemopreventive and therapeutic strategies, particularly for early-stage breast cancer.

The oligo-microarray study by Lavigne et al. (2008) performed on MCF-F breast cancer cells shown that at physiologic concentration (1 or 5 μM) genistein elicited an expression pattern suggestive of increased mitogenic activity, and at pharmacological level (25 μM) it induced a pattern that likely contributes to increased apoptosis, decreased proliferation and decreased total cell number. These results strength former observations of biphasic response of certain cell lines to genistein.

Genistein is also a candidate prostate cancer preventive phytochemical. DNA microarray approach to examine the effects of genistein at concentrations within its physiologic range on global gene expression patterns in androgen-responsive cancer cells shown a concentration-dependent modulation of multiple cellular pathways that are important in prostate carcinogenesis. Interestingly, the androgen receptor (AR)-mediated pathways, in particular, appeared to be modulated by genistein at low concentrations. The regulation of AR-mediated pathways is potentially the most relevant chemopreventive mechanism for genistein administered at physiologic levels (Rice et al., 2007; Takahashi et al., 2004).

Selvaraj et al. (2005) gained insight into signaling pathways that regulate various stages of thymocyte maturation, dependent on estradiol and genistein, and found that the effects of both compounds were similar, although, genistein down regulated more genes than estradiol. Genistein was also shown to induce genes involved in apoptosis, which is a continuous process in the thymus undergoing active thymocyte selection, but at the same time it affected genes which may facilitate thymic release of autoimmune cells.

Microarrays were also used to get closer insight into the influence of genistein on female reproductive organs. The study performed by Naciff et al. (2002) revealed that prenatal exposure to genistein altered the fetal gene-expression pattern of the rat uterus and ovaries in a similar manner as exposition to the estrogenic compounds, such as 17-ethynylestradiol and bisphenol A. Toxicogenomic analysis with use of cDNA microarray applied to determination of testicular mRNA profiles was proposed as a useful tool for evaluation of delayed long-term effects after fetal or neonatal exposure of mice to genistein (Adachi et al., 2004). Although no morphological changes in the testes of genistein-treated mouse were observed, the authors indicated the gene (GeneBank accession No. W49392), which might be useful biological marker, in addition to ERα and AR, for predicting the effects of neonatal exposure to genistein or related compounds.

The long lasting effects of genistein may result from epigenetic influence of genistein on gene expression. The use of differential methylation hybridization (DMH) arrays for screening of the changes in the methylation status of the cytosine guanine dinucleotide (CpG) islands in the mouse genome shown important genistein–induced alterations (Day et al., 2002). The study shown that changes in the methylation pattern reflected potential of genistein for preventing the development of certain prostate and mammary cancers by maintaining a protective DNA methylation profile (Day et al., 2002). The ability of genistein to change the methylation status was observed in several other studies. Genistein was shown to partially demethylate the promoter of the GSTP1 tumor suppressor gene in MDA-MB-468 breast cancer line (King-Batoon et al., 2008). Another study shown the change of methylation status in mice prenatally exposed to genistein (Vanhees et al., 2011). Genistein exposure was associated with hypermethylation of certain repetitive elements, what coincided with a significant down-regulation of estrogen-responsive genes and genes involved in hematopoiesis in bone marrow cells of genistein-exposed mice.

In contrast to studies aiming to establish the role of genistein in homeostasis, the experiments designed for identification of gene expression profiles associated with a therapy, are often carried with suprapharmacological concentrations of a drug. In several studies of this kind, the most of affected genes were not dependent on hormonal regulation (Farivar et al., 2003; Lavigne et al., 2008; Li & Sarkar, 2002). The genes repeatedly found to be down regulated by genistein, were those involved mainly in signal transduction, oncogenesis, cell proliferation, protein phosphorylation and transcription. On the other hand, genistein up-regulated genes were related mainly to signal transduction, protein dephosphorylation, heat shock response, inactivation of mitogen-activated protein kinase (MAPK), apoptosis and cell cycle arrest. Among the genes regulated by genistein there was a number of genes regulated by tyrosine kinases inhibitors, like Gleevec (STI-571), lavendustin and herbimycin (Farivar et al., 2003). Studies on the evaluation of the global transcript profile changes performed on different models could be a valuable approach to determine the similarity in the mode of action of genistein derivatives, comparing to a parent compound. This approach may be useful for description of potential "druggable" targets and determination the safety profile of new compounds.

3. Synthetic derivatives of genistein and their potential medicinal applications

Many opportunities for derivatization of genistein, apparent from its structural formula encourage the synthesis of derivatives with improved pharmacological profile. The chemical basis describing functionalization of three phenolic groups at C-5, C-7 and C-4′ in typical *O*-acylation or *O*-alkylation reactions and skeletal modifications of genistein core, involving C–C bond formation is reviewed elsewhere (Rusin et al., 2010). Multiple examples of promising candidates intended to be used in therapy of different diseases are listed below. Among the diseases potentially treatable with the genistein derivatives are cancer, osteoporosis and metabolic disorders. Other suggested applications of genistein derivatives cover antibacterial and antiparasitic treatment. It must be noted, however, that most of presented derivatives have the status of experimental or investigational drugs.

3.1 Genistein derivatives for treatment of osteoporosis

Hormonal replacement therapy with synthetic estrogens was initially used for prevention of osteoporosis, however serious side effects of hormone replacement therapy stimulated the search for therapeutics, alternative to estrogens. Currently, among agents used in pharmacological prevention of osteoporosis are selective estrogen receptor modulators (SERMs), which function mainly as the antiresorptive agents (Reginster, 2011). Genistein, as a potential antiosteoporotic dietary supplement draw attention for many years, mostly on the basis of epidemiological observations (Knight & Eden, 1996; Messina, 1995). Moreover, genistein was reckoned as a safe supplement, because it did not produce the harmful, estrogen-like effects in the uterus. However, the overall conclusions of genistein supplementation and retardation of bone loss are ambiguous. The randomized, double-blind, and placebo-controlled study by Marini et al. (2007) in osteopenic postmenopausal women revealed decreased bone resorption and increased bone formation in the genistein group. However, the results of this study permit only the conclusion that genistein may prevent progress from the mild disease - osteopenia, to its severe form – osteoporosis. Thus, genistein can be effective rather in chemoprevention than treatment of this disease.

For a long period of time the synthetic isoflavone, ipriflavone (7-isopropoxyisoflavone) was an attractive candidate for a bone-building agent, due to its anabolic, but not estrogenic activity (Gennari, 1997). Ipriflavone inhibits bone resorption mediated by osteoclasts and stimulates activity of osteoblast in cell cultures and in experimental models of osteoporosis *in vivo*. However, clinical studies shown no statistically significant difference in annual percentage change from baseline lumbar spine and bone mineral density between those given ipriflavone and those given calcium (Alexandersen at al., 2001).

In the matter of fact, there is no universal drug for osteoporosis and new treatments, comprising of both new drugs and new drug combinations are still under clinical investigations. Side effects of currently used drugs, which are their obvious drawbacks, accounts for directing attention to phytoestrogens such as isoflavones and their derivatives as more reliable drugs.

Genistein modification aiming enhancement of its antiosteoporetic properties is presented by Wang *et al.* (2005), who synthesized a number of genistein derivatives in which the C-7 or C-4′ hydroxyl groups were variously substituted. Among seventeen novel genistein derivatives the authors found five, which shown increased antiosteoporetic activity when compared to genistein, no acute toxicity and no stimulation of endometrium proliferation in

mouse model of osteoporosis. The derivatives inhibiting bone loss during estrogen shortage contained the 2-hydroxyetylthio motif, which the authors assumed to be a key pharmacophore. The best results were observed for 4',5,7-tri[3-(2-hydroxyetylthio)propoxy] isoflavone.

Useful information on the structural features of genistein derivatives related to antiosteopotic effects may be drown from the work of Zhang et al. (2008b). They discovered two natural derivatives of genistein in the stem bark of *Erythrina variegata* L.: 8-prenylgenistein and 6,8-diprenylgenistein stimulating osteoblastic proliferation, differentiation and mineralization in UMR 106 cells. These derivatives caused significant increase of alkaline phosphatase in cells treated by either 8-prenylgenistein or 6,8-diprenylgenistein for 48 h at the concentration of 10^{-10} M. A structure-activity relationship study indicates that prenylation at of genistein at C-8, but not at C-6, may increase its bone-protective effect.

Wang et al. (2007) presented the strategy, combining nitric oxide and genistein cooperation in inhibiting of a bone loss. The NO donor drugs effectively counteract bone mass loss occurring due to reduced rate of estrogen biosynthesis in postmenopausal women (Wimalawansa, 2000). What is particularly important, the NO donors not only slow down the rate of bone resorption, but also stimulate proliferation of osteoclasts (Hukkanen et al., 2003). In order to find a bifunctional derivative of genistein having both estrogenic properties and being an effective nitric oxide donor Wang et al. (2007) synthesized genistein 7,4'-(nitroxy) butyrate. Its NO-releasing capacity was studied *in vitro* using immature osteoblastic cell line MC3T3-E1 cells. It has been demonstrated that NO is released from the derivative less rapidly and for a longer time than from glyceryl trinitrate (GTN), the classical NO donor, routinely used in medical treatment (Wang et al., 2007). Using MTT assay and flow cytometry it was determined that the compound stimulated growth of MC3T3-E1 cells in a dose- and time-dependent manner, albeit the stimulation was weaker than that observed for an optimal concentration of estradiol. The measurements of the activity of a bone-specific isoform of alkaline phosphatase and the expression of osteocalcin, a specific marker for late osteoblast differentiation, as well as the rate of formation of calcific deposition revealed that the derivative stimulated osteoclast differentiation and mineralization more effectively than genistein, glyceryl trinitrate or combination of the two.

3.2 Genistein derivatives and hypertension

Genistein has been suggested to be protective in cardiovascular diseases. The study *in vivo* on spontaneously hypertensive rats had shown, that genistein reduced systolic blood pressure and enhanced endothelium-dependent aortic relaxation (Vera et al., 2007). Genistein reduced endothelial dysfunction due to increased endothelial nitric oxide synthase (eNOS) activity, associated with increased calmodulin-1 expression and decreased superoxide generation. Nitric oxide (NO) produced by eNOS is a well-known regulatory molecule involved in the modulation of contractility of vasculature thus maintaining vascular homeostasis (Miller & Megson, 2007).

Matsumoto et al. (2005) synthesized bifunctional derivative of genistein, which was aimed to inhibit tyrosine kinases, implicated in development of a hypertension, and release nitrogen oxide to enhance the effect. The authors synthesized two novel genistein derivatives 7-[(4-nitroxy)butyroyl]-genistein and 7-[(4nitrooxymethyl)-(alfa-methyl)phenylpropanyl]-genistein, and assessed their ability to relax rat endothelium-

denuded aortic strips. Both derivatives and genistein itself induced aortic relaxation in the following order: 7-[(4nitrooxymethyl)-(alfa-methyl)phenylpropanoyl]-genistein > 7-[(4-nitroxy)butyroyl]-genistein > genistein. The relaxation induced by the tested genistein derivatives was abolished by a guanylyl cyclase inhibitor, which proved, that genistein derivatives indeed acted as NO donors.

3.3 Genistein and its derivatives for treatment of cystic fibrosis

Genistein was shown to partially activate the defective chloride channels (cystic fibrosis transmembrane regulator, CFTR) associated with cystic fibrosis (CF). Genistein not only partially restored the CFTR activity but also augmented CFTR maturation and increased its localization at the cell surface (reviewed by Węgrzyn et al., 2010). Pre-clinical studies with genistein have provided a basis for clinical trials with CF patients, and a Phase II clinical study is currently underway. So far, genistein derivatives for potential treatment of cystic fibrosis have not been studied extensively. Galietta et al. (2001) generated a combinatorial compound library based on two lead compounds, flavones and benzo[*c*]quinoliziniums, which activate CFTR Cl^{2-} conductance by direct interaction with the CFTR molecule. Among several novel derivatives they identified compounds with high potency to activate CFTR, 7,8-benzoflavones, containing features of both flavones and benzo[*c*]-quinoliziniums.

3.4 Derivatives with antimicrobial and antiparasitic activity

Antimicrobial properties of genistein are described in many papers, although the exact mechanism of this activity remains largely unknown (Hong et al., 2006; Ulanowska et al., 2006; Verdrengh et al., 2004). Cell survival studies suggest that genistein is a bacteriostatic, rather than a bactericidal agent (Ulanowska et al., 2007). There is a suggestion that antibacterial properties of genistein may be mediated by the stabilization of the covalent topoisomerase II-DNA cleavage complex (Verdrengh et al., 2004). The concentration of genistein necessary for bacterial growth retardation depends on the strain, but is regarded as relatively high (100 μM).

Zhang et al. (2008a) reported the derivatization of genistein leading to an increased antibacterial and antifungal activity. They prepared three series of derivatives in which the genistein ring system was linked to the heterocyclic moieties with 2-carbon, 3-carbon or 4-carbon spacers. Among these compounds, five exhibited good antibacterial activities, while one of them also showed notable antifungal activity. The activity of the mentioned derivatives was several fold higher than that of genistein.

The antimicrobial activity of genistein derivatives was also described by Li et al. (2008). They synthesized and tested 14 new deoxybenzoin derivatives of genistein and found that dimeric forms were generally more active than genistein or deoxybenzoins against selected microorganisms.

Several genistein derivatives are indicated for treatment of parasitic diseases. The potential use of genistein derivatives for anti-protozoan therapies were reported by Gargala et al. (2005) and Stachulsky et al. (2006), and, for anti-helminthic treatment by Naguleswaran et al. (2006). Gargala et al. (2005) screened fifty-two dihydroxyisoflavone and trihydroxydeoxybenzoin derivatives for their influence on protozoan parasites life cycle: *Neospora caninum, Sarcocystis neurona and Cryptosporidium parvum*. Two agents selected in this screening: 3′-bromo and 4′-bromo genistein were tested as in *Cryptosporidium parvum*-

infected immunosuppressed gerbils. It was found, that these compounds more effectively abolished fecal microscopic oocyst shedding than two routinely used drugs, nitazoxanide and paromomycin.
Some of genistein derivatives described previously by Gargala (2005) were tested for inhibitory effects on the larval development of tapeworms *Echinococcus sp.* (Naguleswaran et al., 2006). The study shown, that 2'-bromo- and 6'-bromo genistein induced considerable damage in *E. granulosus* protoscolex. The above mentioned genistein derivatives are safe in terms of side effects caused by the estrogen receptor stimulation, because they do not bind to ER. These derivatives were shown to be selective for parasites, without antibacterial activity (Stachulsky et al., 2006), what allows to avoid the development of resistant bacterial strains.

3.5 Derivatives of genistein designed for anti-cancer therapy

Several strategies of genistein modification were applied in order to put its anticancer potential in use. Among them are targeted therapy and chemical modification of a molecule, so that to improve the interaction with molecular targets. Examples of those modifications are described below.

3.5.1 Targeting genistein to cancer cells by conjugation with antibodies or peptide ligands

Major strategy of targeted therapy is to construct two-domain drugs, in which one domain recognizes the target cells, whereas the other one exerts a therapeutic activity. In order to selectively target genistein to intra-cellular kinase domain of epidermal growth factor receptor (EGFR) Uckun et al. (1998) obtained a conjugate of genistein with epidermal growth factor (EGF) via photochemical cross-linking. This conjugate was intended to target cancer cells overexpressing the epidermal growth factor receptor (EGFR). It was expected, that internalization of the conjugate should increase the intracellular concentration of genistein, and lead to efficient inhibition of the EGFR tyrosine kinase activity. In vitro studies confirmed strong proapoptotic activity of a conjugate in human breast cancer cells. (Uckun et al., 1998). Moreover SCID mice bearing tumors of human breast cancer cells (MDA-MB-231), treated intraperitoneally with the conjugate at 100 ug/day for 10 days showed significantly better survival as compared to mice treated with adriamycin, cyclophosphamide or methotrexate (Uckun et al., 1998).
A similar genistein targeting strategy, based on expected local increase of genistein concentration, was used for experimental treatment of leukemias (Ek et al., 1998). Genistein was conjugated to B43 antibody, recognizing the CD19 antigen, present on the surface of B lymphocytes and absent from plasma cells. CD19 is an adaptor protein for Lyn tyrosine kinase, amplifying signals transduction from nonreceptor Src tyrosine kinases. The study was performed on SCID mice bearing human acute lymphoblastic leukemia (ALL) or non-Hodkin's lymphoma shown the conjugate to be more therapeutically effective than cytostatics routinely used for treatment of this kind of leukemias (Ek et al., 1998). In subsequent *in vivo* study performed on cynomolgus monkeys the conjugate administered intravenously shown no toxicity during long term observation (Messinger et al., 1998). These highly encouraging results inclined the authors to perform phase I clinical study (Uckun et al. 1999). The pilot study of B43-genistein in 15 patients with refractory B-lineage acute lymphoblastic leukemia shown that the conjugate was well tolerated by all patients with no life-threatening side effects. There was one durable complete remission and two transient responses.

More recently, Gentile et al. (2003) used genistein-monoclonal antibody approach to treat SW-620 and HT-29 colon cancer cells. The conjugate of genistein and 17.1A monoclonal antibody recognizing an epithelial membrane antigen expressed in colon cancer significantly inhibited cell growth *in vitro* and *in vivo*, and induced apoptosis.

3.5.2 Genistein as a vector selectively targeting cytostatic drugs to ER positive cancers

Interesting therapeutic approach is the use of genistein as a carrier, delivering drugs to cells expressing target recognized by this isoflavonoid. However, some difficulties must be overcome: (1) cytotoxic agent in a conjugate must not produce a loss of genistein binding to the target protein, (2) the target of a cytotoxic agent should be present in the subcellular structure which is likely to be achievable by a carrier, (3) the concentration of the receptor should be high, and its expression should not be down-regulated by a carrier.

An example of the above mentioned strategy was the use of genistein derivative, 6-carboxymethylgenistein (6CG) as a potential vector of a cytostatic drug, daunomycin to estrogen receptor expressing cells (Somjen et al., 2002; Somjen et al, 2003). Although the relative binding affinity of 6-CG was 0.1% to ERb and 0.01% to ERa, respectively as compared to the estradiol, it was shown that 6-CG activated the receptor, which translocated to the nucleus. Transactivation studies proved that 6-CG is a ligand of ERs. In the absence of estradiol 6-CG was found to be an agonist, while in the presence of estradiol it shown moderate antagonist activity for ERa (Somjen et al., 2003). The cytotoxicity of this conjugate was tested against H295R cells. At low daunomycin concentration (0.3-3nM) the cytotoxicity of a conjugate was 10 times higher than that of free drug, what indicated successful targeting of a conjugate. At higher concentration (30nM) the differences were less profound, and no differences were observed between daunomycin and its conjugate with 6-CG, what indicates that the toxicity was a result of non-specific, high intracellular concentration of daunamycin. The targeting of daunomycin conjugated to 6-CG via ER was also confirmed by the experiment with cells devoid of ER, where no differences in toxicity of a conjugate and free daunomycin were observed (Somjen et al., 2003).

3.5.3 Genistein derivatives designed for treatment of hormone dependent cancers

Endocrine treatment with selective estrogen-receptor modulators (SERMs), such as tamoxifen and raloxifene is of major therapeutic value in patients with estrogen-receptor positive tumors. Hormone-dependent breast cancer tumors contain estrogen receptors and tumor growth depends on estrogens. Tamoxifen, a partial nonsteroidal estrogen agonist, is a competitive inhibitor of estradiol, and the prototype of SERMs. However, after long exposure to the tamoxifen the resistance often develops, so designing and synthesis of new antiestrogens for treatment of breast cancer is of much importance.

Very promising trisubstituted derivatives of genistein able to bind estrogen receptor with low affinity were recently described by Davis et al. (2008). Several compounds were able to inhibit cell proliferation in a dose-dependent manner, and compounds containing the bulky 7-phenylmethoxy substituent were toxic for both hormone-dependant MCF-7 cells and hormone-independent MDA-MB-231 cells. Thus, the synthetic tri-substituted isoflavones act on multiple signaling pathways leading to activation of mechanisms of cell-death and ultimately affecting survival of breast cancer cells. A novel genistein derivative exhibiting significantly higher antiproliferative activity than the parent drug was recently obtained by

Kohen et al., (2007) by attaching an *N*-tert-butoxycarbonylo-1,6-diamino-hexane group to C2 of genistein. Although this novel genistein derivative did not show estrogenic activity, its antiproliferative activity was different in estrogen-sensitive cancer cell lines expressing ERα and ERβ mRNA at different ratios. The highest antiproliferative effect measured by radioactive thymidine incorporation was observed for an estrogen-sensitive colon cancer cell line (320DM), and the lowest for an ovarian cancer cell line (A2780). Interestingly, the genistein derivative was more toxic for cells that preferentially expressed mRNA for ERβ, relative to ERα.

3.5.4 High-throughput screening of genistein derivatives

In recent years a search for anticancer cytostatics based on the synthesis of libraries of differently substituted derivatives of genistein, followed by high-throughput screening *in vitro* has been intensified. Although it looks, to some extent, like trusting in serendipity, it allows to empirically find the most promising drug candidates.

Such screening of more than 350 genistein analogues allowed to find several putative drugs for clinical development (Novogen). Among them was isoflaven phenoxodiol, which has been granted fast track status from the FDA to facilitate its development as a therapy for recurrent ovarian and prostate cancers (Silasi et al., 2009). Other compounds: triphendiol, NV-143 and NV-128, which are further derivatives of phenoxodiol, are still investigational drugs.

Phenoxodiol was intended for usage in a therapy of early-stage prostate cancer, late-stage, hormone-refractory prostate cancer, early stage cervical and vaginal cancer, chemo-resistant and chemo-refractory ovarian cancer. It was shown to induce death of cancer cells through plejotropic mechanism: inhibition of the anti-apoptotic proteins FLIP and IAP and the increase of the pro-apoptotic protein BAX, inhibition of tyrosine kinases and topoisomerase II in a dose-dependent manner (Alvero et al., 2006; Kluger et al., 2007; Constantinou et al., 2002, Sapi et al., 2004]. Phenoxodiol was tested in phase 3 OVATURE trial in women with recurrent ovarian cancer. No statistically significant improvement in its primary (progression-free survival) or secondary (overall survival) endpoints were found, so the study was terminated (Howes et al., 2011).

Triphendiol is tested for use against pancreas and bile duct cancers and found to act synergistically with gemcitabine (Saif et al,. 2009). NV-143 was found to be effective against multiple melanoma cell lines, while NV-128, demonstrated efficacy in monotherapy and as a chemosensitizer in non-small cell lung carcinoma (NSCLC) cell lines. Interestingly, NV-128 induced cell death *via* a caspase-independent pathway and autophagic cell death (Alvero et al., 2009). In summary, triphendiol, NV-143 and NV-128 exhibited a good safety profile, they were well tolerated and non-clastogenic. Effective inhibition of tumor proliferation was shown in animal models after oral administration of the tested drugs.

3.5.5 Synthetic glycoconjugates of genistein interacting with mitotic spindle

The effectiveness of microtubule-targeting drugs for the treatment of a broad range of human cancers has been shown in many clinical studies. The search for compounds with similar mechanisms of action to taxanes or vinca alkaloids resulted in the discovery of a number of novel microtubule-targeting drugs, the majority of which are natural products.

Although, genistein was recently shown to interact with interphase microtubules (Mukherjee et al., 2010), its clinical use as an antimitotic drug is rather doubtful due to very high concentration necessary to achieve this effect. However, it seems that chemical

derivatization can help to obtain novel compounds exhibiting increased affinity to microtubules. We found, among the glycosidic derivatives described by Polkowski et al. (2004), that the compound named G21 (Fig. 6), which inhibited cell proliferation at the concentration 10 times lower than genistein, interacted with mitotic spindle (Rusin et al., 2009).

This compound caused remarkable mitotic block at the concentration inhibiting cell proliferation by half, compared to the control. Our observations were recently confirmed by others (Ahmed et al., 2011). Earlier studies shown that the structure of G21 molecule was stable, it did not hydrolyze under *in vitro* cell culture conditions (Ksycińska et al., 2004) and its toxicity against cancer cells was higher than against normal ones (Popiołkiewicz et al., 2005). The continuation of our work on glyconjugates of genistein exhibiting antimitotic activity pointed another molecule, called Ram3 (Fig. 6), as a potent agent affecting mitotic spindle (Rusin et al., 2011) and leading to apoptotic cell death.

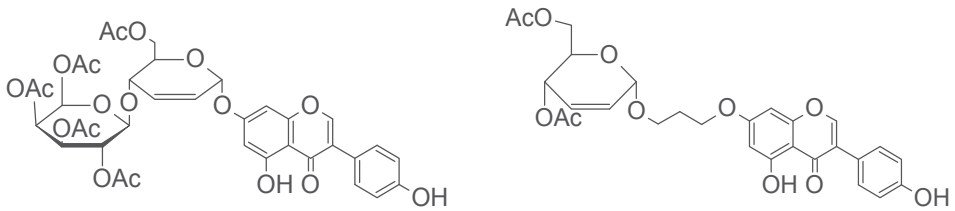

Fig. 6. Structure of genistein glycoconjugates showing antimitotic activity. A. G21, B. Ram3.

4. Conclusion

Many studies concerning the biological properties of genistein analogues demonstrated that the modification of the parent isoflavone may lead to compounds exhibiting not only enhancement of the activities already known, but also revealed novel properties, not observed for the parent compound. The biological activity of the synthetic derivatives of genistein, briefly summarized in this review, indicate that, at least some of them can be viewed as important lead compounds for further modifications. Although most of the derivatives have the status of investigational or experimental drugs, multiple identified molecular targets of genistein identified so far give hope that clinically applicable and target-specific genistein derivatives may appear in the future.

There are obviously open questions, which diseases such genistein derivatives would be addressed for and whether they would be therapeutically useful in monotherapy or - what seems more probable - in combination therapies.

5. Acknowledgment

We would like to acknowledge our colleagues for working together on the glycoconjugates of genistein with antimitotic properties, which is our small contribution into field of genistein derivatives for medicinal applications: Professor Grzegorz Grynkiewicz and Professor Wiesław Szeja for all inspiring discussions and designing the glycoconjugates of genistein, Ms Jadwiga Zawisza for chemical syntheses and all colleagues who carried cytotoxicity assays, dr Magdalena Glowala-Kosinska, Dr Agnieszka Gogler, Ms Aleksandra Gruca and Ms Katarzyna Kujawa.

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Soybean Oil: Production Process, Benefits and Uses in Pharmaceutical Dosage Form

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1. Introduction

Vegetable oils are the most important source of fat in the human diet. Derived from an array of vegetable sources, oils are extracted and processed for a variety of food uses. In the demanding and competitive edible fats and oils marketplace, the product evaluation criteria are important for both customers and suppliers.

Soybean oil is very popular with rich value of Omega 3 and Omega 6. Those fatty acids regulate lipid and cholesterol metabolism and prevent narrowing in artery veins. In addition its high content of vitamin B makes digestion easier and by this feature it prevents chronicle digestion problem and constipation. For those reasons, refined soybean oil is widely used all over the world.

Production of high quality meal and crude oil is the main objective in soybean processing. For achievement, thorough knowledge of the technical system and disciplined operation are required.

The starting point is reception of soybeans at the processing plant. And, the endpoint is meal delivered to the storage silos and oil delivered to the storage tanks.

First of all accurate weight, representative sample, accurate analyses are important criteria for receiving. Receipts, which need drying and cleaning before transfer to storage can be placed in interim storage. Also 'Tramp Iron Removal' part is important for magnets which should be strategically placed in the product stream to protect equipment from damage. Moreover removal of excessive foreign material should be accomplished before transferring soybeans to long-term or primary storage. Than drying equipments recognize the average moisture of the population.

Next primary or long-term storage facilities should include properly placed sensors to monitor the soybean temperature (Smallwood, 2001).

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Soybean dehulling methods differ primarily in the technique used to attain targeted moisture range. There are three types of dehulling methods; "In Conventional Dehulling" soybean tempering followed by cracking, screening and aspiration. "Hot Dehulling", which involves the use of fluidized bed to achieve the desired moisture equilibrium before cracking. "Esher Wyss Dehulling System", in which the soybeans are first cracked into two pieces prior to the use of a fluidized bed to achieve uniform target moisture content.

The purpose of soybean cracking is to provide pieces of the right size to make the best flakes for oil extraction and to remove the hulls. Following cracking and removal of hulls, screening and aspiration is used to separate the hulls from the meats.

Conditioning is used to soften the meats to optimize flaking. In flaking to expose the cell structure sufficiently for penetration of solvent and removal of oil in extraction process, the conditioned meats are fed through a single pair of smooth-surface rolls. With proper soybean preparation, the solvent drains adequately from the flakes at the conclusion of the extraction. In desolventizing step, the purpose is to remove the residual solvent in the wet flakes from the extractor.

In regard to meal quality, the most critical unit operation in soybean processing is toasting. Both urease and trypsin inhibitor which soybean contains can be adequately deactivated by subjecting the meal to the proper combination of temperature and time.

Following toasting, drying step is used to remove the moisture of meal. Forced air cooling is the preferred method for cooling the meal immediately after drying.

First screening, grinding and screening are the typical unit operations in meal finishing. The meal specifications established by American Feed Manufacturers Association (AFMA) are controlled by screening.

The last step for meal is storage of last product. Temperature, time, moisture, infestation, contamination are critical elements of meal storage quality.

If the oil is exposed to air at high temperature, severe oxidative degradation will occur. Thus, a heat exchanger must be included to cool the crude oil to 65 °C before exposing the oil to air in storage tanks. In addition each crude oil storage tank, the filling line should be extended to near the bottom of the tank to minimize oil aeration and oxidation.

Following step is refinery process of crude soybean oil. The operational steps to convert crude soybean oil to usable finished edible fat and oil products are given below. The starting point is reception of crude soybean oil at the processing plant. And the endpoint is deodorized oil loading to the transfer/transportation tank.

Processing sequence begins by the proper receipt and unloading of crude oil. Transporting container must be free of internal contaminates and protect the crude oil from external contamination. Before storage the crude soybean oil has to be sampled and analyzed.

After receiving, crude soybean oil transferred to the storage tanks. Those tanks must be constructed to provide proper isolation from other oils as contaminants.

Degumming is the first processing step for soybean oil which has high amount of hydratable phosphatides. This step provides producing the lecithin and reducing the phosphatide removal load in neutralization. Following degumming, neutralization (caustic refining) are essential part of processing for removing of fatty acids, phosphatides and other materials including protein meal, glycerol, carbohydrates, resins and metals.

The following step of the process, bleaching is used to reduce color pigments and remove oxidation products and residual soap left neutralizing.

To remove sufficient saturated triglycerides or wax from oil, winterization step is used. The process consist crystallization and filtration.

Last step of processing is deodorization. In deodorization process, removal of volatile components including free fatty acids, glycerol, oxidation products, sterol, herbicides and pesticides is done under high vacuum system.

Nitrogen blanketing of the head space above the oil level if possible, agitation to preclude any separation of the triglyceride components, temperature controlling capability, sampling capability are important properties for deodorized oil storing. And tank washing/cleaning at the proper frequency to preclude the accumulation of old oil films on the interior tank surfaces which would eventually reflux into the fresh oil.

During loading, tanks and oil transfer system cleanliness and washing capability are important parameters; also tanks must be conducted before oil loading is initiated.

As a result, for high quality refined soybean oil, the steps that we mentioned above are very necessary if they are performed carefully.

Refinery processing effects the components of oil. Means; fatty acid composition, free fatty acid value, peroxide value, soap value e.g. are depend on criteria of refining process. During refining, under some circumstances fishy odor, trans fatty acid formation and many contaminations may occur. To prevent those issues, refining process has to be performed professionally and attentively (Farhoosh, et al., 2009).

Most studies on the relationship of diet to heart disease have focused on the theory that high levels of serum lipids are related to intake of dietary fats and oils and the reduction in the quality or change in the kind of dietary fat will lead to a lowering of lipid levels in the blood which in turn may help to stop or to slow the progress of these disease.

Soybean oil is classified as polyunsaturated oil which includes about 15 % saturates, 24 % monounsaturates and 61 % polyunsaturates of which 53.2 % is linoleic acid while the linolenic acid content is about 7.8 %. The nutritional advantages of this composition and it's effects in regulating the plasma lipid and eicosanoids bio - synthesis are reviewed on the basis of results from several human clinical trials and studies. These studies have shown that soybean oil effective in lowering the serum cholesterol and LDL levels, and likely can be used as potential hypocholesterolemic agent if used as a dietary fat and ultimately help prevent atherosclerosis and heart diseases (Kummerow, et al., 2007).

Attention also drawn to the ability of soybean oil to regulate the eicosanoids balance in such a manner that reduce the atherosclerotic and thrombotic tendencies which are the main causes of heart attacks and strokes. These positive findings on the effect of soybean oil on the cardiovascular system are attributed to its unique fatty acid composition. Soybean oil provides the optimum linoleic to linolenic acid ratio which is the key to achieve the desired balance between the various categories of eicosanoids that help in prevention of heart diseases.

Soybean oil is a rich source of vitamin E. Vitamin E is essential to protect the body fat from oxidation and to scavenge the free radicals and therefore helps to prevent their potential effect upon chronic diseases such as coronary heart diseases and cancer (Lu and Liu, 2002).

In regard to human nutrition, the U.S. Food and Nutrition Board , recommends an intake of 8 - 9 mg of alpha - tocopherol equivalents/day for adult . Considering an alpha - tocopherol level of 11 mg/100 g in a typical salad and cooking oil made from soybean oil, a 1 - table - spoon (14 g) serving of such a food would contain 1.5 mg of alpha - tocopherol. Thus a single serving of this product would provide about 15-20 % of an adult's recommended daily intake as established by the U.S. Food and Nutrition Board.

Soybean oil is a rich source of essential fatty acids, both linoleic and linolenic acid. These polyunsaturated fatty acids are important key to prevent cardiovascular diseases by

lowering serum cholesterol through reducing lipoprotein (LDL) synthesis and increasing lipoprotein breakdown , as well as by the effect of linolenic acid. Linolenic acid reduces plaque formation and thrombosis by decreasing platelet aggregation, promoting prostaglandin E3 synthesis.

Attention must be drawn to possible adverse effects arising from over consumption of polyunsaturates. Polyunsaturated fatty acids are especially prone to oxidative rancidity and the products of oxidation may contribute to cardiovascular risk. To unfold their health benefits, they must be protected from oxidation by using antioxidants both in the diet and in supplements. For this purpose, the most commonly used naturally occurring antioxidant is vitamin E. According to some researchers the intake of vitamin E should be increased in line with increased intakes of polyunsaturated acids. They have estimated that the more suitable daily ratio will be 0.6 mg alpha - tocopherol /gram polyunsaturated fatty acids.

Drugs must be properly formulated for administration to patients. Especially, lipophilic or low solubility drugs are a major problem in pharmaceutical formulation design. Emulsions are attractive candidates for improving drug solubility. They are well accepted for their ability to incorporate lipophilic drugs, to reduce side effects of various potent drugs, to increase the bioavailability of drugs, and to prolong the pharmacological effects in comparison to conventional formulations. Emulsions are mixtures consisting of one (or more) immiscible liquid phase(s) dispersed in another. Oil-in-water (O/W) or water-in-oil (W/O) emulsions have been widely used in pharmaceutical formulations. They are also used as carriers for the delivery of water insoluble drugs (Duenas-Laita et al., 2009; Han et al., 2010; Ince et al., 2007; Karasulu et al., 2004, 2007; Zhao et al. 2010). Recently, oil-in-water (O/W) submicron-sized emulsions are commonly used for parenteral administration of lipophilic drugs which are a major problem in pharmaceutical formulation (Prabhakar et al., 2011). In addition, emulsions have the potential to achieve sustained drug release, and for site-specific drug delivery by binding ligands for various cell surface receptors to the particle surface (Nishikawa&Hashida 1999). The oils typically used for pharmaceutical emulsions consist of digestible oils from natural sources such as soybean oil. Soybean oil is very popular because it is cheap, healthful and has a high smoke point. Soybean oil does not contain much saturated fat. Like all other oils from vegetable origin, soybean oil contains no cholesterol. Saturated fat and cholesterol cause heart diseases and mainly found in products from animal origin such as milk, cheese and meat products. Soybean oil contains natural antioxidants which remain in the oil even after extraction. These antioxidants help to prevent the oxidative rancidity. Therefore, soybean oil is not only used in food products but is also used as pharmaceutical excipients to produce a novel, biocompatible formulations which are cost effective, non-irritating, and capable of being sterilized before application (Rowe et al. 2006).

2. Nutritional benefits and usage of soybean oil

The dietary fat consumption for about 70% of the population averages 62,5% of the recommended amount for good health. Soybean seed (Fig. 1.) oil is the one of the most preferred oil for an healthy life. The reasons that people's choice are quality, functionality, being precursors of Omega-3, Omega-6 and Vitamin E, having a low price of soybean oil (Mounts et. al., 1986).

Beyond that, the Chinese have included soy protein in their diets for thousands of years. In other parts of the world, dietary protein has come largely from meat. On the basis of economics, it is much less expensive to produce vegetable protein than animal protein. From

the perspective of ecology, production of vegetable protein is much more favorable than animal sources.

Fig. 1. Soybean seeds

Proper intake of essential fatty acids derived from polyunsatureted fats and oils are critical for human growth and health. About 61% of the fatty acid chains of the soybean triglyceride molecules are polyunsaturated. Soybean oil and protein are vital elements in the world food supply (Fig. 2 and 3).

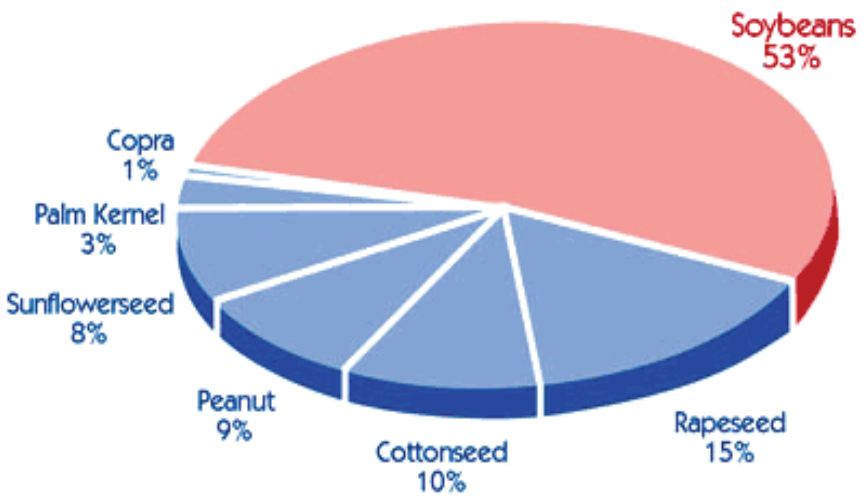

Fig. 2. World Oilseed Production of 2009

Essential fatty acids are required for the human body to produce prostoglandins. Prostoglandins are long-chain fatty acid derivatives synthesized by most cells in the body and affect many of the vital physiological functions. Essential fatty acids are polyunsaturated fatty acids.

Vitamin E (tocopherol) is important for the human body to sustain cardiovascular health. For older males, vitamin E serves as an effective deterrent to prostate cancer. Soybean oil contains more Vitamin E than any other commonly consumed vegatable oil. Tocopherol is a naturel anti-oxidant which serves to retard soybean oil oxidative degredation.

Soybean production, about 24 million metric tons of oil are extracted and largely utilized for human food each year (Fig. 3.). With a fatty acid composition of 61% polyunsaturated and only 15.5% saturated, soybean oil can be utilized in a broad array of products.

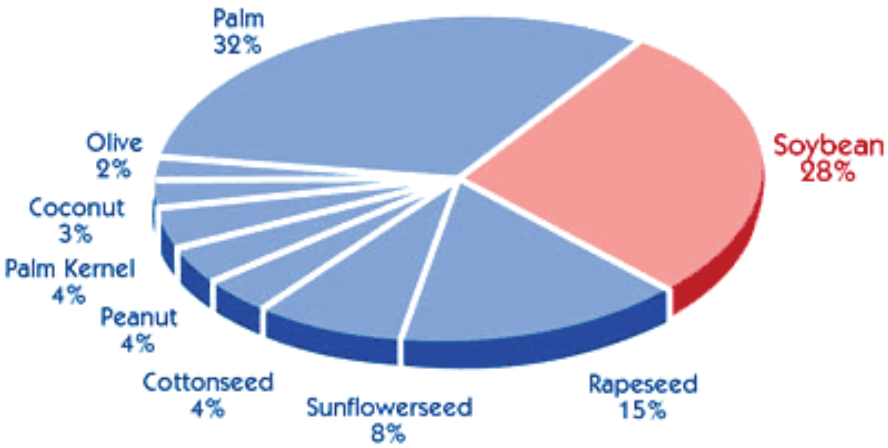

Fig. 3. World Vegetable Oil Consumption of 2009

The neutralized-bleached-deodorized soybean oil is usually used as salad oil, cooking oil, baking fats, confectionary fats, ingredient for margarine and mayonnaise, heavy-duty frying oil if blended with a high stability oil like cottonseed or palm oil. The lightly, lightly too moderately, moderately, moderately too highly and highly-hydrogenated oil is used as frying oil, ingredient for margarine, shortening, confectionery fat and stabilizer applications.

To sum up; soybean oil is rich in polyunsaturated fats, regulates the body temperature, transport fat-soluble vitamins throughout body, rich source of Omega-3 fatty acids and good source of antioxidant Vitamin E.

2.1 Edible oil processing operations

2.1.1 Cold pressed oil

Full pressing is still used on a variety of raw materials for oil extraction particularly when the residual oil left in the meal after pressing is not critical. The very high pressure required to extract the cold pressed oil produces high temperatures which may produce a poorer quality oil and meal (Erickson, 1983).

Today although full pressing is still being used, many full press machines have been converted to pre-pressing or have been replaced with new pre-presses. The modern pre-press machine will remove fifty to sixty percent of the oil contained in the seed, producing a cake that will have the remaining oil removed with solvent extraction. This method of used for high oil content seeds. On the other hand, full pressing is still useful if the high amount of oil is expected from seed.

The operational steps to convert crude vegetable oil to usable finished edible fat and oil products are given in the typical sequence and simply explained in this section. Each sequential step has a specific purpose and is included on the basis of providing the most efficient and cost-effective method for achieving the product quality and yield objectives.

2.1.2 Processes of cold pressing

The whole or de-hulled soybean at field moisture content is fed continuously to the dry extruder. The extruder is set up in appropriate configuration to achieve the desired degree of cooking. Within the extruder barrel, the material is subjected to friction, shear and pressure whereby heat is generated through viscous dissipation of mechanical energy. No external heat source such as steam is required for the process when using dry (autogenous) extruders. The temperature during cold pressing should not exceed 50 °C (Moreau & Kamal-Eldin, 2009).The temperature profile within the extruder barrel can be varied depending upon the intented use of the processed protein meal.

Fig. 4. Cold Pressed Oil Production

Typically, the top temperature at the exit of the extruder barrel is about 150 °C. Lower temperature profiles are used when the meal is intended for use as a functional ingredient in food application. Higher temperature profiles are used when the meal is used directly in feed formulation for various animal species. The average residence time of the material within the extruder is under 30 seconds. The frictional forces within the extruder barrel and the sudden decompression of the material as it exits the extruder cause the rupture of the cell structure and release of oil from the sub-cellular sites.

Soybean exiting the extruder is immediately and continuously conveyed into a continuous horizontal screw press where the oil and meal are separated. The process of extrusion changes the physical characteristics of the soybean from solid particles to an oily meal. The bulk density and compressibility are drastically altered. Hence, a conventional screw press designed to press whole oil seeds will not handle the extruded soybean. The pressing worm and the barrel cage of the press must be re-configured to handle the extruded product. The process of extrusion results in significant increase in the throughput rate of the press over it is rated capacity for whole oil seeds. The oil and meal exiting the press are at elevated temperature. They are further processed or cooled to near room temperature before storage. Pressed soybean oil has high value of tocopherol which imparts oxidative stability to the oil (Fig. 4).

2.2 Why should soybean oil have been refined?

Most crude oils and fats consist for more than 95% of mixtures of triacylglycerols. The non-triacylglycerol fraction contains variable amounts of phosphatides, free fatty acids, oxidation products, unsaponifiable matter (tocopherols, sterols, hydorcarbons etc.) and other so-called impurities the quantities of these minor components in crude oils vary with the oil source, extraction process, season and geographical origins. They can have different effects on the nutritional, functional and organoleptic properties of the oil.

The general objective of refining is to remove those components that have an adverse effect on the overall oil quality with the least possible damage to the triaclyglycerols. At the same time, minor components with a known positive impact have to be retained as much as possible in the oil (Farhoosh, et al., 2009).

Some components like fatty acids, mono and diacylglycerols and phosphatides are the glyceride origin. Although phosphatides protect the crude oil from oxidation, they need to be removed because of their negative effect on the color, taste and oxidative stability of the oil.

Other minor components can be isolated in the unsaponifiable fraction. The main components of the unsaponifiable fraction (0,3-2%) include sterols, tocopherols, hydrocarbons, pigments and vitamins.

The potential possible impact of some components of the unsaponifiable fraction, on health receives a lot of attention. Tocopherols for example are the most important natural antioxidants of phenolic nature protecting the edible oils from oxidation (Lu & Liu, 2002). On the other side, tocopherols are also biologically active substances with a generally accepted vitamin activity in the human body. Phytosterols are desired because they may reduce blood cholesterol levels.

2.3 Processes of refining

2.3.1 Crude vegetable oil receiving and unloading

The processing sequence begins by the proper receipt and unloading of crude oil. The first significant product vulnerability occurs in the transportation means for the crude oil. The transporting container (tank car, tank truck, barge or vessel) must be free of internal contaminates and protect the crude oil from external contamination (Fig.5).

Upon receipt of the crude oil at the processing plant, the oil is sampled and analyzed before unloading to identify oil type and ascertain edibility. The unloading equipment (fittings, hoses, pumps, pipelines, etc.) must be protected to preclude contamination from various sources including rodents, insects and birds (Smallwood, 2001). Finally, if necessary to heat the crude oil to facilitate unloading, proper control must be exercised to avoid overheating.

2.3.2 Crude vegetable oil storage

Storage of crude oil prior to processing is required to build sufficient inventory for efficient processing runs. The kind of storage tanks used and the storage conditions are important considerations. Storage tanks must be constructed to provide proper isolation from other oils and external contaminations. Each storage tank should be equipped with effective agitation to provide uniform mixing of the crude oil components and homogeneous feed to processing. Storage time should be minimized to avoid and analyzed before the start of each processing run to establish proper processing conditions.

Fig. 5. Crude Vegetable Oil Silos

2.3.3 Degumming

Degumming is frequently the first processing step for crude oils like soybean which contain relatively high amount of hydratable phosphatides which are soluble in triglycerides (Karleskind&Wolff, 1996). Hydratable phosphatides can be removed from oil by adding the proper quantity of water, mixing thoroughly, allowing time for the hydration to occur, and separating by centrifugation. For this process, water is injected into the crude oil feed stream in proper proportion to the quantity of water-hydratable phosphatides. After sufficient hold time in a surge tank, the process stream is fed to centrifuges for separation of the hydrated phosphatides (gums) from the oil stream.

The crude oil feed stream is filtered to remove particulate matter if the subsequent gum recovery is for producing lecithin. Properly designed and operated water degumming process can achieve at least 90% of the ideal. Consequently, achieving the typical trading rule specification of 200 ppm phosphorous maximum is usually not a problem.

Degumming provides the advantages of producing lecithin and reducing the phosphatide removal load in caustic refining. If physical refining is subsequently employed, it is essential to degum oils high in phosphatides for both economic and product quality purposes.

With the very best degumming, some water-hydratable phospatides are left in the oil. Further hydration of water-hydratable phosphatides occurs in storage due to atmospheric moisture condensation on the exposed interior tank surface. If the storage tanks are not equipped with the mechanical agitation the hydrated phosphatides settle to the bottom of the tank over time. Each individual tank truck, tankcar or barge shipment of degummed oil drawn from a given static storage tank will have a different phosphorous levels. Consequently, degummed oil storage tanks should be equipped with a properly specified and installed mechanical agitator to sustain product consistency.

If emulsions are formed in the degumming process and are still present in the product stream at the inlet of the centrifuge, efficient separation will be difficult (Fig. 6). Depending upon the centrifuge back-pressure setting, the emulsion will be separated as either part of the degummed oil (light phase) or part of the wet gums (heavy phase). If the emulsion is part of the degummed oil phase, the phosphatide level will be high. If the emulsion is part

of the wet gums, the oil loss will be high. Formation of emulsions must be avoided to in the degumming process to achieve high-quality and high-yield oil. Use of hard water and turbulent flow conditions may cause emulsification.

Fig. 6. Degumming Separator

Lab-scale degumming tests can be done to guess proper degumming process. When degummed oil quality problem are encountered, first thoroughly check the actual process to determine any detectable anomalies (Karleskind&Wolff, 1996). If processing condition deviations are found make a proper corrections and determine if the oil quality problem is corrected by the changed conditions. If the cause of the problem is not readily apparent, use the lab test to identify the problem cause. The lab tests are relatively fast and can be controlled. It is a much more efficient method than testing on a plant scale.

2.3.4 Neutralization

The purpose of caustic refining (neutralization) is to remove free fatty acids, phosphatides and other materials including protein meal, glycerol, carbohydrates, resins and metals. In addition, some color reduction (pigment removal) is achieved.

Crude oil, degummed oil or mixtures of crude and degummed oil are fed to the refining process. Caustic soda (sodium hydroxide) is fed in the proper strength and quantity to react with the free fatty acids and phosphatides to form soapstock and hydratable gums.

Having polar and hydrophilic properties, the soapstock produced is effective in removing some of the other impurities from the oil stream. Soapstock and other impurities are separated from the oil by centrifuges.

The processing endpoint for caustic refining varies according to the quality of the crude or degummed oil. It could be free fatty acid, phosphatide or color level. The impurity most difficult to remove in meeting the in-process oil specification determines the refining endpoint.

Caustic soda is not completely selective in reacting with the targets of free fatty acid and phosphatides; therefore, some triglycerides are hydrolyzed and saponified (broken down and converted to soap). Thus, selecting the proper strength caustic soda and the caustic-oil exposure time are key considerations to maximize the yield of refined oil (Smallwood, 2001). Some processors pretreat crude oil with phosphoric acid to hydrate the phosphatides that are not water hydratable. Thus, the caustic soda addition requirement in refining is reduced by the amount which would have been needed to hydrate the gums. Refining loss is slightly improved due to less exposure of triglycerides to caustic soda.

Usually, the most significant cost factor in neutralizing is oil loss. Oil loss occurs in two ways; saponification loss and separation loss. Saponification loss is consequence of caustic soda being in contact with neutral oil. While caustic soda is much more reactive free fatty acid saponification and phosphatide hydration, some reaction occurs with the triglycerides (hydrolysis and subsequent saponification of the free fatty acids). By keeping the caustic soda strength, the contact time and the contact temperature low, saponification of triglycerides can be minimized.

The second occurrence of oil loss is in the separation (Fig. 7) of the refined oil (light phase) from the soapstock (heavy phase) produced in caustic refining. The magnitude of the separation loss is a function of specific gravity difference between the light and the heavy phase, the presence of an emulsion (a third phase), the separation method, the design and condition of the separator, and the temperature of the product stream through a centrifuge. To achieve the lowest separation loss, the process design should reflect minimum product stream passes through a centrifuge.

Fig. 7. Neutralization Separator

The advantage of continuous neutralization is the very short contact time between oil and soda, which reduces the risk of saponification. A good separation between soap and oil phases after neutralization depends largely on the correct execution of phospholipids 'conditioning', as the surfactant properties of phospholipids favor the emulsion. Possibly, similar effects are produced by the presence of considerable quantities of mono and diglycerides and glycerides whose chains are heavily oxidized.

2.3.5 Bleaching

The implications of bleaching are substantial and are often misunderstood or underrated. The common misconception about bleaching is to consider the purpose limited to reducting the color pigment level. Removal of oxidation products, residual soap left from caustic refining and metals along with further reduction of the phosphatide content are the functionally-essential requirement.

Bleaching is known as a mass transfer process that involves the partial removal of the oil-soluble, non-triglyceride components from the oil stream by changing the physical state to a solid separating the solids from the oil stream by filtration. Specifically, the non-triglyceride components in a liquid state are changed to a solid state by adsorption on to the surface of the solid bleaching earth particles added to the oil stream.

To optimize the performance of edible oil bleaching, the process design and the operating variables involved must be selected to drive the equilibrium toward maximum adsorption within the required product quality and operational equipment restraints.

Increasing temperature improves adsorption. The oil temperature for bleaching is limited to the maximum allowable for the amount of air (oxygen) exposure occurring in the process. The air exposure is determined by the vacuum capacity of the bleaching vessel, the mechanical condition of the system (air entry), and operating practices, the maximum allowable temperature is in the range of 104 to 116 °C (220 to 240°F) with a vacuum level of 50 mm Hg (absolute). In the case of bleaching at atmospheric pressure (about 760 mm Hg), the maximum allowable temperature is 65 °C (150 °F).

Time is a primarily factor in the context of the probability that the undesirable, non-triglyceride components will come in contact with a bleaching earth surface feature that is capable of adsorption. In the bleaching vessel prior to filtration, the probability of adsorption occurrence is expended in about 20-25 minutes. After about 20-25 minutes, the rate of adsorption becomes asymptotic.

Proper bleaching vessel (Fig. 8) design must include sufficient agitation to keep the bleaching- earth particle in suspension and uniformly dispersed. Vessel design must assure plug flow of the oil column from top inlet to bottom outlet for providing 20-25 minutes of residence time for the total oil stream.

The surface condition of the clay particles is a critical factor in bleaching. Adsorption occurs at points on the clay crystal surface where a sharp edge, striation, or boundary is exposed. Natural clay mined for use as bleaching earth contains other material that can interfere with adsorption (Omar, et al., 2003). Often deposits of clay contain basic compounds such as calcium carbonate that can cover a significant portion of the clay crystal surface features capable of adsorption.

Acid-activated clay has been treated with a mineral acid to react with and dissolve the basic compounds on the surface of the clay particles. The activation significantly improves the performance of the bleaching earth by increasing the exposure of surface features capable of adsorption.

The mechanism of bleaching is adsorption using natural or acid-activated clay. The process is carried out by adding the proper amount of clay to the refined oil stream. Typical bleaching conditions include a minimum of 20-25 minutes contact time between the clay and the oil, a temperature range 104 to 116 °C (220 to 240°F) and a vacuum of 50 mmHg absolute. Subsequently, the spent clay is filtered out of the bleached oil.
Moisture enhances adsorption within a specified range that varies with the type of bleaching earth used. Most vendors can offer guidance on the optimum oil moisture content for the bleaching earth supplied. If this information is not available, laboratory scale bleach test can be performed on oil samples with varying moisture content to determine the optimum level.

Fig. 8. Bleaching Step

Quality of the oil feed to the bleaching process is another critical determinant of bleaching performance. If impurities are present in the oil which covers the clay surface features necessary for adsorption, the bleaching effect mass transfer will be proportionally reduced. Impurities like phosphatides, soap and polymerized oil are especially detrimental to bleaching by coating the clay particle surface features.
From the bleaching earth slurry tank the oil with bleaching earth is pumped through an oil to oil heat exchanger (economizer) to gain heat from the hot oil exiting the bleaching vessel. The design parameters for this oil to oil heat exchange (equipment type and product stream velocity) are critical to preclude fouling and frequent cleaning. From the oil to oil heat exchanger, the product stream passes through the final heat exchanger to for heating by steam to the targeted bleaching temperature.
On exit from the final heat exchanger, the oil-bleaching earth slurry flows to the vacuum bleaching vessel. Typically, the bleaching vessel is installed with the long dimension in the vertical position. Oil enters near the top of the vessel through nozzles to evenly distribute the flow over the top surface of the oil column. In the top of the vessel, a demister pad is installed to condense and reflux oil droplets in the vapor stream pulled from the vessel by the vacuum. To keep the bleaching earth uniformly dispersed in the column of oil in the

vessel, a multi-impeller top-entering mechanical agitator is used. Baffling is installed from the vessel walls between agitator impeller stages to maximize plug flow of the oil column from top inlet to bottom outlet. The baffling minimizes the chance of short circuit flow to assure that the residence time for all of the oil is at least 20-25 minutes in the vessel.

After exiting the bleaching vessel, the oil-bleaching earth slurry passes through the economizer then to a pre-coated filter.

After each bleaching filter cleaning, clean (filtered) bleached oil is pumped to the pre-coat and sufficient diatomaceous earth (filter aid) is added to and mixed with the oil to provide a protective film over the all of the filter media surface area in the clear filter. The filter aid is uniformly deposited on the filter media by completely filling the filter with the oil-diatomaceous earth slurry and re-circulating the stream until complete deposition occurs.

Filter pre-coating as described above offers both product quality and operating economic advantages; oil quality is enhanced by better removal of solids from the product stream, oil recycle on start-up a cleaned filter can be minimized or eliminated and filter media is protected from direct contact with fouling substances like soap, phosphatide and polymerized/oxidized oil. The life of the filter is thus improved for each operating cycle and the number of uses before washing of the media is required.

In recent years, most equipment vendors have replaced mechanical agitation, with steam sparing to maintain bleaching earth dispersion in the vacuum bleaching vessel. The justification for this design change has been on the argument that the moisture addition enhances adsorption. From the author's perspective, careful analysis reflects that the economics of this change are not favorable and that mechanical agitation remains as the preferred solution. It is outside of the scope of this presentation to probe the details of this matter.

Using filtration leaves requires the use of safety filters generally equipped with paper, filtration cardboard or cellulose products. The selection of a filter is based on the type of installation (continuous or discontinuous flow) local factors (cost or labor) and goal (primary or safety filtration). The plugging reduces the filtration flow and that plugging is slower if the flowing speed into the porous wall is low.

Metal cloth or leaf supports the filter cake made of a mix or filtering and bleaching earths that formed the porous wall. The gaps between the metallic cloth mesh is such that a layer of cake has to be spread before starting to filter. This type of filter always allows the passage of traces of earth; thus, a safety net must double it.

A polished filter is provided for the oil stream before entry to bleached oil storage. A bag-type filter is usually preferred. Pressure drop can be continually monitored across the polish filter to detect anomalous bleaching earth content or when it is necessary to routinely replace the filter bags.

Installation of an in-line turbidity monitor can be beneficial to detect to presence of bleaching earth particles and divert/recycle the product stream before ether bleached oil in storage is fouled.

To transport the bleached oil to storage, a transfer pump is required in the scheme unless gravity flow is possible to the storage tanks, which is usually not the case. If closed filtration systems are used, it is not permissible to connect the transfer pump directly to the filter discharge. If that were to be done, a hydraulic pulse would be transmitted back to the filter and disrupt (crack) the filter cake, which would result in bleaching earth particles passing through the filter media and the oil would be fouled. For closed filter systems, it is necessary to discharge the oil from the filter after passing through the economizer into a hydraulic-

impulse break tank that is under atmospheric pressure. If nitrogen is available, the bleached oil in the hydraulic- impulse break tank must be kept under nitrogen blanket to preclude oxidation. In the case that nitrogen is not available, the hot oil from the economizer must be cooled to at least 40˚C-65°C before entry to the hydraulic-impulse break tank, the pre-coat tank, or bleached oil storage tanks. Furthermore, for open filtration systems, the bleached oil-spent bleaching earth slurry must be cooled to 40˚C-65°C before filtration to preclude oil oxidation at the filter outlet, where atmospheric exposure occurs.

An in-line colorimeter is installed in the oil pipeline to bleached oil storage. The signal from the colorimeter is feed to a control loop for adjusting the feed rate of bleaching earth addition to the slurry tank. With this capability significant economic advantage can be gained from the fact that as the bleaching earth filter cake thickness increases, the adsorption probability is increased, which results in improved bleaching performance. Thus, over the life of the filter cycle, the amount of bleaching earth required to meet the specified endpoint (color in most cases can be continually reduced). At some point in the filter cycle, the amount of bleaching earth addition may drop below the level necessary to sustain adequate cake porosity for maintaining the needed flow rate. To avoid this occurrence, the control system is designed to initiate and ramp up the addition of the sufficient diatomaceous earth (filter aid) to sustain the needed filter cake porosity. Careful economic analysis is needed to assure that the operating parameters selected are advantageous.

The processing endpoint for bleaching is determined by the most difficult of the factors to achieve in meeting the bleached oil specification (color, soap, oxidation products, metal or phosphatide). In theory, properly bleached oil should have a zero peroxide value (Bailey, 1996). In practice, it does because the sample taken for analysis is usually exposed to the atmosphere (air); thus the presence of peroxide will be found typically < 1.0 meq/kg.

With physical refining, caustic refining is by-passed; thus bleaching along with phosphoric acid pretreatment are the only tools available to clean up the oil stream prior to the final processing step of deodorization.

2.3.6 Winterization

The purpose of winterization is; to produce a product higher in unsaturated, to remove sufficient saturated triglycerides or wax from an oil in order that the liquid fraction will remain clear and brilliant at room temperature for aesthetic purposes and isolate the saturated fraction for use in formulating products to achieve specific functionality.

In most cases, the winterization process is a less sophisticated form of a dry fractionation. The oil is cooled slowly and kept at a low temperature for a long time to allow the waxes to crystallize. After stabilization, oil temperature is normally increased to about 15^0C to allow easier filtration by lowering the viscosity. In order to facility crystallization and filtration some filter aid is usually added.

There are different types of filters (Fig. 9) that can be used to remove the waxes and filter aid from the oil.

Moreover, crystals appear and create turbidity then a settling prejudicial to the oil's commercial image. This additional treatment is required to obtain oil that will remain clear and shiny, regardless of storage conditions. This process is 2 step processes, containing a cooling that causes crystallization at an elevated melting point and a separation of the formed crystals. A cooling down to 5-6^0C (41-43^0F) causes the oversaturation desired and a crystal growth (Gomez, et al., 2002). The latter consists in maintaining the mixture at this

low temperature until complete crystallization of the waxes and the formation of crystal sufficiently large not to hinder the second step that is separation. Originally, wax crystals were barely left to naturally decant during the winter storage of oil.
The winterization process is accomplished by cooling oil to a temperature sufficiently low to solidify (crystallize) the saturated triglyceride fraction or wax. After crystal formation is complete, the gently agitated oil is transferred through a filter to separate the fractions (liquid and solid).

Fig. 9. Winterization filters

2.3.7 Deodorization

Deodorization is the final step in the processing sequence of converting crude vegetable oil or fat to an acceptable product for human consumption. In the deodorization process, the remaining impurities are either removed or reduced to a sufficiently low level for the production of acceptable flavor and functional edible oils and fats. While oils and fats do not harbor the growth bacteria and other organisms, one of the benefits of deodorization is to completely sterilize final product (Bockisch, 1998).
With deodorization, relative bland flavor and odor, essentially complete removal of residual herbicide and pesticide residue, low free fatty acid content, removal of oxidation products (zero peroxide content), low moisture content (about 0,05%), color reduction through heat bleaching of the carotene pigments improvements achieved.
Usually the last step of in the actual processing sequence, deodorization is utilized to remove volatile components including free fatty acids, glycerol, oxidation products, sterols, herbicides and pesticides. Heat bleaching of carotene and xanthophylls color pigment is achieved. Normally, a chelant, like citric acid, is injected into the deodorized oil stream for reacting with any residual metals. The metal salts formed are subsequently removed from the oil by filtration.
Deodorization is carried out in batch (Fig. 10), semi-continuous or continuous systems. The deodorization principles are the same regardless of the system used. By heating the oil up to a temperature in the range (230-260°C) under a vacuum of 2 to 10 mm Hg absolute, the non-triglyceride components and steam (vapor steam) are removed from the deodorization vessel by the vacuum system.

As previously mentioned, product sterilization occurs in the deodorization process as the consequence of the high temperature and the injection of live steam in to the product stream. Like bleaching deodorization is a mass transfer process that involves the partial removal of the oil-soluble, non-triglyceride components from the oil stream by changing the physical state. In deodorization, the non-triglyceride components are changed from a liquid to a vapor state with subsequent removal of the vapor by vacuum. Conditions are established to raise the partial pressure of the non-triglyceride components from transformation to the vapor state. Mass transfer is an equilibrium-type phenomenon. The variables involved in the deodorization-process mass-transfer equilibrium (vaporization of the non-triglyceride components) are; temperature, pressure (vacuum level), height (thickness) of the oil column, time (operating rate), stripping steam addition rate, molecular weight (kind) of the oil fed to the process, quality of the oil fed to the process.

Fig. 10. Deodorization step

To optimize the performance of deodorization, process design operating variables involved must be selected to drive the equilibrium toward maximum vaporization of the non-triglyceride components while minimizing the removal of the oil. By increasing temperature, the partial pressure of each component fraction is increased in relation to the molecular weight. Oil temperature in the range of (230-260°C) is common for deodorizing vegetable oils. The temperature at the top of the range is applicable to most of the highly polyunsaturated oils (Brekke, 1980). If the high temperature is not applied during refining fishy odour may occur. For this reason, performance of the deodorization process has to be done attentively.

By reducing the pressure (increasing the vacuum level), the partial pressure required for transformation to the vapor state is reduced relation to the molecular weight of each component fraction.
Deodorization efficiency is inversely proportional to the height of the oil column. The first deodorizer vessels constructed were batch type with the oil column height in the range of 2 to 4 meters high. The time required to deodorize a batch of oil ranged between 1 and 4 hours. For the most part, tray-type deodorizers have replaced batch deodorizers with about one meter of oil depth in each tray. With tray-type deodorizers, the deodorization time is less than 1 hour. Finally thin-film deodorization has been developed with oil film on the packing approaching one-molecule thickness. The deodorization time for thin-film deodorizers is measured in a few minutes.
Live steam is injected at the bottom of the oil column, bottom of each tray, or bottom of the packed thin film column by means of a sparge ring or other nozzle configuration. The quantity of steam added ranges from 1,5 to 3,0% of the oil-feed rate by weight. Steam addition of 2,0% of the oil-feed rate by weight is the most common practice. The purpose of the steam is to provide a highly-volatile vapor stream to strip out the less-volatile non-triglyceride components.
The molecular weight of the triglycerides in the oil feed stock affects the deodorization conditions. Shorter fatty acid chain triglycerides with lower molecular weight are easier to deodorize than longer fatty acid chain triglycerides.
Feed-stock quality has a profound impact on deodorization performance. The capacity (operating rate) of deodorization is largely determined by the amount of non-triglyceride components in the feed-stock. Each deodorizer is designed to handle a specific vapor load. The operating rate cannot exceed the vapor removal capacity of the process. Thus, as the proportion of non-triglyceride fraction of the feed stock increases, the deodorization rates reduced to not exceed the maximum vapor removal capacity.
Deaeration is accomplished by the product stream flowing counter to the injection of live steam in a vessel under vacuum (about 3 mm Hg). Deaerator vessels are designed with either a packed or open column. The product stream enters the deaerator vessel near the top while the live stream (stripping steam) is injected above the bottom oil reservoir. A level control loop is provided to maintain the desired oil level in the deaerator bottom reservoir. In the top of the deaerator, a demister pad is installed to condense and reflux any oil droplets entrained in the vapor stream exiting the vessel. Oil is removed from the deaerator vessel by means of a centrifugal pumped located at an elevation sufficiently below the bottom of the deaerator to maintain a net-positive-suction head. To preclude air entry into the hot, deaerated oil stream, the aerator discharge pump is usually magnetically coupled to the drive motor to eliminate the mechanical seal, or the pump is equipped with a double mechanical seal. From the deaerator, the product stream passes through an oil to oil heat exchanger for additional heating. Various designs are used to achieve this energy conservation. The heat exchange is accomplished by means of a heat recovery tray in which a tube bundle is inserted for the product stream to pass through prior to entry into the top tray of the deodorizer.
In the top tray of the deodorizer, the product is heated to the targeted deodorization temperature by heat transfer from high-pressure steam or a heat transfer fluid circulated through the tube bundle or coils in the tray.
The product stream exits the heating tray and subsequent trays by means of an overflow pipe that provides the flow path to the tray below. In the top heating tray, live steam is

added through a sparger to provide effective oil agitation and achieve uniform heating of the oil.

Beginning in the top heating tray, following in the two deodorizer trays, and culminating in the heat recovery tray, effective heat bleaching of the carotene pigments occurs with oil temperature >250^{0}C. Heat bleaching is both a time temperature phenomenon. Thus, a minimum of 20 minutes residence time at the elevated temperature is needed to maximize heat bleaching. Proper deodorization process design includes sufficient holding capacity time in the trays identified above the heat bleached.

From the third tray (second deodorizer tray), the product stream overflows to the heat recovery tray previously described. Through heat transfer to the incoming product stream, some heat reduction is attained in the deodorized oil stream. Live steam is added through a sparger to provide effective oil agitation and achieve uniform heat transfer from the deodorized oil.

Similar to the deaerator, oil is pumped from the deodorizer vessel by means of a centrifugal pumped located at an elevation sufficiently below the bottom of the vessel to maintain a net positive suction head. To preclude air entry into the deodorized oil stream, the deodorizer discharge pump is usually magnetically coupled to the drive motor to eliminate the mechanical seal.

The empty deodorizer trays for stock change or for shut down of the operation, each tray is equipped with a bottom discharge pipe to the tray below. There is a valve in each drain pipe to actuate the tray emptying operation. For stock change each tray is emptied in sequence to maintain a complete break of the two feed stocks.

To improve oil quality, a chelating agent is injected into the product steam after the outlet from the deodorizer vessel discharge pump. The use of chelating agents to improve deodorized oil quality is presented in a separate paper.

For deodorized oil, a chelating agent enhances oil stability in two ways. First, if pro-oxidant metals like copper or iron are present, the acid will react to form a metal salt that is subsequently removed by filtration. Second, the residual chelant in the oil has some anti-oxidant attributes. It is common practice to add the chelating agent to deodorized oil on exit from the deodorization process prior to polish filtration.

Citric acid is the most commonly used chelating agent. Citric acid can be purchased in 100% or less concentrations. Whatever concentration is purchased the amount added to the deodorized oil should be equivalent to the recommended amount. To dissolve citric acid, addition of hot water at a temperature near the boiling point is recommended. The amount of water added to the citric acid can not exceed an amount that would result in the moisture content of the deodorized oil being above 0,05%. The temperature of citric acid can not exceed 104 ^{0}C. Above 104 ^{0}C, citric acid starts to decompose. Thus, the maximum temperature for the deodorized oil at the point of citric acid addition can not exceed 104 ^{0}C (Smallwood, 2001).

One of the advantages in using citric acid as a chelating agent is the tolerance for variation in the addition rate. There is a wide variation in the addition rate the impact on the oil quality is relatively minor.

For handling citric acid, two identical make-up/feed tanks are recommended. The tanks should be constructed of 316-grade stainless steel and be equipped with an external heating jacket a mechanical agitator and a graduated sight glass in the sight wall. With this capability while citric acid is fed to the deodorized oil from one tank, a new supply can be prepared in the other tank. The purpose of the side-wall graduated sight glass is to observe

the rate of this appearance for calibrating the metering pump which transfers the citric acid to the deodorized oil stream.

Phosphoric acid is a much better chelant than citric acid for 2 reasons; first of all, it is purchased in a liquid state and, thus, is much easier to handle. Second, phosphoric acid has much better antioxidant attributes than citric acid. Addition of phosphoric acid is much simpler than citric acid.

The transfer line from the feed tank to the injection point must be constructed of 316-grade stainless steal for both citric and phosphoric acid. If not failure will occur and a serious safety hazard will result from the acid leakage.

The deodorized oil is pumped through a polish filter (usually a bag-type filter) to remove any sediment from the oil prior to transfer to storage. Usually, two polish filters are installed in parallel to enable the use of one while the other is out of service for replacing the bags or other type of filter elements.

The deodorized oil is pumped through a polish filter to remove any sediment from the oil prior to transfer to storage. Usually, two polish filters are installed in parallel to enable the use of one while the other is out of service for replacing the bags or other type of filter elements.

On the start-up of the process, the product stream is initially recycled until proper operating conditions are achieved and the product quality is acceptable. Then, the fully deodorized oil stream is diverted to storage.

Vacuum for the deodorization process is obtained by a system comprised of four stages of steam ejectors and two stages of barometric condensers. Some designs utilize vacuum pumps in place of steam ejectors in the last two stages or final stage.

Most deodorizer processes include a vessel and equipment to condense and recover the fatty acid and other less volatile components from the vapor stream. The less volatile components are condensed by circulating the cooled condensate back to the condensing vessel for spray application to the incoming vapor stream. To maintain the needed balance of deodorizer distillate that is recycled for condensing the less volatile vapors, the excess quantity is continually pumped to a storage tank a heat exchanger is used to cool the deodorizer distillate before recycle or transfer to storage. Cooling water is used to cool the distillate. A single pass, tube and shell heat exchanger is the most reliable for distillate cooling. Plate heat exchangers are often used in this service due to superior energy efficiency.

Properly deodorized oil will have free fatty acid content under 0.05% and have no oxidation products as indicated by peroxide value (zero expected). The flavor of deodorized oil is relatively bland (typically weak nutty or buttery).

Deodorization is the final major processing step to produce acceptable quality edible oil products. To meet finished product quality objectives the deodorization process must be operated with in prescribed temperature and vacuum limits, have relatively clean interior metal surfaces in contact with the product stream and receive feed stock within acceptable quality limits. Direct exposure of air to the product stream results in both several oxidation of the oil and rapid deposition of oxidized oil on the metal surfaces. Air leakage into the system that does not directly contact the product stream. But contacts the product the-coated surfaces results in rapid deposition of oxidized oil build up of oxidized oil on the deodorization system interior surfaces impacts product quality in two ways. First oxidized oil film on the interior surfaces will slough off into the product stream. Second, build up of

oxidized oil on the entrainment separators and demister pads will result in increase oil loss and decrease vacuum level due to increase pressure drop. Furthermore any air leakage in to the deodorizer system will increase the non condensable load and reduce the system vacuum. Finally oxidize oil build up on heating cooling coil surfaces will decrease the heat transfer efficiency and accordingly increase the system energy input requirements. In conclusion there are three cardinal rules for achieving effective edible oil deodorization; establish and meet proper feed stock quality limits, assure that the deodorizer system is air tight via proper design, installation, maintenance, operating and routine testing and inspect and clean the deodorization system interior surfaces at the proper frequency.

Each edible oil processor should independently determine the proper cleaning frequency according to need. Deodorizer system cleaning on a six month frequency is the industry norm. With an effectively designed and executed cleaning capability, the practice of cleaning too soon instead of waiting too long yields the best product quality and operating expense results.

The scope of the deodorizer system cleaning should include all the elements which impact functional performance, product quality and operating cost. If present, routinely clean the following elements of the deodorization system; deareator heat exchanger, deaerator vessel, deodorizer heat exchangers, deodorizer vessel internals, distillate recovery system, system piping from inlet to outlet and barometric condensing system.

Also the computer-based process control has many benefits for vegetable oil refineries. More precise and responsively controls can be applied while using materials like caustic soda, sulfuric acid, bleaching earth etc. Also temperature, pressure catalyst additions and hydrogen gas input can be programmed and controlled. Problems which are occurred in process system are detected easily and solved quickly by using computer-based process control.

2.3.8 Deodorized oil storage

If not transferred directly from deodorization to a tank car for shipment, deodorized oil is held in storage tanks prior to loading for bulk shipment by truck or transferring to package filling (packaging). Effective deodorized oil storage requires the following to assure product quality:

1. Nitrogen blanketing of the head space above the oil level if possible.
2. Agitation to preclude any separation of the triglyceride components.
3. Temperature control capability.
4. Sampling capability
5. Tank washing/cleaning at the proper frequency to preclude the accumulation

Of old oil films on the interior tank surfaces which would eventually reflux into the fresh oil. Nitrogen Blanketing of edible oils is essential for, oxidative and flavor stability, extended storage period and maintenance of nutritive value. Whether refined or not, all oils are sensitive to heat, light and exposure to oxygen. Exposure to above conditions leads to the rancidity in edible oils. Rancid oil has an unpleasant aroma acrid taste, and its nutrient value is greatly diminished. Also, rancid oils are injurious to health. To delay the development of rancid oil, a blanket of nitrogen, an invert gas is applied to the vapor space in storage container immediately just after production and also in the packing containers. This is referred to as Total Nitrogen Blanketing. Care for quality of edible oils during storage is especially important for refined oil products. Major sources of deterioration may be brass sampler, reheating and contamination in pipe line and by absorption of oxygen.

Oil storage tanks (Fig. 11), should be carefully inspected on a quarterly frequency and thoroughly washed at least annually. Failure to maintain clean tanks will result in poor oil and product quality. Means for effective and rapid tank washing should be included in the total system.

Fig. 11. Refined Oil Silos

2.3.9 Deodorized oil loading

The final and perhaps most vulnerable step in edible oil processing for bulk shipment are loading the products in tank cars and trucks. High quality products improperly loaded will result in poor quality products received by the customer (end user). Tank car/truck cleanliness is the starting point in oil loading. Tank car/truck washing capability must be provided to assure consistent, high quality cleaning. Thorough inspection of the tank car/truck must be conducted before oil loading is initiated. The oil transfer system itself (hose, nozzles, pipelines, etc.) must be clean and properly protected. The critical consideration in transferring oil from storage to the point of application is to avoid the introduction of air. Nitrogen sparing is recommended to minimize or eliminate air exposure during loading. Bottom filling of tank car/truck using a loading nozzle extends through the top hatch to the bottom of the tank is advisable. A protective cover around the open top hatch during loading is essential. Finally, security seals properly attached to all access openings to the tank car/truck are mantadory after loading and sampling are completed.

2.4 Uses in pharmaceutical drug delivery systems: Why is soybean oil necessary for pharmaceutical dosage form?

In pharmaceutical preparations, soybean oil emulsions are primarily used as a fat source in total parenteral nutrition (TPN) regimens. Although other oils, such as peanut oil, have been used for this purpose, soybean oil is now preferred because it is associated with fewer adverse reactions (Rowe et al. 2006). An emulsion is a dispersion of two or more immiscible

liquids stabilized by a surfactant or emulsifier coating the droplets and preventing coalescence by reducing interfacial tension or creating a physical repulsion between the droplets. Two common types of emulsions are found in parenteral drug delivery systems. Water in oil emulsions (W/O) is used in sustained release of steroids and vaccines by intramuscular injection. Oil in water (O/W) or lipid emulsions can be administered by a variety of parenteral routes (for example subcutaneous, intramuscular and intra-arterial) but are predominantly injected intravenously in parenteral nutrition applications. Lipid emulsions have been used in parenteral nutrition for more than four decades as a life-saving treatment by the intravenous infusion of fat in patients who cannot orally consume or metabolize food properly. The use of fat or lipid emulsions in parenteral feeding provides an insulin independent energy source and essential fatty acids necessary for metabolism. The emulsion is usually admixed with amino acids, carbohydrates and electrolytes as part of a therapy programme known as total parenteral nutrition. The stability of these lipid emulsions based on soybean oil dispersed with egg lecithin in parenteral nutrition has demonstrated a shelf life of 24 months at room temperature. Consequently, the stability and safety record of these types of lipid emulsions offers an attractive drug delivery system for lipophilic candidates in a drug development programme (Amidon, 1995; Aungst, 1993; McNiff, 1977; Medina 2001).

Emulsions containing soybean oil have also been used as vehicles for the oral and intravenous administration of drugs; drug substances that have been incorporated into such emulsions include vitamins, peptides, proteins, hormones, poorly water-soluble active substances, fluorocarbons and insulin (Chansri, et al. 2006; Ganta et al. 2008; Hwang, et al. 2004; Fukushima, et al. 2000; Nishikawa et al. 1999; Rowe et al. 2006; Tamilvanan, 2009; Terek et al., 2006). In addition, soybean oil has been used in the formulation of many drug delivery systems such as liposomes, microspheres, dry emulsions, self-emulsifying systems, microemulsions, and nanoemulsions and nanocapsules. Soybean oil may also be used in cosmetics and is consumed as edible oil. As soybean oil has emollient properties, it is used as a bath additive in the treatment of dry skin conditions (Constantinides et al., 2008; Karasulu, et al., 2007; Rowe et al. 2006).

An interesting study was performed with a new lipid emulsion based on soybean oil, medium chain triglycerides, olive oil and fish oil (SMOFlipid) for safety, tolerance, metabolic and clinical efficacy in surgical patients. It is concluded that SMOFlipid is clinically safe and well tolerated in postoperative patients. There are indications that SMOFlipid may be associated with a better liver tolerance and a shorter length of hospitalization (Metres, 2006).

The oral route is the preferred route for chronic drug therapy. Numerous potent lipophilic drugs exhibit low oral bioavailability due to their poor aqueous solubility properties. Self-emulsifying drug delivery systems (SEDDS) have been previously described in the literature as homogeneous mixtures of natural or synthetic oils, solid or liquid surfactants, or alternatively, one or more hydrophilic solvents and co-solvents. The principal characteristic of these systems is their ability to form fine oil-in-water (O/W) emulsions or microemulsions upon mild agitation following dilution by aqueous phases. This property renders SEDDS as good candidates for the oral delivery of hydrophobic drugs with adequate solubility in oil or oil/surfactant blends. Lipid-based drug delivery systems are commonly studied for the enhancement of oral bioavailability of hydrophobic and lipophilic drugs (Han et. al., 2010; Zhao et al., 2010).

An injectable microemulsion of arsenic trioxide (As_2O_3-M) was prepared for intratumoral injection and the suppressive effect of As_2O_3-loaded microemulsion on human breast cancer cells MCF-7 was compared with those of a solution of the drug (Karasulu, 2004). Microemulsion was made up of soybean-oil as oil phase, a mixture of Brij 58 and Span 80 as surfactants, and absolute ethanol as co-surfactant and bi-distilled water containing As_2O_3 solution as the aqueous phase. Microemulsion formulation containing of $5x10^{-6}$ molar (*M*) As_2O_3. The formulation was physically stable for 12 months at room temperature when kept in ampule forms, as well as after autoclaving at 110°C for 30 min. It was clearly demonstrated that As_2O_3-M had significant cytotoxic effect on breast cancer cell lines and the cytotoxic effect of As_2O_3-M was significantly more than that of regular As_2O_3-solutions. Even ~3000 times diluted microemulsion formulation loaded with $5x10^{-6}$ *M* As_2O_3 showed cytotoxic effect. According to the in-vitro cytotoxicity studies, it can be concluded that when As_2O_3 was incorporated into the microemulsion (As_2O_3-M), which is a new drug carrier system, it suppresses tumour cell growth on multiple tumor lines.

The most significant problem of microemulsion systems is the lack of biological tolerance of excipient such as surfactant, cosurfactant and oil. Karasulu et al. (2007) have examined microemulsion of methotrexate (M-MTX) and solution of the drug (Sol-MTX) on a model biological environmental model. In this formulation, microemulsion was made up of soybean-oil, Span 80, Cremophore EL isopropyl alcohol and NaOH solution. For this purpose a gastrointestinal cell culture model, the Caco-2 cell line, was used to investigate the cytotoxic effects of the polymeric carrier and its effect on the cell monolayer integrity. After an incubation of the cells with Sol-MTX for 3 days, the Caco-2 cell proliferation was significantly inhibited in a dose-dependent manner to an extent of 38.11 ±3.90 % at the highest concentration of 40 ng/75μL. The differences between the viability of cells for M-MTX and Sol-MTX were found to be significantly different when applied to ANOVA according to 2x8 factorial randomized design (p:0.016; for α: 0.05, power : 0.695). Therefore, by using M-MTX in the therapy, low cytotoxic effect on normal cells and low side effects may be expected. In the other study, the formulation of microemulsion containing pilocarpin was development using Brij 35P, Span 80 as surfactant and propanol as cosurfactant, and soybean oil as the oil phase (Ince et al. 2007). The test microemulsion was non-irritant in rabbit eyes and a prolonged pharmacodynamic effect was observed in vivo compared to the drug administered as a simple solution or gel.

Lipid emulsions composed of egg phosphatidylcholine, cholesterol and soybean oil were the optimized carriers for All-trans-retinoic acid (ATRA) delivery. The delivery of ATRA by emulsions can reduce the elimination of ATRA from the blood circulation and preferentially accumulate retention of ATRA in the liver can successfully suppress the progression of liver metastasis in mice injected with colon carcinoma cells. These findings indicate that the effective delivery and retention of ATRA in hepatocytes by emulsion is an efficient approach for the treatment of liver metastasis (Chansri, 2006).

Pharmaceutical specifications of soybean oil are listed at Table 1. Soybean oil is a clear, pale-yellow colored, odorless or almost odorless liquid, with a bland taste that solidifies between -10 and -16°C (Rowe et al. 2006).

3. Conclusion

Soybean oil has a variety usage in nutrition such as, salad oil, cooking oil, baking fats, confectionary fats, ingredient for margarine and mayonnaise and also heavy-duty frying oil

if blended with high stability oil like cottonseed or palm oil. Having a high value of Omega-3, Omega-6 and Vitamin E and low value of price makes soybean oil an vital nutriment for daily consume.

Both cold pressing and refining of soybean oil are commonly used and beneficial processing systems. In cold pressing while high temperature have not been used, the deodorization step of refining needs high temperature and pressure to eliminate of impurities comes from extracted raw material. Refining has more steps than cold pressing, like, degumming, neutralization, bleaching, winterizing and deodorizing. Moreover conditions of crude oil receiving and unloading, storage, refined oil storage and loading steps effects the quality and hygiene of last product.

Soybean oil has been used in the pharmaceutical formulations. In conclusion, soybean oil is not only used in food products but is also used as pharmaceutical excipients to produce a novel, biocompatible formulations which are cost effective, non-irritating, and capable of being sterilized before application.

Test	JP 2001	PhEur 2005	USP 28
Identification	-	+	-
Characters	-	+	-
Specific gravity	0.916–0.922	≈ 0.922	0.916–0.922
Refractive index	-	≈ 1.475	1.465–1.475
Heavy metals	-	-	≤ 0.001%
Free fatty acids	-	-	+
Fatty acid composition	-	+	+
Acid value	≤0.2	≤0.5	-
Iodine value	126–140	-	120–141
Saponification value	188–195	-	180–200
Unsaponifiable matter	≤1.0%	≤1.5%	≤1.0%
Cottonseed oil	-	-	+
Peroxide	-	≤10.0 or ≤5.0[(a)]	+
Alkaline impurities	-	+	-
Brassicasterol	-	≤0.3%	-
Water	-	≤0.1%[(a)]	-

[(a)]In soybean oil intended for parenteral use.

Table 1. Pharmacopeial specifications for soybean oil

4. Acknowledgment

The authors would like to thank the management of *ZADE Edible Oil Refinery & Cold Pressed Oil Plant* for assisting on the preparation of this review.

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14

Beneficial Effects of Bioactive Peptides Derived from Soybean on Human Health and Their Production by Genetic Engineering

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1. Introduction

Soybean [*Glycine max* (L.) Merrill] is one of the oldest cultivated crops of the Far East. For centuries, the Oriental people, including the Chinese, Japanese, Koreans, and Southeast Asians, have used soybean as a staple source of dietary protein and oil. It is one of the five commercial food crops, besides rice, wheat, barley, and millet. Soybean is very important for vegetarians and vegans because of its high protein content and abundance of vitamins, minerals, and fiber. Because of its versatility, it can be prepared into a variety of foods, including fermented and nonfermented foods.

Asians consume about 20–80 g of traditional soy foods daily, the most common being tofu, miso, and tempeh (Fournier et al., 1998; Messina & Flickinger, 2002), whereas Americans consume only about 1–3 g daily (Cohen et al., 2000; Fournier et al., 1998), mostly in processed forms such as soy drinks, breakfast cereals, and energy bars. Soybean-based foods have generated much interest because of the evidence that consumption of large amounts of soybean can lower the risk of chronic diseases such as cardiovascular disease and cancer; this effect is particularly noticeable among Asian populations because of their high soy food intake (Kulling et al., 2001; Persky et al., 2002; Tikkanen & Adlercreutz, 2000). In addition, consumption of soy foods may reduce the risk of osteoporosis and help alleviate menopausal symptoms (Genovese & Lajolo, 2002; Messina, 2000; Persky et al., 2002), which are major health concerns for women.

Soybean-based foods contain an array of biologically active compounds that can confer important health benefits such as antioxidant effects (Setchell, 1998; Tsai & Huang, 1999). These phytochemicals include saponins, phytates, protease inhibitors, phenolic acids, and lecithin, all known for their anticancer potential (Cohen et al., 2000; Messina & Flickinger, 2002); phytosterols, which have hypocholesterolemic effects; isoflavones, which are known for several health benefits (Fukui et al., 2002); and omega-3 fatty acids, which have well-recognized cardioprotective effects. Among these compounds, isoflavones have attracted the most attention (Messina & Flickinger, 2002; Messina & Loprinzi, 2001).

The proteins β-conglycinin (7S globulin) and glycinin (11S globulin) constitute up to 90% of the total soy protein (Gianazza et al., 2003). Evaluation of these dietary proteins is very interesting because their hydrolysis by proteases produces peptides with biological activities. A growing

body of scientific evidence has revealed that many food proteins and peptides exhibit specific biological activities in addition to their established nutritional value. Bioactive peptides present in foods may help to reduce the worldwide epidemic of chronic diseases. Functional proteins and peptides are now an important category within the nutraceutical food sector.
Many bioactive peptides have been isolated from soybean. Numerous studies of the enzymatic or chemical hydrolysis of soybean proteins have demonstrated their functional properties and physiological effects such as antimicrobial, antifungal, anticancer, anti-obesity, antihypertensive, anti-inflammatory, hypocholesterolemic, immunostimulatory, and antioxidant activities. Further, many gene expression systems such as *Escherichia coli* and *Pichia pastoris* have been employed to produce recombinant proteins and peptides. In this chapter, we review the physiological properties of bioactive peptides derived from soybean proteins and discuss the production of soybean peptides by genetic engineering.

2. The actions and beneficial properties of bioactive peptides from soybean

Bioactive peptides are usually encrypted in the amino acid sequences of food proteins (Korhonen & Pihlanto, 2003). Such peptides are inactive within the sequences of precursor proteins, but limited hydrolysis (chemical or fermentation) may unravel the compact protein structures and thereby expose the amino acid residues and patches for inhibiting oxidation.
As summarized in Figure 1, soybean-derived bioactive peptides have many beneficial properties, including hypolipidemic and hypocholesterolemic effects, hypotensive effects, improvement in arterial compliance and endothelial function, insulin resistance, and weight loss in obesity (Erdman, 1995; Friedman & Brandon, 2001; Hermansen et al., 2001; Merritt, 2004; Sirtori et al., 1995).

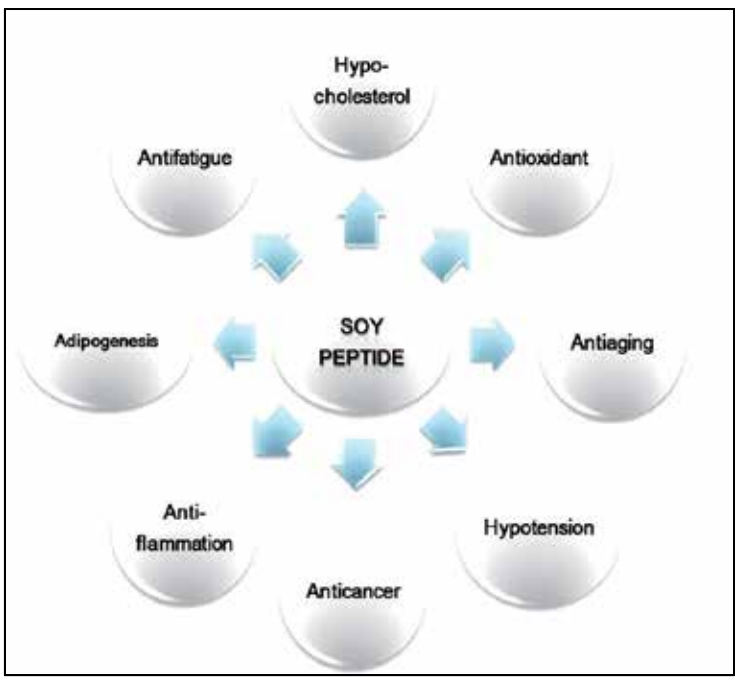

Fig. 1. Beneficial properties of bioactive peptides from soybean.

2.1 Hypocholesterolemic activity

The US Food and Drug Administration has approved health claims concerning the role of soy protein in reducing the risk of coronary heart disease; only people with soy allergy (about 0.5% of the global population) should avoid eating foods containing soy protein. However, a recent randomized, double-blind, parallel-intervention trial showed no significant effect of soy supplementation (30 g soy protein, 9 g cotyledon fiber, 100 mg isoflavones) on blood lipid levels compared with a placebo (30 g casein) in hypercholesterolemic subjects after 24 weeks (Hermansen et al., 2005). Therefore, the biological efficacy of a soy protein-rich diet remains controversial. In addition, the final mechanism responsible for plasma cholesterol reduction remains to be elucidated, although nutrigenomic analysis has revealed significant differences in hepatic gene expression concerning lipid and energy metabolism, transcription factor regulation, and antioxidant enzyme production in animals fed soy protein compared with those fed casein (Nagasawa et al., 2002; Takamatsu et al., 2004).

Several studies in cells and validated animal models have suggested that the mechanism of action of soy protein is related to the direct activation of low-density lipoprotein receptors or gene expression in liver cells (Khosla et al., 1989; Lovati et al., 2000; Manzoni et al., 2003), or modulation of both synthesis and catabolism of low-density lipoproteins by specific proportions of dietary amino acids corresponding to soy protein (Lovati et al., 1987; Sirtori et al., 1993; Tovar et al., 2002; Vahouny et al., 1996). Lovati et al. (1996, 2000) demonstrated that the smaller major peptide (i.e., 7S globulin) from soy protein, devoid of isoflavone components, inhibits apolipoprotein B secretion and sterol biosynthesis in HepG2 cells. Dietary soy protein hydrolysates not only inhibit the absorption of dietary lipids and increase the absorption of dietary carbohydrates but also augment postprandial energy expenditure, accompanied by a postprandial increase in the oxidation of dietary carbohydrates, in type II diabetic mice (Ishihara et al., 2003).

2.2 Anti-obesity activity

Recently, the incidence of obesity has dramatically increased. Obesity is considered a serious problem associated with the development of major human metabolic diseases. This phenomenon occurs as a result of an imbalance between energy intake and energy expenditure that leads to adipocyte accumulation (Aoyama et al., 2000a; Aoyama et al., 2000b). Therefore, low-energy diets such as a high-protein diet, which helps maintain body protein during restricted energy intake, are used for treating obese patients with diabetes (Hwang et al., 2005).

Obesity is caused by increased adipocyte hyperplasia and hypertrophy as well as fat storage, which induces the transformation of pre-adipocytes into adipocytes (Caro et al., 1989). Nagasawa et al. (2003) reported that soy protein isolates can lower the triglyceride content and fatty acid synthase mRNA level in adipose tissue. Therefore, they suggested that soy protein isolates control the gene expressions in adipose tissue and effectively regulate adipogenesis. Further, the anti-obesity benefits of the black soybean peptide, such as reduction in body weight and adipose tissue weight, have been observed.

Kim et al. (2007) synthesized a tripeptide from soy protein and investigated its activity. In Western blot analysis, the synthetic peptide exhibited a similar inhibitory effect to 5-aminoimidazole-4-carboxamide riboside (AICAR), which inhibits the expressions of adipogenic markers and transcription factors for adipogenesis (Haro et al., 1996; Kim et al., 2007).

2.3 Anti-inflammatory activity

Peptide-induced anti-inflammatory activities include modulation of inflammation, binding of toxins, and neutralization of bacteria and fungi. Inflammation associated with skin conditions is often a result of bacterial involvement, partially because of lipopolysaccharide released from the outer membrane of Gram-negative bacteria and lipotechoic acid released from Gram-positive bacteria. Innate-immunity peptides such as defensins and LL-37 are known to bind and neutralize bacterial debris including lipopolysaccharide and lipotechoic acid, resulting in the downregulation of pro-inflammatory cytokines (Mookherjee et al., 2006). Another example is the suppression of propionibacterium acne-stimulated cytokine release by granulysin-derived peptides (McInturff et al., 2005).

Oligopeptide-10 (Granactive Oligopeptide-10; Grant Industries, Elmwood Park, NJ, USA), a synthetic peptide that binds to lipotechoic acid, has been developed for topical anti-acne treatment. It has also shown potential in mitigating symptoms associated with yeast and fungal colonization, such as in dandruff, seborrheic dermatitis, and tinea pedis (Howell, 2007). The combination of oligopeptide-10 with a peptide capable of downregulating cytokine-mediated responses involving interleukin-6 and interleukin-8 may have multiple applications, particularly for sensitive skin. Such a product would combine the benefits of binding pro-inflammatory toxins and reducing redness. Recent findings also suggest that a lack of modulation of peptides of the innate system may contribute to various skin conditions (Schauber & Gallo, 2009). Notably, mounting evidence has shown that overexpression of a pro-inflammatory component of LL-37 is an important contributor to the development of rosacea (Yamasaki et al., 2007).

2.4 Anticancer activity

Soy protein may also have anticancer effects. Azuma et al. (2000) and Kanamoto et al. (2001) demonstrated that feeding of an insoluble, high-molecular-weight protein fraction prepared from a proteinase-treated soybean protein isolate suppressed colon and liver tumorigenesis induced by azoxymethane and dietary deoxycholate in experimental animals. Bile acid plays a critical role in liver and colon tumorigenesis. The authors hypothesized that the high-molecular-weight protein fraction exerted these protective effects against colon and liver tumorigenesis by interfering with the enterohepatic circulation of bile acids, thus inhibiting their resorption in the intestine and increasing their fecal excretion. Peptides found in the feces of animals fed with the high-molecular-weight protein fraction were rich in hydrophobic amino acids; therefore, the authors suggested that this protein fraction forms nondissociable complexes with bile acids in the intestine through hydrophobic binding, which are then excreted via feces. High-molecular-weight protein fractions may therefore be used in functional foods to prevent the tumor-promoting activity of bile acids in the liver and colon.

2.5 Antioxidant activity

A high level of reactive oxygen species causes DNA, protein, lipid, and carbohydrate damage in the host (Murosaki et al., 2000). Hydrolysates of soy proteins contain antioxidant peptides (Chen et al., 1998). These peptides show high activity against the peroxidation of linoleic acid, paraquat-induced oxidative stress in rats, and scavenging effects on peroxynitrite, active oxygen, and free radical species (Takenaka et al., 2003). They may therefore help prevent some free radical-related diseases.

2.6 Hypotensive activity

The angiotensin-converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) plays an important role in regulating blood pressure. It increases blood pressure by converting inactive angiotensin I to potent vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin (Yang et al., 1970). Therefore, inhibition of the angiotensin-converting enzyme reduces the activity of angiotensin II and increases the bradykinin level, thus lowering blood pressure.

Soybean is a valuable source of inhibitors of the angiotensin-converting enzyme (Ahn et al., 2000; Shin et al., 2001). Inhibitors of this enzyme are now widely used as antihypertensive agents, causing a fall in blood pressure comparable to that produced by thiazides, and calcium antagonists (Pool et al., 1989). Many peptides isolated by the hydrolysis of food proteins have inhibitory activity against the enzyme and reduce blood pressure after oral administration (Ahn et al., 2000; Je et al., 2004; Yamamoto, 1997). Daily use of food with such peptides may be effective in maintaining blood pressure at the normal level.

2.7 Anti-fatigue activity

Exercise-induced fatigue is a listlessness resulting from excessive exertion or "overexercise," leading to diminished bodily and mental functions. It is attributable to the following factors. First, free radicals cause metabolic disturbances; both normal and exhaustive exercises can cause an increase in free radicals in hepatic tissues, resulting in hepatocyte damage (Gul et al., 2006; Voces et al., 1999). Second, exercise causes the production and accumulation of products of metabolism, such as lactic acid. Third, exercise promotes the mobilization and consumption of energy sources such as glycogen (Ikeuchi et al., 2006). In addition, overexercise diminishes the brain's supply of oxygen, which leads to fatigue. Soybean peptides purified by ultrafiltration and gel chromatography have been shown to extend the swimming time of mice, effectively delay increases in their blood lactate level, and enhance the storage of hepatic glycogen.

Soybean peptides are rich in amino acids such as leucine (23.6%), valine (12.22%), lysine (10.01%), isoleucine (8.8%), and phenlyalanine (8.75%). In addition, soybean peptides contain 4.74% aspartate and 5.55% glutamate, which help in performing exercise (Guezennec et al., 1998) and delaying fatigue (Marquezi et al., 2003). A supply of amino acids, especially branched-chain amino acids, can improve exercise ability and markedly retard the catabolism of proteins in muscle during exercise (Blomstrand & Newsholme, 1992). Bazzarva et al. (1992) indicated that the amount of amino acids, especially alanine, glycine, valine, gamma-aminobutyric acid, isoleucine, threonine, serine, and tyrosine, in the plasma decreases rapidly during endurance testing.

Red mold rice contains alanine, valine, and serine, implying that consumption of this rice can enhance exercise ability via its constituent amino acids. In addition, red mold rice contains gamma-aminobutyric acid and glutamic acid, which have positive effects on the nervous system and could help in executing exercise (Guezennec et al., 1998). Marquezi et al. (2003) indicated that aspartic acid is helpful in oxidative deamination and can lower the blood ammonia concentration, thus delaying fatigue.

2.8 Anti-aging activity

Many protein hydrolysates have been used in cosmetic formulations for decades because of their ability to stimulate skin firmness, tone, and elasticity as well as counteract skin aging. Until now, these peptides were poorly studied and their efficacy was poorly demonstrated.

During aging, the epidermis and dermis become thin; therefore, an efficient anti-aging product should be able to stimulate the metabolism of senescent fibroblasts and keratinocytes in order to increase the quantity of extracellular matrix components such as collagen and glycosaminoglycans. A parallel study performed on an in vitro skin-equivalent model and with human volunteers demonstrated the efficacy of a specific soy biopeptide against aging. This biopeptide induced a significant increase in glycosaminoglycan synthesis both in vitro and in vivo after a one-month treatment. This new cosmetic ingredient was also able to stimulate collagen synthesis in vitro and in vivo (Andre-Frei et al., 1999).
Enzyme-inhibitory peptides directly or indirectly inhibit the activity of enzymes. Protein or peptides naturally extracted from soybean seeds can inhibit the formation of proteinases (Centerchem, Inc., Norwalk, CT, USA). Soy protein is frequently used as an anti-aging, skin-moisturizing, anti-solar, cleansing, and hair-promoting agent. In a randomized, double-blind, placebo-controlled study (Sudel et al., 2005), soy extract and placebo creams were applied to the volar part of the forearm of 21 healthy women; the papillary index increased to a significantly greater extent with the soy extract cream than with the placebo cream (4.56 vs. 3.76 arbitrary units, $P < 0.05$). Another study with a pseudorandomized design in 10 Caucasian women indicated the superiority of 2% soy biopeptide emulsion to a placebo in terms of collagen synthesis and stimulation of glycosaminoglycan contents (Andre-Frei et al., 1999).

3. Bioactive peptides produced by gene expression systems

In the recent years, various small peptides isolated from proteolytic hydrolysates of food proteins have shown a variety of physiological activities (Fiat et al., 1993), such as modulating the autonomic nerve system, activating the cell immune mechanism, and improving cardiovascular system function. These discoveries are valuable for exploiting functional foods and developing new medicaments.
The expression of heterologous proteins in microorganisms by genetic recombination is still the high point in the development and exploitation of modern biotechnology. An optimal expression system can be selected only if the productivity, bioactivity, purpose, and physicochemical characteristics of the protein of interest are taken into consideration, together with the cost, convenience, and safety of the system itself. *E. coli* has a typical prokaryotic expression system. Itakura et al. (1977) successfully expressed somatostatin, a mammalian peptide hormone, in *E. coli*, realizing the in vitro expression of a foreign gene in prokaryotic cells. The cost of the bioactive substance produced in medium was similar to that of the protein extracted from the brains of 500,000 sheep. The event is therefore regarded as a milestone in genetic engineering. To date, reformed *E. coli* has been used extensively as a cellular host for foreign protein expression because of its rapid growth, capacity for continuous fermentation, and relatively low cost. Many commercial expression systems designed for various applications and compatibilities are available. The T7-based pET expression system (Novagen, Merck KGaA, Darmstadt, Germany) is the most commonly used in recombinant protein preparation. In general, three forms of foreign proteins can be expressed in *E. coli*: fusion proteins, secreted proteins, and inclusion bodies. *E. coli* cannot perform the post-translational modifications (e.g., *N*- and *O*-linked glycosylation, fatty acid acylation, phosphorylation, and disulfide-bond formation) often required for proper folding of the secondary, tertiary, and quaternary structures as well as for the functional characteristics of the protein of interest. Obviously, these modifications

can affect the bioactivity, function, structure, solubility, stability, half-life, protease resistance, and compartmentalization of the protein (Jung & Williams, 1997).
Figure 2 shows strategies to obtain bioactive peptides from soybean. To the best of our knowledge, the use of genetic engineering instead of other synthetic methods to obtain a protein or peptide is both time-saving and cost-effective. We have successfully used a commercial *E. coli* expression system to express recombinant lunasin (Liu & Pan, 2010). In the following section, we present a representative method to obtain recombinant lunasin by using an *E. coli* expression system.

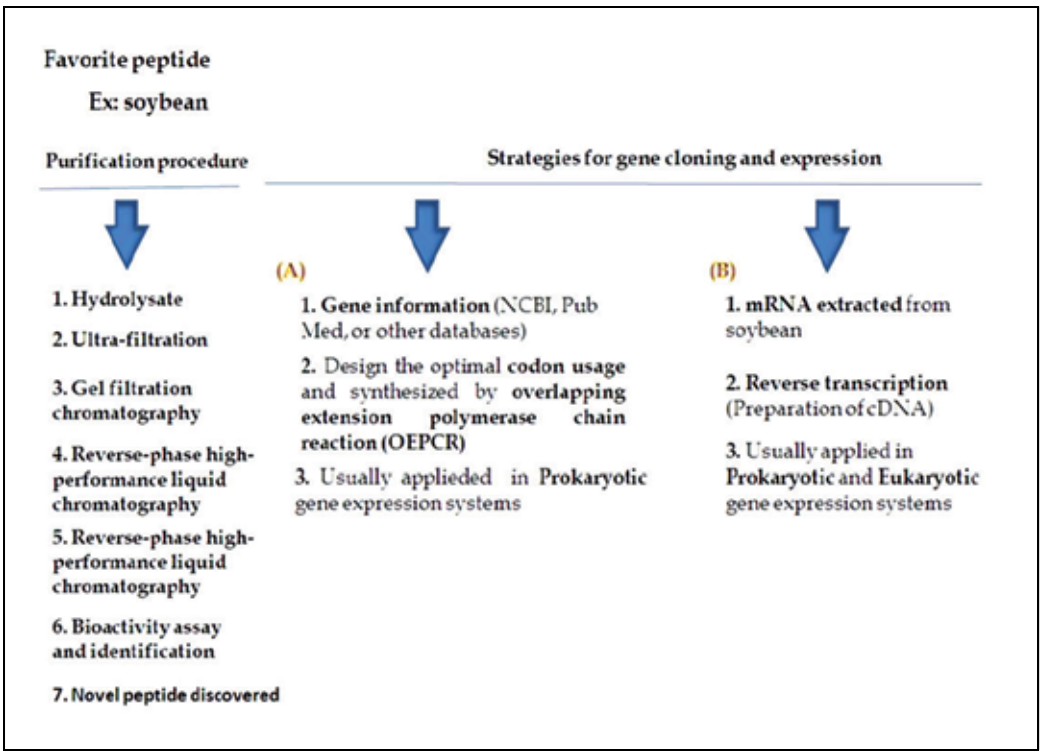

Fig. 2. Strategies for the purification and cloning of bioactive peptides from soybean.

4. Recombinant lunasin

4.1 Bioactive lunasin in current research

Lunasin is a 43-amino acid peptide with nine aspartate residues (D) at the C-terminus, an Arg-Gly-Asp (RGD) cell adhesion motif, and a conserved region of chromatin-binding proteins (Odani et al., 1987a, 1987b). Previous studies showed that lunasin suppresses carcinogenesis triggered by chemical carcinogens and oncogenes both in vitro and in a mouse skin cancer model (Galvez et al., 2001). Hsieh et al. (2010) recently evaluated the anticancer properties of lunasin in breast cancer by using a xenograft mouse model. The US Food and Drug Administration recommends the consumption of 25 g of soy protein per day to lower cholesterol levels and reduce the risk of heart disease (Xiao, 2008); this supplies approximately 250 mg of lunasin. Whether this dose is sufficient for chemoprevention still needs to be determined.

In a human feeding trial for detecting the presence of lunasin in plasma after soy protein consumption, lunasin was found in the circulation, a key requirement for its bioactivity in the target organs (Dia et al., 2009). Recently, some studies showed that lunasin can reduce low-density lipoprotein and total cholesterol levels by directly inhibiting gene expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase, which reduces cholesterol biosynthesis, and increasing low-density lipoprotein receptor expression, which enhances clearance of plasma low-density lipoprotein cholesterol (Galvez, 2001). In 2001, Galvez applied the *Pichia* yeast expression system for large-scale production of lunasin. Besides, lunasin and lunasin-like peptides isolated from defatted soybean flour can inhibit inflammation in lipopolysaccharide-induced RAW 264.7 cells by suppressing the nuclear factor kappa B pathway (de Mejia & Dia, 2009). In addition, synthetic lunasin has antioxidant properties such as linoleic acid oxidation-inhibitory and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical-scavenging activities (Hernández-Ledesma et al., 2009). Recently, lunasin from soybean was found to promote apoptosis of HT-29 colon cells by activating mitochondrial pathways and inducing nuclear clustering (Dia & de Mejia, 2010).

4.2 Experimental design involving genetic synthesis

Figure 3 shows the experimental design for obtaining recombinant lunasin.

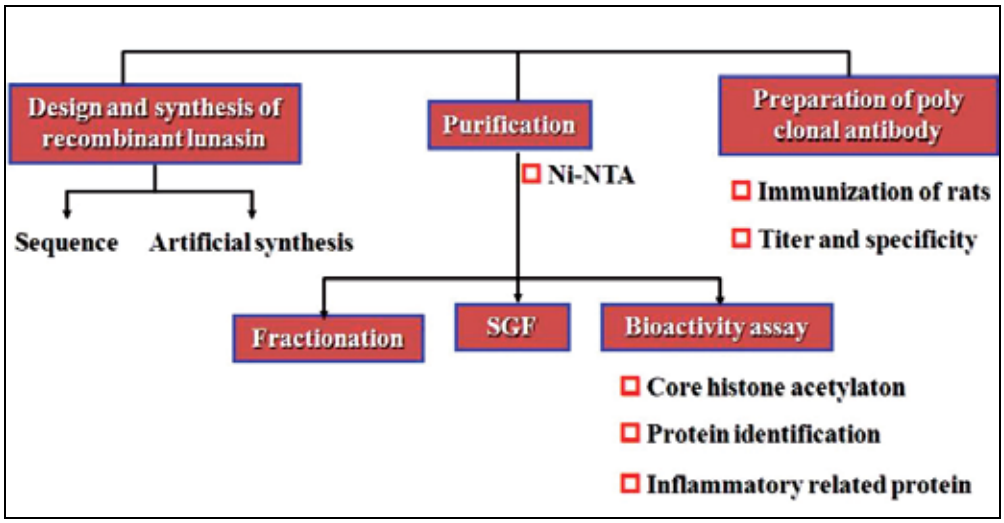

Fig. 3. Flow chart of a method for obtaining recombinant lunasin via *E. coli* (Liu & Pan, 2010).

In the experiment, the lunasin sequence from soybean was codon-optimized for high expression in *E. coli* by changing the original sequence of lunasin (Odani et al., 1987b) to highly frequent codons used in the host. As shown in Figure 4, synthesis of the lunasin gene was achieved by an overlapping extension polymerase chain reaction strategy using four oligonucleotides.

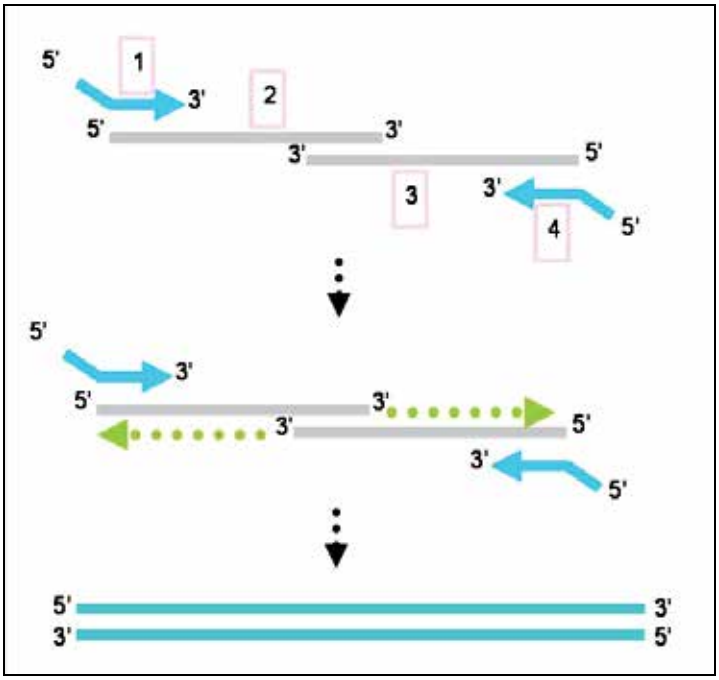

Fig. 4. Schematic of the overlapping extension polymerase chain reaction. Primer 2 and 3 were used as template primers; primer 1 and 4 were used as forward and reverse primers.

4.3 Purification and production

Lunasin is a polypeptide derived from the small subunit of soybean cotyledon-specific 2S albumin. The current research is based on extraction by plant or chemical synthesis of this polypeptide (Galvez & de Lumen, 1999). The purification of lunasin from plant sources is tedious. To date, lunasin has been isolated from various plants such as soybean (Odani et al., 1987b), barley (Jeong et al., 2002), herbs (*Solanum nigrum* L.; Jeong et al., 2007a), wheat (Jeong et al., 2007b), and amaranth (*Amaranthus hypochondriacus*; Silva-Sanchez et al., 2008). Natural lunasin has been extracted and purified by ion exchange chromatography and immuno-affinity column chromatography. Commercial soy products contain reasonable amounts of lunasin, ranging from 5.48 mg of lunasin per gram of protein (defatted soy flour) to 16.52 mg of lunasin per gram of protein (soy concentrate) (Jeong et al., 2003). The lunasin content of extracts from different varieties of barley ranges from 5.93 to 8.71 mg of lunasin per gram of protein (Jeong et al., 2002) and 36.4 mg of lunasin per gram of protein in *Solanum nigrum* L extract (Jeong et al., 2007a). Wheat and amaranth extracts have 3.95–17.2 mg lunasin per gram of protein (Jeong et al., 2007b) and 11.1 µg lunasin per gram of protein (Silva-Sanchez et al., 2008), respectively.

Some heterologous eukaryotic proteins such as human insulin-like growth factor II, alpha-interferon, and interleukin-2 have been expressed in *E. coli* (Dijkema et al., 1984). In our experiment, recombinant lunasin containing *his*-tag at the C-terminus was expressed in soluble form, which could be purified by immobilized metal affinity chromatography (Figure 5). After 4 h, the expression level was above 4.73 mg of recombinant *his*-tagged lunasin per liter of Luria–Bertani broth. This method does not affect the bacterial growth

and expression levels. Consequently, it is easier to use an *E. coli* genetic expression system to express a heterologous protein than to extract the protein from plants.

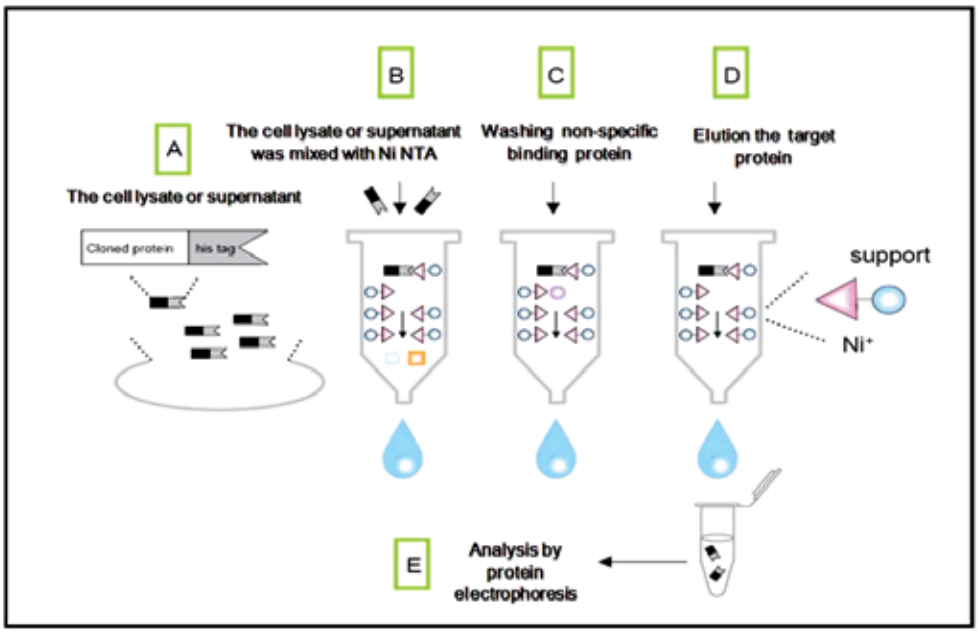

Fig. 5. The flow chart of recombinant lunasin purified by IMAC. (Liu & Pan, 2010)

4.4 Bioactivity

For detecting the bioactivity of the recombinant peptide from *E. coli*, we used M10 and Raw 264.7 cells to evaluate the levels of histone acetylation and anti-inflammation. We found that recombinant *his*-tagged lunasin expressed by *E. coli* decreases acetylation in M10 cells by approximately 26.6%. The results of a histone acetylation assay depend on the cell model and source of lunasin. Galvez et al. (2001) suggested that 2 μM of lunasin isolated from soybean can efficiently prevent histone acetylation in C3H and MCF-7 cells. Jeong et al. (2002) reported that *N*-butyrate-treated NIH3T3 cells show an approximately 18-fold decrease in H3 acetylation after treatment with lunasin extract from barley seeds. In MCF7 cells, H3 acetylation can be completely inhibited by lunasin (Jeong et al., 2002). Recombinant *his*-tagged lunasin is a 92-amino acid peptide, almost double that of native lunasin (43-amino acid residues) obtained from plants. The extra amino acid residues at the N- and C-terminals do not appear to affect the bioactivity of the recombinant protein.

Macrophage activation is associated with a significant proportion of lipopolysaccharide-induced inflammation. During this process, active macrophages produce cytotoxic inflammatory mediators, including reactive oxygen and nitrogen intermediates, hydrolytic enzymes, lipid mediators, and inflammatory cytokines (Laskin & Pendino, 1995). In lipopolysaccharide-induced macrophages, nitric oxide is mainly produced via inducible nitric oxide synthetase (Knowles & Moncada, 1994), which is synthesized via the nuclear factor kappa B pathway, similar to tumor necrosis factor-alpha and other pro-inflammatory cytokines such as interleukin-1beta, interleukin-6, and interleukin-12. Dia et al. (2009) were

the first to indicate that lunasin isolated from defatted soybean flour has some anti-inflammatory activity in RAW 264.7 cells; they found lunasin can inhibit the cyclooxygenase 2–prostaglandin E2 and inducible nitric oxide synthetase–nitric oxide pathways. De Mejia and Dia et al (2009) reported that lunasin and lunasin-like peptides purified from defatted soybean flour inhibit inflammation in lipopolysaccharide-induced RAW 264.7 macrophages by suppressing the nuclear factor kappa B pathway. Chemically synthesized lunasin also has some antioxidant and anti-inflammatory properties (Hernández-Ledesma et al., 2009). Recombinant lunasin produced from *E. coli* can inhibit the release of pro-inflammatory cytokines (tumor necrosis factor-alpha and interleukin-1beta) and nitric oxide production. (Liu & Pan, 2010)

5. Conclusion

We have presented several opportunities for the industrial exploitation of soy-derived bioactive peptides to enhance health and prevent disease. In this chapter, we have introduced the physiological properties of bioactive peptides from soybean and provided an example on the expression of a recombinant peptide by genetic engineering. Choosing the right expression system requires adequate time and makes the difference between success and failure. Once the protein is expressed, a suitable purification scheme can be planned. As shown in Table 1, many bioactive peptides from soybean have been identified and can be applied in genetic expression systems to express important genes.

Biological properties	Peptides	Reference
Hypocholesterol peptides	WGAPSL Ile-Ala-Glu-Lys Leu-Pro-Tyr-Pro	Zhong et al., 2007 Nagaoka et al., 2001 Kwon et al., 2002
Anticancer	X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr	Kim et al., 2000
ACE inhibitory peptides	Asp-Leu-Pro	Wu and Ding, 2001
Hypotensive	Tyr-Val-Val-Phe-Lys Pro-Asn-Asn-Lys-Pro-Phe-Gin Asn-Trp-Gly-Pro-Leu-Val Ile-Pro-Pro-Gly-Val-Pro-Tyr-Trp-Thr Thr-Pro-Arg-Val-Phe RPLKPW	Kodera and Nio, 2002 Korhonen and Pihlanto, 2003
Antihypertensive	PGTAVFK	Kitts and Weiler, 2003
Immunostimulating peptide	MITLAIPVNKPGR MITLAIPVN MITL	Yoshikawa et al., 2000
Antioxident peptide	LLPHH	Korhonen and Pihlanto, 2003
Adipogenesis inhibitor	Ile-Gln-Asn	Kim et al., 2007

Table 1. Some examples of bioactive peptides derived from soybean

6. References

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15

Soy Isoflavones as Bioactive Ingredients of Functional Foods

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1. Introduction

Soybean (*Glycine max*) is one of the most important agricultural commodities. It contains considerable amounts of nutrients (proteins, fatty acids, minerals, vitamins) and constitutes a well known source of bioactive phytochemicals with health-promoting effects, including oligosaccharides, lectins, trypsin inhibitors, saponins, phytates, phytosterols, and isoflavones (IFs). This legume contains the highest amount of IFs, in the range of 0.1-0.4% dry weight (Liu, 2006), the main species being daidzin (4',7-dihydroxyisoflavone), genistin (4',5,7-trihydroxyisoflavone), and glycitin (7,4'-dihydroxy-6-methoxy-isoflavone), the β-glucosides of daidzein, genistein, and glycitein, respectively. These compounds may be found in the free or the conjugate forms: glucosides, acetylglucosides, and malonylglucosides. In the acetylglucoside form, IFs are named as 6''-*O*-acetyldaidzin, 6''-*O*-acetylgenistin, and 6''-*O*-acetylglycycitin. In the malonylglucoside form, their respective names are 6''-*O*-malonyldaidzin, 6''-*O*-malonylgenistin, and 6''-*O*-malonylglycitin.
Since IFs are phytoalexins, their concentrations increase in times of plant stress, such as reduced moisture, and are influenced by the environmental conditions. The IF levels and distribution of isomers in soybeans depend largely on the genotypes of the soybean variety, the crop year and environmental factors such as the location and sowing/harvesting periods (Eldridge & Kwolek, 1983; Kitamum et al., 1991; Wang & Murphy, 1994a; Aussenac et al., 1998). Total IF content in soybean has been described in the range of 300 μg/g to greater than 3,000 μg/g among the United States Department of Agriculture (USDA) soybean germoplasm collection (www.ars-grin.gov/var/apache/cgi-bin/npgs/html). Considerable data on the IFs content of foods are available from USDA (1999) and Jackson & Gilani (2002). In general, the amounts of IFs range from *ca*. 1 to 4 mg/g in soybeans and 0.5 to 2.6 mg/g in traditional soy foods such as tofu (Wang & Murphy, 1994b).
A universal definition for the term "Functional Food" has not been established, and various countries and groups of countries apply different meanings to the term. However, it is accepted that functional foods are aimed to promote health and well being of the consumers. While in some countries foods that supply high amounts of nutrients are considered as functional, as well as natural unprocessed foods such as fruits and vegetables, there is a trend towards the use of the term exclusively for those foods that exert beneficial effects beyond their nutrients only. Consequently, the bioactive components should impart

health benefits beyond basic nutrition. The beneficial effects of functional foods are attributed to the presence of putative bioactive compounds, and their benefits should be demonstrated by means of adequate laboratory and clinical trials. The consumers have the right to know what bioactive compounds are contained in a functional food, the amounts per serving and how it should be consumed, as well as if the compounds are absorbed and exert their action via blood distribution into target organs or if they act locally in the gastrointestinal tract, among other issues. It is the role of the governmental agencies to analyze, accept or reject the proposed health claims, based on the significant scientific agreement standard of evidence available (ADA, 2009; ILSI Europe, 2010)

The bioactive properties of soy IFs have been evaluated by a variety of *in vitro*, *in vivo* and clinical studies providing the rationale for their use in the formulation of various functional foods directed towards the reduction of risk factors of chronic diseases such as cancer, cardiovascular diseases, hypertension, osteoporosis, and neurodegenerative diseases. A series of concentrated and purified soybean products are found in the market, including pure forms of the putative health-promoting IFs. However, the results of the studies designed to demonstrate the beneficial effects of soy IFs represent a challenge due to a series of complications that may lead to unresolved issues. The aim of this chapter is to describe some of the considerations that should be taken into account when analyzing the formulation of novel functional foods containing soy IFs in order to be able to sustain their beneficial effects and accept health claims to communicate them to the consumer.

2. Bioavailability of soy IFs

2.1 Absorption of soy IFs

The absorption of IFs differs among populations due to factors such as the composition of the intestinal microflora (Xu et al., 1995), dietary habits, and ethnic background (Zubik & Meydani, 2003). The first phase of IF absorption, up to one hour, is impaired in lactose malabsorbers, which suggests a role for lactase, but overall this is compensated by microbial hydrolysis, and Tamura et al. (2008) observed that total absorption was not significantly affected by lactose malabsorption. The bioavailability of IFs is further influenced by their chemical form in foods, their hydrophobicity and susceptibility to degradation, and the food matrix (Birt et al., 2001). Izumi et al. (2000) and Kano et al. (2006) found a greater bioavailability of daidzein and genistein, but not their glucosides, whereas Setchell et al (2001) reported a more efficient use of glucosides. Other studies reported that the absorption of aglycones and glucosides was similar (Tsunoda et al., 2002; Richelle et al., 2002; Zubik & Meydani, 2003). It seems that IF aglycones are absorbed faster than glucosides, due to their greater hydrophobicity and a smaller molecular weight, whereas glucosides have lower absorbability and must be converted to aglycones.

The absorption of IFs is highly dependent on their chemical form, and the β-glucosides require hydrolysis to aglycones to be absorbed by the gut and exert their potentially protective effects (Setchell et al., 2002a; Zheng et al., 2003). In fact, free IFs reach peak plasma levels before the corresponding glycosylated forms (Setchell et al., 2001). Hydrolysis occurs along the entire length of the intestinal tract by the action of both the brush border membrane and the bacterial β-glucosidases and β-glucuronidases (Day et al., 1998; Manach et al., 2004), while β-glucuronidases and sulphatases participate in the reabsorption process of the hepatic conjugates and biliary excretion (Xu et al., 2000). The aglycones and bacterial metabolites are absorbed from the intestinal tract to undergo enterohepatic recycling

(Sfakianos et al., 1997). IFs undergo extensive biotransformation catalyzed by hepatic cytochrome P450 (Kulling et al., 2001), producing metabolites that exert antioxidant activity (Rüfer & Kulling, 2006).

The colonic microflora plays important roles in the metabolism of IFs. Daidzein may be metabolized to form two IFs: equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) and O-desmethylangolensin (Xiao, 2008). Only near 30% soy consumers produce equol (Lampe et al., 1998; Setchell et al., 2002b). The ability to produce equol may be determinant of the beneficial effects of IFs, since subjects that are able to form this metabolite exhibit enhanced responses to diets containing IFs (Duncan et al., 2000; Setchell et al., 2003a; Akaza et al., 2004; Vafeiadou et al., 2006).

De Pascual et al. (2006) determined the effect of food matrix on the levels of IFs attained in serum and urine in healthy postmenopausal women given an oral dose of three different foods containing 50 mg IFs on three separate occasions. They observed a lower total urinary recovery of genistein following ingestion of juice (61%) in comparison with solid foods (66% and 70% for bars and cookies, respectively). The levels of daidzein were not altered by food matrix and none of the volunteers appeared capable of converting this precursor to equol. Serum peak genistein concentrations were attained earlier following consumption of liquid matrix, although the differences were not statistically significant. When the authors compared the IF concentrations after the technological processing of the different test foods, they only found differences in aglycone levels.

Bacterial species of bacteroides, bifidobacteria and lactobacilli have the highest β-glucosidase activity (Xu et al., 1995). Furthermore, an increase of the intestinal β-glucosidase activity with the chronic ingestion of soy has been observed (Wiseman et al., 2004). Nielsen & Williamson (2007) summarized data from 16 studies on factors affecting the bioavailability of IFs, reporting that it increases with a rapid gut transit time and low fecal digestion rates and decreases with dietary fiber. Dietary prebiotics (such as fructooligosaccharides, FOS) increase the intestinal bioavailability and affect the metabolism of IFs in rats (Uehara et al., 2001), and increase microbial production of equol in mice (Ohta et al., 2002). However, Sung & Choi (2008) assayed different doses of IFs in rats (up to 500 mg/kg diet) and observed that an increased plasma equol level did not offer additional protection to that provided by FOS against colon carcinogenesis. These results indicate that equol production is not directly related to a health promoting effect, since an inhibitory effect on colon cancer in a favorable gut ecosystem was not observed. Moreover, Larkin et al. (2007) observed that the addition of neither probiotic bacteria nor resistant starch (prebiotic) to a soy diet significantly affect IFs absorption or metabolism. The authors propose that since the probiotics *L. acidophilus* and *B.bifidus* do not increase β-glucuronidase activity in the human gastrointestinal tract, the lack of effects observed in the study may indicate that β-glucuronidase activity is more important than β-glucosidase activity in IFs bioavailability.

An important factor affecting the efficiency of cellular uptake by passive diffusion is the affinity of the IF molecules to the cellular membrane. Increased order of affinity to liposomal membranes have been reported as genistin = daidzin < daidzein < genistein < flavonoid aglycones (Murota et al., 2002).

2.2 Bioavailability of IFs

One of the requisites a functional food must comply is the demonstration of the bioavailability of the putatively active compounds it contains in order to allow their

adequate distribution in body tissues and target cells in physiological concentrations. This is currently assessed by measuring the bioactives in blood (plasma) and/or their urinary excretion. In fact, the rate of excretion of a compound in urine is usually directly proportional to the systemically bioavailable fraction. In general terms, although the oral bioavailabilities of flavonoids are low (Hu, 2007), the concentrations of their phase II metabolites, in particular glucuronides in the body are still appreciable and some of these metabolites are also demonstrated to be bioactive (Zhang et al., 2007).

IF levels in urine and plasma have been widely used as biomarkers of IF intake (Yamamoto et al., 2001; Nagata et al., 2006). Franke et al. (2009) reported a good correlation between peak concentrations of plasma daidzein and genistein and their concentrations in the first 24 h urine following soy consumption. Elimination of IFs is predominantly via the urine (mainly equol), although total recovery by mass balance is less than 50%, suggesting that unrecognized metabolites are being formed. In populations that consume small to moderate amounts of soy only occasionally, adherence should be assessed because urinary IFs reflect primarily the intake within the past 48 h. Turner et al. (2003) emphasize the role of the gut microflora on the bioavailability of IFs by stating that studies involving the metabolism of functional food components should focus on the bacteria inhabiting the small intestine, where absorption takes place, in order to facilitate the manipulation of factors influencing their activity and hence bioavailability of IFs.

Setchell et al. (2003a) observed that IFs are absorbed relatively quickly, attaining maximum serum concentrations from 2 to 8 h after ingestion, with means for daidzein and genistein of 6.1 and 5.0 h, respectively. The bioavailability, obtained from the area under the curve (AUC) of plasma concentration *vs*. time, showed a curvilinear relationship with increasing levels of IFs ingested, especially in the dose range of 0.4–1.8 mg/kg body weight. The authors attribute the reduced systemic availability to reduced absorption of IFs with increasing levels of intake. The pharmacokinetics and systemic bioavailability of β-glycosides has been found to be greater than the corresponding aglycones in dose-normalized AUCs, and relatively small proportions of aglycones appear in plasma even after an ingestion of high amounts of these compounds (Setchell et al., 2001). The actual composition of most IF–rich ingredients to be used in functional foods and dietary supplements are not completely known, and it would be very advantageous to direct the chemical composition of the formulation towards the expected clinical effect expected. A review of the analytical methods available for the analysis of IFs in foods, supplements and biological samples was recently published by Hsu et al. (2010).

Zhou et al. (2008) demonstrated that the absorption, biotransformation, and excretion of genistein show a nonlinear dose-dependent relationship at high doses in rats. They observed that genistein (free and glucuronidated) can be detected in plasma within 5 min after oral administration, indicating that this compound can be quickly absorbed and metabolized in the gastrointestinal tract. The authors reported that the primary form of genistein in plasma is the glucuronidated form due to the action of the gut microflora (Sfakianos et al., 1997). In effect, the majority of metabolite is genistein-7-O-β-D-glucuronide; other metabolites include genistein-4′-O-sulfate and genistein-4′-O-sulfate-7-O-β-D-glucuronide, which are formed in smaller amounts (Yasuda et al., 1996; Prasain et al., 2006). Since glucuronidated genistein can be further deconjugated by glucuronidase in the intestine, the released genistein can be absorbed, metabolized, and excreted for a second time (enteric recycling and enterohepatic circulation). Thus, the exposure time of the body to genistein is prolonged. However, its bioavailability is low, due to its poor absorption and its significant first-pass metabolism (glucuronidation and sulfation) (Chen et al., 2005).

Urinary IFs are frequently used as biomarkers of their potential bioavailability from foods or supplements rich in these compounds (Faughnan et al., 2004). Urine is easy to collect and contains 100-fold higher concentrations of IFs as the plasma levels (Setchell et al. 2001, 2003b). It is usually collected for 24 h and correlates well with serum assessment of systemic bioavailability of IFs (Setchell et al. 2003a). Additionally, quantitative assessment in urine provides information on the extent of intestinal metabolism of IFs and subject compliance in intervention trials.

Daidzein and genistein are the two main IFs excreted in the urine after soy foods are consumed, and daidzein is always excreted in greater amounts than genistein in the urine of adults (Kirkman et al., 1995). However, only a small proportion of dietary IFs are excreted in urine (1–25%). Therefore, they may be not absorbed from the gut, absorbed and released in bile followed by faecal excretion, or metabolized by gut microflora or the liver (Scalbert & Williamson, 2000).

On the other hand, Lampe et al. (1999) observed higher urinary lignan and phytoestrogen excretion in individuals consuming higher amounts of fruits and vegetables as well as sex differences in IF excretion, since men exhibit higher urinary excretion of genistein and daidzein. In fact, the urinary recovery appears to be influenced by gender and the food matrix (Lu & Anderson, 1998), with longer half-lives for daidzein and genistein in females compared with males. Moreover, the production of equol differs among postmenopausal women populations, and Chanteranne et al. (2008) classified the population of different countries in three groups, according to the magnitude of equol production, as high, medium and very low. The authors observed that French volunteers were the main equol producers (42%), in opposite to Italy (30%) or the Netherlands (21%). In each country, daidzein concentrations in plasma were lower than those of genistein, which reflect the ingested proportions, and the reverse was observed in urine.

As stated by Martin et al. (2008), collectively current data suggest that the bioavailability of soy IFs is in the range of 20-30% in both animals and humans, reaching plasma concentrations in the range of high nanomolar to low micromolar in both animals and humans.

2.3 Types of studies

In order to substantiate health protective functions claims, appropriate human-intervention trials and other clinical studies must be made. Intervention studies are quite complex, since they demand the participants to restrain completely from certain foods or drinks and to be randomly assigned to consume a test product or placebo even for several years, among other requisites. To overcome the need for long-term intervention studies, alternatives include the use of validated biomarkers to predict certain disease risk factors, which represents a challenge. In the latter years, metabolomic techniques that help to identify the response of each individual to the dietary intake of bioactives constitute a promising method for studying mechanisms of action (Gibney et al., 2005; Fardet et al., 2008).

Many conflicting results have been obtained stating the relationship between soy IFs and health-related endpoints. This is often associated to a reductionist approach to the study, assuming that the effects of feeding a soy food reflect the activity of one or a few related soy components; that the activity of a purified soy component reflects the effects of eating whole soy foods; or that soy foods equal IFs that are either estrogenic or antiestrogenic, which explains the biological effects observed. Animal studies demonstrate that these assumptions are false (Naciff et al., 2005; Badger et al., 2008; Chen et al., 2008; 2009; Singhal et al., 2009).

Bioavailability and potential modes of action of various soy constituents differ, and most ingredients act via multiple mechanisms (van Ee, 2009). In addition, soy constituents may potentially interact synergistically in maintaining/obtaining study endpoints.

In vitro assays are usually performed to explore bioactivity. The main flaw of these studies is due to the use of supraphysiological levels of isolated IFs or mixtures of IFs in cell cultures, over- or underestimating *in vivo* effects (Stevenson & Hurst, 2007). Therefore, the relevance of these assays to human situations is uncertain and the results should be interpreted with caution, although they may be helpful in determining the mechanisms by which soy IFs may exert their effects at cellular and molecular levels (Erdman et al., 2004). On the other hand, animal models are not completely comparable to humans. IF metabolism in rodents and nonhuman primates differ markedly from that of humans (Gu et al., 2006). Moreover, animals may be fed with very high amounts of soy, at levels exceeding what can be administered in clinical trials. If the IFs are administered to animals by injection, they would bypass the gastrointestinal tract and liver and may also exceed the exposure of human consumption.

The bioavailability of IFs depends upon factors such as solubility, partition coefficient, permeability, metabolism, excretion, target tissue uptake, and disposition of the bioactives (Karakaya, 2004), all of which make the results of the studies controversial. It is critical that IF blood levels (total and aglycone) be evaluated and be comparable to the blood levels observed in human populations consuming IF-containing products. Besides, the intestinal microflora of animals may be more efficient at producing equol and consequently, results of studies in these species may not predict the effect of soy consumption in humans.

In the US, the National Institutes of Health (NIH) launched the Justification for Clinical Research Guidance (http://grants.nih.gov), while the Food and Drug Administration (FDA) published the Clinical Trials Guidance Documents (http //www.fda.gov/RegulatoryInformation/Guidances/ucm122046.htm), which establish the variables to be taken into consideration in cell culture, animal and clinical studies as well as product characterization, stability, and analytical methods used to determine product integrity, among others. For instance, although epidemiological studies of Asian populations provide the background for many clinical studies, the type of soy Asians consume often differs from that consumed by other populations. Also, few dose-response trials have been conducted in humans, so it is difficult to estimate with confidence the threshold amount of soy needed to exert various physiologic effects *in vivo*.

The NIH commissioned the National Center for Complementary and Alternative Medicine and the Office of Dietary Supplements to review the evidence-based scientific reports on the effects of soy intake, through the Agency for Health Care Research and Quality (AHRQ) (Balk et al., 2005). The document, titled "Effects of Soy on Health Outcomes," summarized the formulations of soy products and/or soy food used in clinical trials and the current evidence of the health effects of soy and its constituents on cardiovascular disease, menopausal symptoms, endocrine function, cancer, bone health, reproductive health, kidney function, cognitive function, and glucose metabolism. Specifically, the NIH launched the Soy Research Guidelines, a document that addresses the following items to be considered in a study: 1) The need for sound justification for studying the health effects of soy in humans; 2) Approaches to understanding and ensuring product composition and integrity; 3) Methods for assessing exposure to non-study soy and intervention adherence; 4) Some appropriate analytical methods to test the products; 5) The importance of understanding how soy is processed and how it acts in the body: and 6) The role that genetic makeup may play in the health effects of soy (Klein et al., 2010).

An important source of conflicting results is the use of diverse forms of soy products. The soybean contains 12 forms of IF isomers, including the 3 aglycones, their respective β-glycosides, and 3 β-glucosides, each esterified with either malonic or acetic acid. The type and concentrations of these isomers in foods will vary depending on the plant part from which they are derived and the method by which they are processed (Coward et al., 1993; Erdman et al., 2004; Choi & Rhee, 2006). For instance, some soy products are designed to be very bland and are made from soy flour that has been treated with hot aqueous ethanol. Since this solvent extracts the IFs, these products are essentially IF-free (Barnes, 2008). Total IF content has been reported in the range of 60 to 340 mg/100 g for soy ingredients such as defatted and whole soy flours (90-95% glycosylated), soy protein isolates (20-55% aglycones) and textured soy proteins (90-95% glycosylated, but 15-25% acetylglycosides) (Genovese & Lajolo, 2010).

Since the IF content of foods is often reported without indicating whether it refers to aglycone or glycoside, Klein et al. (2010) propose the use of the term "aglycone IF equivalents" to describe the bioactive form of IFs, since cleavage of the glycosides is probably required before the compounds can be absorbed. As a result, IF values could be converted to aglycone equivalents if desired.

3. Health claims

3.1 Cardiovascular health

The beneficial effects of fruits and vegetables have been largely ascribed to polyphenols, since these bioactives affect dyslipidemia and atherosclerosis; endothelial dysfunction and hypertension; platelet activation and thrombosis; the inflammatory process associated with the induction and perpetuation of cardiovascular diseases (Fraga et al., 2010).

In spite of the great amount of scientific reports demonstrating the beneficial effects of IFs in a variety of assays *in vitro, in vivo* and clinical studies, as well as their mechanisms of action, the only health claim currently approved by the US FDA relates the intake of soy protein to the protection of cardiovascular health, establishing that an amount of 25 g of soy protein should be consumed on a regular daily basis (FDA, 1999). The exact mechanism by which soy lowers blood lipids remains unclear, but in 1999 the FDA approved the health claim stating that the inclusion of soy protein into a diet low in saturated fat and cholesterol may reduce the risk of coronary heart disease by lowering blood cholesterol levels. Due to the inconsistency of the results of the studies available, the National Center for Complementary and Alternative Medicine and the Office of Dietary Supplements, both of NIH, reviewed the evidence-based literature and published a report through the Agency for Health Care Research and Quality (AHRQ) (Balk et al., 2005).

The European Food Safety Authority analyzed the claimed effect for soy protein stating that "reduces blood cholesterol and may therefore reduce the risk of (coronary) heart disease" (EFSA, 2006). Clinical studies were provided to sustain the claim, most of which were randomized controlled trials. Meta-analyses and a review of possible mechanisms by which soy protein might exert the claimed effect were all examined. However, most of these studies were not appropriately designed to test the effect of soy protein *per se*, but were conducted using either soy protein isolate (SPI, by definition contains 90% protein) or soy foods containing other constituents that may exert an effect on blood cholesterol in human intervention studies (e.g., fat and fatty acids, fiber, IFs). The Panel considered that the design of the studies on SPI did not address the effects of the food constituent that is the subject of

the health claim on LDL-cholesterol concentrations. Then new intervention studies were included in a new meta-analysis which aimed to address the effects of soy protein *per se* on blood cholesterol concentrations, and there was a statistically significant dose-response relationship between the intake of IFs and the decrease in total and LDL-cholesterol concentrations. One study was designed to assess the effects of IF-containing and of IF-free SPI on biomarkers of cardiovascular risk, including blood lipids. No significant differences were observed between the SPI with no IFs (or the SPI with IFs) and the control group with respect to changes in total or LDL-cholesterol concentrations during the study. The EFSA Panel concluded that this study did not support an effect of the protein component of soy on LDL-cholesterol concentrations.

In weighing the evidence, the EFSA Panel took into account that the results from human intervention studies identified as being controlled for the macronutrient composition of the test products did not support an effect of the protein component of soy on LDL-cholesterol concentrations, and that the proposed mechanism by which the protein component of soy would exert the claimed effect is not supported by available scientific evidence. Consequently, a cause and effect relationship was not established between the consumption of soy protein and the reduction of LDL-cholesterol concentrations (Scientific Opinion on the substantiation of a health claim related to soy protein and reduction of blood cholesterol concentrations pursuant to Article 14 of the Regulation (EC) No 1924/2006, published: 30 July 2010).

The "Dietary Approaches to Stop Hypertension" (DASH) study (Sacks et al., 2001) showed that blood pressure levels may be lowered with a healthy eating plan that includes fruits and vegetables, is low in total fat, saturated fat, and cholesterol. Antihypertensive effects of soy IFs have been reported for over a decade, although the results of the clinical studies and the ability of specific dietary compounds to lower blood pressure is still controversial. Nestel et al. (1997) observed that IFs improve systemic arterial compliance. A cardioprotective effect of genistein was observed in association with its ability to lower blood pressure in postmenopausal women (Teede et al., 2001). However, the same authors (Teede et al., 2006) did not report any beneficial effects on arterial function after three months of soy protein dietary supplementation containing IFs in hypertensive men and postmenopausal women.

A number of the cardiovascular protective actions of IFs have been associated to their effects on thromboxane A_2 (TxA_2), a pro-atherogenic metabolite of arachidonic acid, since the stimulation of TxA_2 receptors activates a series of cell signals involved in the development of atherogenesis (Huang et al., 2004). Genistein inhibits TxA_2-mediated platelet responses (Nakashima et al., 1991), acting as an antagonist of TxA_2 receptors, and the same action has been described for equol (Munoz et al., 2009). These molecules compete with TxA_2 receptors and decrease their density (Garrido et al., 2006). Genistein and daidzein inhibit platelet adhesion and aggregation (Sargeant et al., 1993; Gottstein et al., 2003; Borgwardt et al., 2008) and inhibit the secretory activity of platelets (Guerrero et al., 2005; Munoz et al., 2009).

IFs also exert anti-inflammatory effects, and Huang et al. (2005) observed a 66.7% reduction of TNF-α in postmenopausic healthy women who consumed soymilk containing 112.2 mg IFs for 16 weeks, while Chan et al. (2008) observed a reduction of C-reactive protein with IFs supplement in patients with ischaemic stroke, improving their endothelial function. Soy IFs have been shown to inhibit TNF-α induced NF-κB activation (a transcription factor that regulates genes involved in inflammation, cytokine response, and cell proliferation and survival) in cultured human lymphocytes, growth control and its supplementation in

healthy men was shown to prevent NF-κB activation by TNF-α in blood lymphocytes (Davis et al., 2001). Choi et al. (2011) demonstrated that soybean and two Korean traditional fermented soybean products modulate inflammation-related NF-κB activation in Sprague–Dawley rats fed a high-fat diet. The authors report that the expressions of NF-κB related proinflammatory genes, notably COX-2, iNOS, and that of the adhesion molecule VCAM-1, increased with the feeding of a high-fat diet, but that soybean and fermented soybean products modulated these gene expressions.

Besides, several studies have shown that endothelial nitric oxide synthase (eNOS) expression is increased following treatment with dietary soy (Mahn et al., 2005) or genistein (Squadrito et al., 2003; Si et al., 2008), increasing nitric oxide (NO) production, thus improving vascular function. Joy et al. (2006) showed that rapid activation of eNOS with IFs includes interaction between multiple signaling pathways, involving activation of the ERK1/2 pathway, and activation of the PI3 kinase/Akt pathway (Tissier et al., 2007), resulting in phosphorylation of eNOS and subsequent association of eNOS with heat shock protein 90, which participates in the activation of this enzyme. Mann et al. (2007) reported that an important aspect of the vascular response to IFs involves increased expression of components of cellular antioxidant mechanisms, since IFs may amplify NO signaling increasing NO bioavailability by directly quenching reactive oxygen species. Moreover, IFs also reverse vascular contraction through inhibitory interactions with a number of vascular constriction mechanisms (Joy et al., 2006).

Other actions of soy IFs have been reported that may aid in the cardiovascular protection, such as a decrease of body fat in older ovariectomized mice (Naaz et al., 2003), and rats (Kim et al., 2006), an inhibitory effect on the enlargement of adipose tissue (Ørgaard & Jensen, 2008), and the reduction of fasting blood glucose and lipid levels (Park et al., 2006), contributing to prevent obesity-associated diseases. However, care should be taken when describing these actions, since many of the studies have been performed *in vitro* or animal studies using concentrations that are unexpected to be reached from a dietary intake of IFs.

3.2 Bone health and menopausal symptoms

IFs possess estrogenic activity in animals at concentrations lower than 0.1 μM based on the direct interactions between IFs and estrogen receptors (ERs): ERα and ERβ, providing these polyphenols the ability to act as estrogen agonists or antagonists (Messina, 2010a). This chapter does not describe the effects of soy IFs on circulating levels of estrogens and other hormones in women, and the extensive review and meta-analysis of the literature to examine these effects published by Hooper et al. (2009) is recommended. Since a variety of functional foods especially formulated for women are marketed considering the putative beneficial effects of IF intake on bone health and menopausal symptoms, these subjects are briefly described.

Epidemiological Asian studies have found that postmenopausal women with the highest intake of IF-rich soy foods have the highest bone mineral density (BMD) in the lumbar spine compared with women with low intakes (Somekawa et al., 2001; Mei et al., 2001). The evidence of a bone health protective effect of IFs is associated to their ability to bind selectively to estrogen receptors (Kuiper et al., 1997). These compounds stimulate osteoblasts and inhibit osteoclast activity *in vitro*, effects that are consistent with reduced bone turnover (Rassi et al., 2002; Chen et al., 2003). Besides, animal studies have shown bone-sparing effects of soy protein or IFs (Setchell & Lydeking-Olsen, 2003), while short-term human studies have demonstrated that IFs can reduce bone loss in postmenopausal

women (Potter et al., 1998; Scheiber et al., 2001). In a model of ovariectomized rats, Al-Nakkash et al. (2010) after 2 weeks of genistein treatment (250 mg/kg body weight) observed increased uterine weight, femur weight, and femur-to-body weight ratio, estrogen-like effects that were not associated to oxidative stress. According to Lydeking-Olsen et al. (2004), soy foods with IFs can prevent bone loss of the lumbar spine in postmenopausal women, who may otherwise lose 1.5–3% of bone/year. This prevention of bone loss, if continued into old age, could translate into a decrease in lifetime risk of osteoporosis and a lowering of fracture rates.

The North American Menopause Society (2000; 2006) stated that "the role of IFs in the management of short-term menopausal symptoms as well as diseases related to menopause/aging is still uncertain…" These reports, however, were retired. In a review on the subject of the anti/proestrogenic effects of IFs in breast cancer, Hasler & Kundrat (2002) emphasize that clinical trials are needed in order to clearly establish the potential beneficial effects. Although a series of subsequent papers report beneficial effects of the intake of soy IFs on BMD (Atkinson et al., 2004; Kreijkamp-Kaspers et al., 2004), the situation remains unclear. Tests have been performed using pharmaceutical forms also, and Marini et al. (2007) administered 54 mg/day tablets of purified genistein to postmenopausal women, evidencing a significant increase in BMD in certain locations *vs*. placebo.

More recently, the North American Menopause Society (2010) stated that the evidence of the benefits of the consumption of IFs on bone health is weak, regardless of the food source. In agreement with this statement, the EFSA analyzed the claimed effect of IFs on bone health, related to bone mass, BMD, and bone structure, all of which contribute to bone strength. However, although significant effects on markers of bone turnover and/or on spine BMD have been described in some short-term randomized trials in relation to the dietary intake of soy IFs, longer-term interventions do not support a sustained effect of soy IF intake on markers of bone health. The Panel also took into account the lack of a clear dose-response relationship between the dietary intake of soy IFs and the claimed effect, and the different results obtained depending on the source and nature of the IFs used. In conclusion, it stated that the evidence provided was not sufficient to establish a cause and effect relationship between the consumption of soy IFs and the maintenance of BMD in post-menopausal women (EFSA Scientific Opinion on the substantiation of health claims related to soy IFs and maintenance of bone mineral density (ID 1655) pursuant to Article 13(1) of Regulation (EC) No 1924/2006, published: 1 October 2009).

3.3 Antioxidant effects

The antioxidant capacity of IFs has been observed mostly *in vitro* (Ruiz-Larrea et al., 1997; Rüfer & Kulling, 2006). However, as it has been described to polyphenols in general, IFs may act as indirect antioxidants by up-regulating endogenous antioxidant enzymes (Stevenson & Hurst, 2007; Kampkotter et al., 2008). Borras et al. (2006) observed that genistein at low micromolar physiological levels up-regulates the expression of longevity-related genes in a manner similar to 17 β-estradiol, involving interactions with estrogen receptors, activation of ERK1/2 and NFκB and up-regulation of longevity-related gene expression. *In vivo*, Wiseman et al. (2000) observed that dietary soy IFs decrease $F(_2)$-isoprostane concentrations (a biomarker of oxidation) and increase the resistance of LDL to oxidation in humans. Among the various beneficial effects attributed to their antioxidative properties, dietary soy IFs have been described as neuroprotective in transient focal cerebral ischemia in male and ovariectomized female rats. Consequently, IFs may protect the brain

via increases in endogenous antioxidant mechanisms and reduced oxidative stress (Ma et al., 2010).
With regard to properties such as "protection of DNA, proteins and lipids from oxidative damage", claimed as antioxidant health, the target population was assumed to be individuals performing physical exercise. In the context of the proposed wording, the EFSA Panel assumed that the claimed effect refers to the protection of DNA, proteins and lipids from oxidative damage caused by free radicals that are generated during physical exercise. However, no conclusions could be drawn from the two human studies provided for the scientific substantiation of the claimed effect because they do not distinguish between the effects of soy protein (which is the subject of the health claim) and those of soy IFs on lipid peroxidation. On the basis of the data presented, a cause and effect relationship was not established between the consumption of soy protein and the protection of DNA, proteins and lipids from oxidative damage (Scientific Opinion on the substantiation of health claims related to soy protein and contribution to the maintenance or achievement of a normal body weight (ID 598), maintenance of normal blood cholesterol concentrations (ID 556) and protection of DNA, proteins and lipids from oxidative damage (ID 435) pursuant to Article 13(1) of Regulation (EC) No 1924/2006, published: 19 October 2010).

3.4 Cancer

It has been suggested that a high intake of soy products in Asian populations may have contributed to cancer protection, mainly by lowering the risk at certain locations such as colorectal and breast cancer. The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including free radical scavenging, the modification of enzymes that activate or detoxify carcinogens, and inhibition of the induction of the transcription factor activator protein-1 (AP-1) activity by tumor promoters (Shih et al., 2000). Soy constituents have also been shown to have other anticancer effects, including the inhibition of DNA topoisomerases I and II, proteases, tyrosine kinases, inositol phosphate, and angiogenesis, as well as the boost of immune response and antioxidative effects (Adlercreutz & Mazur, 1997; Taylor et al., 2009).
Genistein is an effective inhibitor of DMBA-induced DNA damage in MCF-7 cells by inhibiting CYP1A1 and CYP1B1. In fact, it inhibits recombinant human CYP1A1 and CYP1B1 (Chan & Leung, 2003). IFs could reduce xenobiotic-induced CYP1A1 and 1B1 mRNA expression through interference with xenobiotic responsive elements (XRE)-dependent transactivation (Moon et al., 2006). XRE are enhancer elements located in the promoter regions of xenobiotic responsive genes, which include genes encoding for CYP1A1 and 1B1, and their expression can be regulated through pathway involving aryl hydrocarbon receptor (AhR). However, Kishida et al. (2004) reported that dietary soy IFs had no effect on the hepatic mRNA abundance of CYP1A1 and 1A2 in rats, determined by real-time quantitative RTPCR. This indicates that dietary IFs may not be able to induce CYPs in either the transcriptional step or through post-transcriptional mRNA stabilization.
Genistein also exerts anticancer properties by modulating genes that regulate the cell cycle and apoptosis (Sarkar et al., 2002; Banerjee et al., 2008). As an antioxidant, genistein decreases reactive oxygen species levels, and it also induces the expression of the antioxidant enzymes superoxide dismutase (SOD) and catalase, which are associated with AMP-activated protein kinase (AMPK) and phosphatase and tensin homolog deleted from chromosome 10 (PTEN) pathways (Park et al., 2010). Genistein has been proposed as a natural alternative to estrogen replacement due to its ability to act via estrogen receptor-

dependent mechanisms, utilizing the phosphatidylinositol 3-kinase /Akt pathway (Tissier et al., 2007). The PTEN protein is a lipid phosphatase and has been suggested to act as a tumor suppressor owing to its inhibition of the PI3K/Akt signalling pathway, and genistein has been reported to promote apoptosis in mammary epithelial cells by inducing PTEN (Dave et al., 2005), accompanied by a decrease in mammary tumorigenesis.

3.4.1 Colorectal cancer

Evidence from *in vitro* and animal studies has implicated soy and soy IFs in colorectal cancer protection. Moreover, a series of *in vivo* studies performed in laboratory animals have shown that soy diets inhibit chemically induced colon tumorigenesis, and IFs may be the putative bioactives playing a role in this inhibitory process (Raju et al., 2009). However, care should be taken when the results of these studies are analyzed, since interactions between soy protein and IFs may occur as well as different effects from purified extracts of IFs and other components such as saponins, in their anticancer effects. Soy saponins may be important anticancer compounds present in soy due to their ability to inhibit tumor cell growth without altering normal colon morphology (MacDonald et al., 2005).

The human studies reported present a series of methodological limitations, particularly with regard to dietary measurement issues, such as incomplete assessment of soy intake, inadequate quantification, and inappropriate time period for cancer prevention as well as inadequate adjustment for confounders (Spector et al., 2003). Yang et al. (2009) in a population-based, prospective cohort of Chinese women with high but varied soy intake found that the risk of colorectal cancer decreased with increasing soy food intake, mainly in postmenopausal women. The authors reported that the risk was 30% lower among women in the upper third of soy food intake level compared with women in the bottom third of soy food intake level.

More recently, Yan et al. (2010) developed a meta-analysis of 11 epidemiologic studies that assessed the association of soy consumption with colorectal cancer incidence in humans. In two of these studies soy intake was found to be associated with a significant reduction in colorectal cancer risk in women, whereas in the other nine studies no such association was observed in either women or men. The authors conclude that consumption of soy foods is not associated with the risk of colorectal cancer or colon or rectal cancer separately.

3.4.2 Breast cancer

An inverse relationship between soy consumption and breast cancer incidence has been observed in Asian populations and attributed to the high intake of soy foods. However, it is noteworthy that Japanese incidence rates of breast cancer have increased markedly during the last decades, along with Westernization of the diet and culture, while simultaneously breast cancer has become the number one type of cancer among Japanese women (http://www.mc.pref.osaka.jp/ocr/). The results of the epidemiological studies published are confusing, and evidence suggests that plasma or serum concentrations of IFs may be a more sensitive predictor of the possible protective effects of soy foods against breast cancer than the assessment of dietary IFs intake (Messina & Wu, 2009), mainly due to the marked differences in IF metabolism that exist among individuals, as well as differences in sources of IFs, differences in biological response to IFs among ethnic groups, interactions with background diet, among others. Consequently, plasma IF levels are more reliable biomarkers compared with dietary IFs intake for evaluating the potential of soy to reduce breast cancer risk.

One of the more intriguing subjects is that the protective effect of soy IFs observed in Asian studies results from lifelong exposure or exposure to IFs early in life. Although the preclinical models have limitations, these studies provide useful information that supports clinical and epidemiological studies, as is the case of the observation of a protective effect from early life exposure to soy IFs against breast cancer (Russo & Russo, 2006; Warri et al., 2008; Lee et al., 2009). Undoubtedly, the most conclusive evidence of health-promoting effects of IFs is obtained through well designed clinical trials, and a better understanding of the mechanisms by which IFs may affect the development of breast cancer is still needed.
It is noteworthy that a controversial question remains associated with the roles of IFs on cancer prevention. In moderation, IFs may exert beneficial effects; however, for some cancers, increased risk has been reported when IF intakes are high (Gee et al., 2000; Daly et al., 2007). In fact, Petrakis et al. (1996) demonstrated that consumption of soy protein isolate had stimulatory effects on the breast tissue of premenopausal women, reporting greater numbers of hyperplastic epithelial cells, while Allred et al. (2001) reported that soy protein isolates stimulated the growth of MCF-7 tumors in a dose-dependent manner as the concentration of genistein increased. Consequently, the IFs have paradoxical effects that should be taken into consideration when dosage and timing of administration are defined. For example, prepubertal exposure to genistein appears to be protective against the development of breast cancer, but consumption of the IFs in either pure form or in soy protein isolate, after development of an estrogen-dependent breast cancer may enhance the growth of that tumor (Allred et al., 2001). Shu et al. (2009) in a population based study of breast cancer survival demonstrated that soy food intake was associated with improved breast cancer survival. The authors showed that women who had the highest level of soy food intake and did not take tamoxifen had a lower risk of mortality and a lower recurrence rate than women who had the lowest level of soy food intake and used tamoxifen, suggesting that high soy food intake and tamoxifen use may have a comparable effect on breast cancer outcomes.

4. Patents

Patents related to soy and/or IFs may be classified into two main groups: a) those dealing with processes developed to obtain extracts and other forms as ingredients suitable to the formulation of functional foods, dietary supplements and/or nutraceuticals, and b) those dealing with the health-promoting effects of innovative products. Among the first group, a series of diverse processes have been developed, and just a few are mentioned in order to exemplify the diversity of subjects covered. The patented processes include, among a wide variety, an encapsulated soy extract that includes IF derivatives: daidzin, glycitin, genistin, daidzein, glycitein and genistein, encapsulated with cyclodextrins or combinations of various oligosaccharides (PCT/US2007/083323); a process to produce a composition containing a high concentration of aglycone IFs using microorganisms which are generally recognized as safe (GRAS), that can express or produce β-glycosidase on a soy-based substrate (Serial No. 358938, Taiwan); a soy protein isolate that has increased amounts of IFs and saponins and a high Nitrogen Solubility Index ("NSI") produced by a process that involves ultrafiltration and the avoidance of isoelectric precipitation. The soy protein isolate has at least about 90.0 wt % protein of total dry matter; an IF content of at least about 1.0 mg/g IFs of total dry matter, and a NSI of at least about 75% (US Patent 7,306,821). Methods of producing from natural soybeans, soybean materials (i.e. tofu dregs, soy molasses) and

other plant sources are also described (Appl. No. 11/622,468, Hong Kong), as well as the recovery of conjugated IFs of residues and sub-products of food industries based on the use of soy and its derivatives, including foods containing IFs and from genetically modified *Aspergillus oryzae* ATCC 22786 (RIB 430), involving a process of conversion of conjugated IFs (malonate and acetates), in glucosylated IFs, which through fermentative and enzymatic processes are transformed into aglycones (US Patent Application 20100048689).

A series of companies have developed various soy-based products marketed to the treatment of various diseases and exhibit expanded patent portfolios, that include uses such as US patent 6,399,072 for the "Method of Preparing and Using Isoflavones for the Treatment of Alcoholism" and US patent 6,391,310 describing the "Method of Preparing and Using Isoflavones for the Treatment of Neurological Symptoms". One of the developed products claims a series of benefits that are a direct result of their patented, natural concentration process (US Patent 6,482,448 "Soy formulas and their use for promoting health"). Products presented as protein shakes or protein bars provide about the same amount of soy IFs found in 6 cups of a typical soymilk (~160 mg of soy). In the US, these products as well as others available should be labeled "*These statements have not been evaluated by the Food and Drug Administration. (Name of brand) ® foods and dietary supplements are not intended to diagnose, treat, cure or prevent any disease. Individual results vary.*"

A series of patents describing the applications of IFs in the prevention of diseases have been published, mainly in the late 1990's and early 2000's, including situations such as cancer (Thurn & Juang, 1999), heart disease (Potter et al., 1999), macular degeneration (Jenks, 1999), as well as to prevent hair loss and maintain hair integrity (Segelman, 2000), to improve deficient skin conditions (Lanzendorfer et al., 1999), to inhibit Alzheimer's disease and related dementias, for preserving cognitive function (Clarkson et al., 1999), to inhibit gram-negative bacterial cytotoxicity (Fleiszig & Evans, 1999), and to treat cystic fibrosis (Hwang et al., 1999), among others.

Many patents deal with the relief of menopausal symptoms, as is the case of preparations such as a composition claimed for the relief and/or prevention of climacteric and menopausal disorders affecting women in pre-, peri- or post-menopause, comprising soy IFs and viable lactic acid bacteria aimed to enhance the absorption of soy IFs (US Patent 7,025,998). In this case, as in most of the patents available, the compositions are provided in dosage forms for oral administration (which constitute actual nutraceutical products, since they are presented as pharmaceutical dosage forms and are not consumed as part of the normal diet, a requisite for a functional food), and some commercial preparations announce pharmacological effects. This is the case of a soy formulation claimed to "promote the health of an individual, preferably utilizing the soy formulations, dietary supplements, food products and/or pharmacological compositions of the invention" (US Patent Application 20030021859). A list of US patents related to soy and soy IF can be seen at http://patft.uspto.gov/while in Europe the list is available at http://www.epo.org/patents/patent-information.html, a website that also allows to search Asian patents at http://www.epo.org/patents/patent-information/east-asian.html. The Google website http://www.google.com/patents may also be visited.

Additionally, due to all the available information about soy IFs that in many cases may be confusing to the consumer, the NIH's Office of Dietary Supplements published the fact sheet available at:

http://ods.od.nih.gov/Health_Information/Information_About_Individual_Dietary_Supplements.aspx

5. Functional foods containing soy IFs

A series of soy based functional foods are currently marketed in many countries, most of which attribute beneficial effects to their IFs content. The so-called functional soy foods include a variety of products e.g. those traditionally fermented such as miso, tempeh, natto, tofu. It should be noted that fermentation of soybeans changes the amount of IFs in them. Amounts of IFs in the fermented soybean products miso (bean paste) and natto (fermented soybeans) are significantly different than those in unfermented soybeans (Fukutake et al., 1996). The amount of genistein in the fermented soybean products is higher than in soybeans and soybean products such as soymilk and tofu. There is a wide variety of IF composition in the different soybean products commercially available as a source of bioactive IFs (Nurmi et al., 2002).

Setchell et al. (2001) analyzed 33 phytoestrogen supplements and extracts available at that time and observed differences in the content from that claimed by the manufacturers. Novel fermented products include those fermented by probiotics that have the potential to reduce the levels of some carbohydrates responsible for gas production in the intestinal system and to change the bacterial composition towards a healthy population (Champagne et al., 2009). The most traditional concentrated and purified soy IFs are produced mostly from soy molasses, soy germ, and defatted soy flakes (Liu, 2004). Other soybean functional foods include dairy products that highlight the absence of cholesterol, lactose and milk proteins; bakery products; substitutes for meat, poultry or fish; and beverages (Jooyandeh, 2011).

Boniglia et al. (2009) observed different ''fingerprints" in 14 soy-based dietary supplements intended to help alleviate perimenopausal and menopausal symptoms on sale in Italy, probably on account of different sources of the soy raw materials and the methods of processing and preparation of extracts. These authors reported total IF levels ranging from 33.75 to 80.00 of the values given by the manufacturers, while Stürtz et al. (2008) quantified the intact IFs (glycosides forms) in different supplements and observed values higher than those declared on the labels. These and other studies show that the IF contents are extremely variable and many times far below the values (34–150 mg) that appear to have some beneficial effects. For this reason it is important to standardize the amount of IFs present in these products. To overcome these inconsistencies, after analyzing a series of commercial products, Collison (2008) recommended an analytical method to be adopted as Official First Action for analysis of total soy IFs in dietary supplements, dietary supplement ingredients, and processed soy foods containing at least 0.5 mg/g total IFs.

One important question that should be addressed when selecting a functional food is the variety and amount of bioactive ingredients it contains. Functional foods containing IFs should be effective to provide the benefits by consuming regular amounts of the products in the diet. Slavin et al. (1998) in a dose-response study suggest that the IFs in soy-protein isolate are bioavailable at amounts as low as 9 mg/day, or about the amount found in 28.4 g tofu (standard serving size), while Frank et al. (1999) reported mean total IF levels ranging from 35 ppm (soy milk) to 7,500 ppm (dietary supplements) in a variety of 25 soy-based foods and dietary supplements. It is important to keep in mind that health outcomes in relation to soy intake may be dependent, to some extent, on the timing and duration of soy exposure, the hormonal status of the individual, the tissue(s) affected, and the amount and/or composition of the soy consumed, and adverse effects should also be reported (Song et al., 2007). While traditional soy foods are comprised of a unique and complex blend of protein, lipids, vitamins, minerals, IFs, and other bioactive compounds that may act

individually and/or synergistically to exert healthful physiologic effects, there are many IF supplements or nutraceuticals currently available that manufacturers claim contain 1000 mg of genistein per dosage form (usually tablets) but for which the efficacy is unknown (Reinwald et al., 2010). These products should not be considered as part of a regular diet, however in many countries they are sold as nutritional aids.

Additionally, all functional foods should be innocuous and secure. Thus, toxicological studies are a requisite for functional ingredients. The NOAEL (no observed adverse effect level) of genistein has been established in 50 mg/kg body weight/day by McClain et al. (2006), who carried out hazard analyses for acute, subchronic and chronic safety of genistein in rats. The US Center for the Evaluation of Risks to Human Reproduction of the National Toxicology Program expert review panel expressed negligible concern for adverse effects in the general population of consuming dietary sources of genistein, concluding that under current exposure conditions, adults would be unlikely to consume sufficient daily levels of genistein to cause adverse reproductive and/or developmental effects (Rozman et al., 2006). Klein & King (2007) reviewed the literature on the potential genotoxicity or cellular effects of high doses of genistein (> 5 μM) *in vitro*, an amount that is not likely to be found *in vivo* due to the low uptake and bioavailability of this compound.

6. Conclusions

Consumers demand the food industry to produce functional foods that contribute to maintain health and prevent diseases, mainly those related to the aging process. However, it is of major importance to provide adequate information in order to protect the consumer's interests by using adequate health claims that are based upon solid scientific support obtained by means of *in vitro, in vivo* and clinical studies that substantiate the bioactivity of the functional ingredients contained in the products.

Many health benefits have been attributed to soy IFs. However, to date there is only one accepted health claim related to dietary soy intake: it refers to soy protein, and is not associated to the various bioactive phytochemicals that are contained in this legume, including IFs. As stated by Messina (2010b) in an extensive review on the evidence of the health-protective effects of soy intake, there is almost no credible evidence to suggest that traditional soy foods exert clinically relevant adverse effects in healthy individuals when consumed in amounts consistent with Asian intake.

Since the actual Western dietary IFs intake is low, an increase in dietary intake of IFs constitutes an interesting target and an alternative is the design of functional foods. A wide diversity of soy ingredients have been developed as a source of IFs that may be used as functional food ingredients. However, the various processes used affect the IF content and profile. The validation of the health claims associated to the dietary intake of putative bioactive IFs is a crucial issue. However, a thorough understanding of their bioavailability from different food products is critical to achieve the desired biological efficacy of these compounds. Clinical studies should be performed, although they may be costly and highly complex due to the various factors involved. Biological effects require sufficient delivery of IFs from the site of administration (the gastrointestinal tract) to the sites of target organs and receptors. One major factor is the bioavailability of IFs, which depends upon factors such as solubility, partition coefficient, permeability, metabolism, excretion, target tissue uptake, and disposition of the bioactives. Moreover, the poor bioavailability of polyphenols makes it even more difficult to conduct relevant but smaller clinical trials because large exposure differences are expected among the participants.

In spite of a myriad of patented processes and products containing soy IFs, including functional foods currently available for health-conscious consumers, most of the metabolic mechanisms of the specific health benefits attributed to IF consumption have not been clearly established and require further research. Consequently, no health claims should be declared for functional foods containing IFs, since they still lack scientific sound evidence.

7. References

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16

Soybean Oil: How Good or How Bad in Comparison with Other Dietary Oils in the Context of Colon Cancer

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1. Introduction

Soybeans (*Glycine Max*) are one of the oldest crops cultivated around the world, and in Asia soyfoods have been consumed for more than 1000 years. The soybean was popularized because of its high content of protein and good percentage of lipids. World consumption of soybeans in 2008 was over 221 million metric tons, with approximately 50% of this supply coming from US production, where over 77 million ha are planted annually. Soybeans are desired in the market as a source of protein and oil. Nowadays, soy has a huge economic importance because the beans are very versatile and can be used not only for human and animal nutrition but also for industrial products (Clemente & Cahoon 2009).

Many beneficial effects of soy components such as soy protein, isoflavones, phytosterols, inositol hexaphosphate and saponins have been suggested, including the beneficial role for inhibition of tumor development in animal models (Hawrylewicz, Zapata et al. 1995). This has stimulated, in only the last 30 years, soybean introduction into western cultures and diets as different sub-products (Golbitz 1995). These products are usually fermented (tempeh, miso, soy sauces, natto, fermented tofu) or non-fermented (fresh soybeans, whole dry soybeans, soy nuts, soy sprouts, whole-fat soy flour, soymilk, tofu, soy protein, soy oil). One of the most used nonfermented subproduct is the soybean oil. The oil represents approximately 20% of the grain and is influenced by both genotype and environmental factors, and in some countries this is the main sub-product utilized in the diet (Clemente & Cahoon 2009).

Soybean oil is a representative source of lipids in human nutrition. There are proposed mechanisms that explain lipids involvement in cancer development, especially colorectal cancer (CRC). Thus, comparing soybean oil composition with other oils is a discussion that is relevant in the context of CRC, once this is a public health concern.

2. Cancer epidemiology

Cancer is a major public health problem in the United States (US) and in others parts of the world (Jemal, Siegel et al. 2010). According to GLOBOCAN 2008 statistics, CRC is the third

most incidental type of cancer in men and the second in women worldwide and accounts for 8% of all cancer deaths, making it the fourth most common cause of death from cancer. Almost 60% of the cases occur in developed regions (Ferlay, Shin et al. 2010).

Changes in the incidence of CRC have been observed worldwide, with a statistically significant increase in its incidence from 1983-87 to 1998-2002. Europe, Slovakia, Slovenia and Czech Republic have shown the largest increase (>45% for men and 25% for women). In countries of Western Europe (France, Italy, England, Germany and Switzerland) the CRC incidence has remained stable or increased only slightly, except for Spain which presented large increases. In Asia, the largest increases were observed in Japan, Kuwait and Israel, and some areas with large increases in incidence, such as India, Thailand and Kuwait, still maintained relatively low incidence rates. In China, incidence increased by 50% in Shanghai compared with 10% in Hong Kong. In contrast, the incidence rates decreased significantly for the US population of both sexes, as well as for Canadian and New Zealand women. In general the increase was largely confined to economically transitioning countries and stabilized in the majority of developed countries, decreasing only in the US. The technology available for CRC screening can influence incidence rates since techniques such as colonoscopy are able to reduce this incidence through the removal of precancerous polyps. In the US, colonoscopy has been the most used screening test, which may be contributing to the decreased incidence rates (Center, Jemal et al. 2009; Murphy, Devesa et al. 2011).

Colorectal cancer is considered a multifactorial disease. The fact that cancer incidence and mortality rates vary dramatically worldwide, and that population migrating from a low to high-incidence country will present approximate rates of the new region, indicate that environmental and lifestyle factors are important determinants in cancer rates (Willett 2001). It is estimated that 25% of the cases are related to hereditary factors while 75% are related to other risk factors such as smoking, obesity, sedentarism, aging and dietary habits (Wallace & Caslake 2001). The adoption of a western lifestyles and behaviors such as high fat diets, physical inactivity and increase in obesity prevalence has been pointed out as one of the environmental factors associated with genetic vulnerability that could explain the increase in CRC incidence rates in economically transitioning countries (Center, Jemal et al. 2009).

Diet is definitely a modifiable exogenous influence on etiology of CRC (Labianca & Beretta 2010). One of the dietary factors suspected of playing a role on the incidence of CRC is fat intake. The suspicion that fat intake is related to cancer incidence is resultant from the observation that there are large differences in the fat consumption per capita around the world and also differences in cancer rates (Willett 1998), however these data are still controversial. One of the limitations of epidemiological studies is the fact that they do not provide proof of an association between fat and CRC incidence due to measurement error in food frequency questionnaire-based dietary assessment, which can cause attenuation of risk estimations (Flood, Velie et al. 2003). Many animal studies have shown the importance of the type and amount of fat on carcinogenesis (Coleman, Landström et al. 2002; Dwivedi, Natarajan et al. 2005; Boateng, Verghese et al. 2006), while studies assessing human dietary patterns suggest a decreased risk of CRC associated with a higher frequency of low-fat foods (Flood, Rastogi et al. 2008) or a higher risk associated with a western dietary pattern, characterized by greater intakes of red and processed meats, sweets and desserts, high-fat dairy foods, french fries and refined grains (Fung, Hu et al. 2003). Thus, public health campaigns promoting healthier dietary patterns are one of the measures that should be

taken to help prevent cancer (Jemal, Bray et al. 2011). Thus, understanding how fats, more specifically fatty acids, can play a role in carcinogenesis, must be considered to direct these public health campaigns to prevent increases in cancer incidence.

3. Production, intake and oils composition

From an evolutionary perspective, the combination of our ancient genome with the nutritional qualities of recently introduced foods may underlie many chronic diseases of western civilization, including cancer (Cordain & Eaton 2005). One of the main changes in the human diet refers to the type and quantity of fatty acids and antioxidant content of foods. In the last 20 years, significant changes have occurred in the consumption of lipid sources, with reduction of animal fat intake and increase in the intake of vegetable oils (Simopoulos 2008).

The advent of the oil-seed processing industry significantly raised the total intake of refined vegetable oils. During the period from 1909 to 1999 there was a significant increase in the use of these oils, representing roughly 17.6% of energy intake in the western diet. This resulted in a direct increase in the level of ω-6 polyunsaturated fatty acids (PUFAs) at the expense of a lowered level of ω-3 PUFA since vegetable oils are inherently richer in the first (Cordain & Eaton 2005). The utilization of grains in animal diets and oil hydrogenation has also contributed to the increased level of linoleic acid (LA, 18:2, ω-6) in the diets (Simopoulos 2008).

The major oilseeds supplied and distributed around the world are copra, cottonseed, palm kernel, peanut, rapeseed, soybean and sunflower seed. The soybean, from a commodity viewpoint is number one in terms of production, importation and exportation, while for the production of vegetables oils it is the second seed most used, behind palm oil (Figure 1). Soybean oil is the second most consumed oil, and from 2006 to 2011 its consumption increased by 17.8%. The main countries consuming vegetable oils are China, EU-27, India, US, Indonesia and Brazil (Figure 2), and soybean oil is particularly most consumed in China, US, Brazil, India, EU-27 and Argentina (Figure 3).

The hallmark of the increase in oilseed consumption relates to the changes in the proportion of fatty acids in the diet. The main fatty acids in concern are from ω-3 and ω-6 family. Alpha-linolenic acid (ALA, 18:3 ω-3; found in oils, nuts, seeds), eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) are members of ω-3, these last two mainly found in animal sources (such as fish, fish oil), while the most common fatty acids from ω-6 family are LA (mainly vegetable sources) and arachidonic acid (AA, 20:4 ω-6; mainly from animal sources) (Roynette, Calder et al. 2004; Russo 2009). In a typical American diet, 89% of the PUFA consumed is LA, while 9% is ALA, being that vegetable oils are the main sources of both. This large difference between ω-6 and ω-3 fatty acids in the western diet is implicated in the increase of the ω-6:ω-3 ratio, especially when fish and fish oil intake is low. On the other hand, in Europe over the last two decades a 50% increase in LA intake has been observed (from 10 to 15g/day) and a 100% increase in ALA (from 1 to 1.9g/day) (Russo 2009), reducing this ratio. According to Russo (2009), the proportion 1:1 (ω-6: ω-3) presumed to be consumed by ancient civilization has been replaced by 15:1 - 20:1 in some moderns civilizations of western regions (Simopoulos 2008).

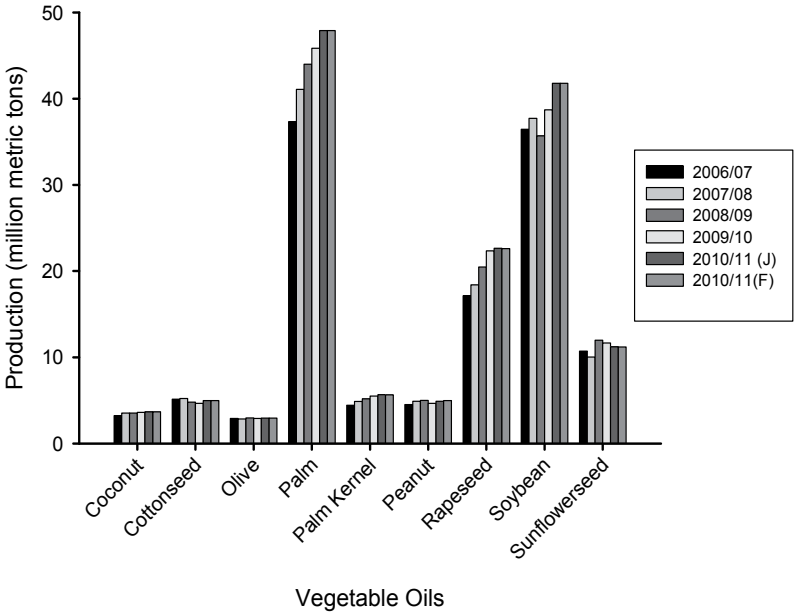

Fig. 1. Major vegetable oils produced (million metric tons) from 2006 to January (J) and February (F) of 2011 (Source: USDA).

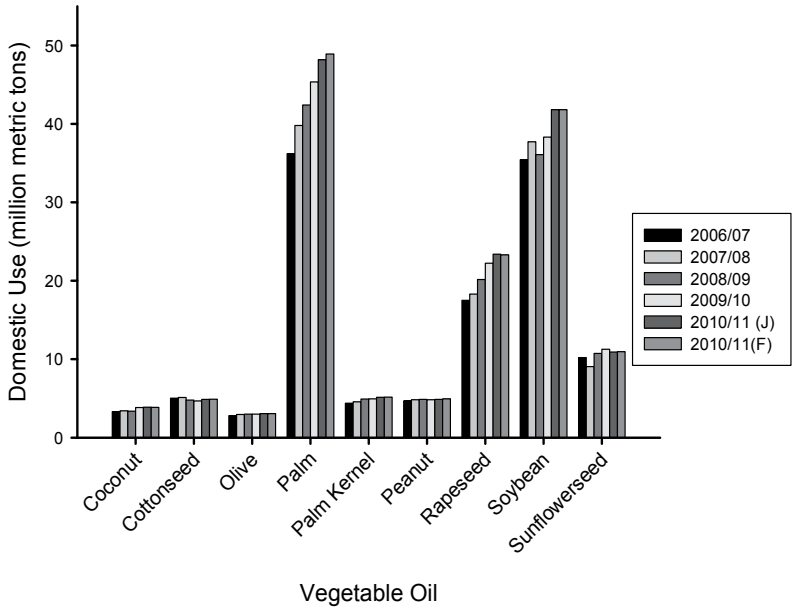

Fig. 2. Domestic consumption of major vegetable oils (million metric tons) from 2006 to January (J) and February (F) of 2011 (Source: USDA).

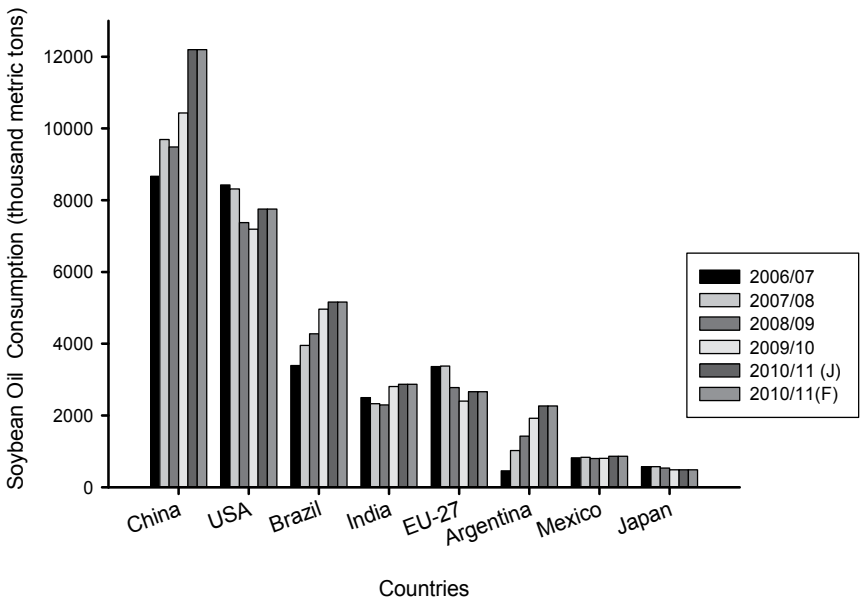

Fig. 3. Domestic consumption of soybean oil (thousand metric tons) from 2006 to January (J) and February (F) of 2011 (Source: USDA).

Considering the economic importance and the high dietary intake of soybean oil, it should be discussed its composition in comparison with other oils of importance in human nutrition (Table 1). The major fatty acid in sunflower, soybean and corn oils is the LA. The canola oil has approximately 21% of LA, while palm, flaxseed, olive oil have less than 16%, and fish oil less than 3%. Vegetables oils only present ω-3 in the form of ALA. Flaxseed has a high level of ALA (more than 50%), while the other oils has less than 7%. The soybean and canola oil have similar quantities of ALA and the highest content after flaxseed, showing levels 7-21 fold higher in comparison to other oils than flaxseed. The palm oil, is together with soybean oil, the most consumed oil, but it should be highlighted that it has 3 to 4-fold more saturated fatty acids in comparison with other vegetable oils, what may be a disadvantage considering the role of saturated fats on cardiovascular risks. The fish oil, although is from animal source, is included in this table because is the only source of oil directly providing EPA and DHA, which accounts for more than half of its fatty acid composition. Its ALA content is low and soybean oil has 8 times more ALA than fish oil.

4. Importance of PUFAs in human nutrition

The effects of oils and fats in human nutrition have been largely discussed in the last decades, emphasizing the reduced intake of saturated and trans fatty acids and the increase in ω-3 intake (Wortman, Miyazaki et al. 2009).

ALA and LA are considered essential because they are not synthesized in the human body due to the lack of enzymes capable of inserting double bonds in the third and sixth carbons counted from the methyl terminal (Roynette, Calder et al. 2004). The LA is converted into AA. In mammals, the essential fatty acids ALA can be converted to EPA and DHA, but the endogenous rate of conversion is unknown. It is believed that this conversion might be low,

approximately 5-10% of the ALA is converted to EPA and 2-5% to DHA in healthy adults (He 2009).

Fatty Acids	Concentrations[a]							
	Soybean oil (1)	Corn oil (2)	Sunflower oil (2)	Canola oil (2)	Palm oil(2)	Olive oil (1)	Flaxseed oil (1)	Fish oil (1)
C14:0	ND	ND	0.07	0.06	0.79	ND	ND	5.86
C15:0	ND	ND	ND	ND	ND	ND	ND	0.22
C16:0	7.75	12.12	6.10	4.59	36.77	11.30	6.45	10.76
C17:0	ND	ND	ND	ND	ND	ND	ND	0.50
C18:0	3.01	2.18	3.42	2.21	4.61	2.96	4.35	3.60
C20:0	ND	0.49	0.26	0.57	0.35	0.38	ND	0.48
C22:0	0.34	0.18	0.67	0.30	0.10	0.12	ND	ND
C24:0	ND	0.19	0.25	0.15	0.08	ND	ND	ND
∑ AGS	**11.10**	**15.20**	**10.80**	**7.90**	**43.1**	**14.70**	**10.80**	**21.42**
C16:1	ND	0.12	0.08	0.2	0.14	1.09	ND	6.60
C18:1 ω-9	21.36	33.04	25.15	61.14	39.86	74.01	18.00	9.98
C20:1	ND	0.23	0.18	1.11	0.24	0.25	ND	1.08
∑ MUFA	**21.36**	**33.4**	**25.4**	**62.6**	**40.1**	**75.35**	**18.00**	**17.66**
C20:2	ND	ND	ND	ND	ND	ND	ND	3.05
C18:2 ω-6	60.75	49.94	62.22	20.87	15.69	8.74	12.71	2.78
C20:4 ω-6	ND	ND	ND	ND	ND	ND	ND	0.73
C22:2 ω-6	ND	ND	ND	ND	ND	ND	ND	0.87
C18:3ω-3	6.96	0.96	0.32	6.78	0.83	0.75	58.47	0.80
C20:5ω-3	ND	ND	ND	ND	ND	ND	ND	24.01
C22:6 ω-3	ND	ND	ND	ND	ND	ND	ND	19.86
∑ PUFA	**67.71**	**50.90**	**62.6**	**28.4**	**16.6**	**9.50**	**71.18**	**52.10**
Totalω-6	60.75	49.94	62.22	20.87	15.69	8.74	12.71	4.37
Totalω-3	6.93	0.96	0.32	6.78	0.83	0.75	58.47	44.63
ω-6/ω-3	*8.77*	52.0	195.0	3.01	18.9	11.65	0.22	0.10

[a]Fatty acid concentration in soybean, corn, sunflower, canola, olive, flaxseed and fish oils. The identification of the methyl esters of the fatty acids was performed by gas chromatography. Values are in g/100 g. *ND= not determined. Source: (1) (ROSA, SALES et al., 2010) (2) (TACO, 2006).

Table 1. Concentration of fatty acids in the oils used in the diets (percent).

The importance of PUFAs in human nutrition relates to their role in modulating inflammatory responses, which in turn is related to immunological resistance, metabolic disturbances, thrombotic events and neoplasia (Martin, Almeida et al. 2006; Hirayama, Speridião et al. 2006). The ω-6 and ω-3 fatty acids are not metabolically interconvertable but because both share enzymatic pathways and usually play opposite effects, their balance or proportion seems to be important (Whelan & McEntee 2004).

According to Willet (2001), non-cancer outcomes can aid in interpreting findings for cancer, especially when an association is observed with coronary heart disease, and any actual dietary decisions should be made in the light of not just cancer, but how dietetic variables relate to cardiovascular disease (CVD) and other important conditions. Evidences suggest that when increasing ω-3 intake (EPA and DHA), the incidence of CVD is reduced, but

opinions are still divergent on whether the same effects can be obtained with the reduction of ω-6 intake. It is worthy to note that there are ethnic differences in the percentage of deaths from CVD when comparing Europe and the US (45%) with Japan (12%) which are coincident with significant differences in platelet phospholipid EPA concentration (0.5% *vs* 1.6%), while AA concentration is similar (26% *vs* 21%) (Russo 2009). Thus, the moderate consumption of fatty acids from the ω-3 family, especially from fish oil, plays an important role on the reduction of CVD risks. Considerable attention has been directed to evaluate the action of ALA, EPA and DHA in the prevention and treatment of diseases in general. The American Heart Association (AHA) recommends the use of 1g/day of ω-3 (EPA plus DHA) from fish oil or fatty fish to patients with CVD (Lavie, Milani et al. 2009).
In 2007, Von Schacky and Harris proposed the "ω-3 index" as a new risk factor for cardiac disease. This index is defined as the percentage of EPA plus DHA of the total fat content in erythrocytes, reflecting the individual ω-3 status. Although the authors have shown evidences that support the clinical efficacy of this index, it hasn't been validated as a biomarker. The PUFA content in cell membranes depends on diet content and its proportion of ω-6 and ω-3 fatty acids, although an optimal proportion still hasn't been established (von Schacky & Harris 2007).

5. PUFAs and colorectal carcinogenesis

Biological effects on CRC promotion by ω-6 PUFA or suppression by ω-3 PUFA has been suggested by experimental and epidemiological studies (Nkondjock, Shatenstein et al. 2003; Moreira, Sabarense et al. 2009; Rosa, Sales et al. 2010; West, Clark et al. 2010). These effects include alteration in properties of cancer cells (proliferation, invasion, metastasis, and apoptosis) and host cells (inflammation, immune response and angiogenesis) (Larsson, Kumlin et al. 2004; Roynette, Calder et al. 2004).
These effects are mediated by various mechanisms. It has recently been shown that polymorphisms in fatty acid metabolism-related genes are associated with CRC risk. Although their contribution to CRC is not fully explained, they reinforced the key role of eicosanoid signaling in colon carcinogenesis (Hoeft, Linseisen et al. 2010). These eicosanoids are end-products of cell membrane fatty acids mobilization. The PUFA, LA and ALA are mobilized from cell membrane phospholipids through the action of phospholipases A_2 and C enzymes. They then compete for desaturases and elongases enzymes for conversion to their metabolites, AA and EPA respectively. These enzymes have a greater affinity for ω-3 fatty acids, such that ω-3 are preferentially metabolized when its intake in the diet is high. This leads to a "competitive inhibition" of ω-6 PUFA metabolism, decreasing LA desaturation and AA concentration after ALA, EPA or DHA supplementation. On the other hand, increases in ω-6 will reduce EPA and DHA conversion (Roynette, Calder et al. 2004).
After mobilization from the cell membrane and AA or EPA formation, these fatty acids are subsequently metabolized into eicosanoids by the action of cyclooxygenase (COX) and lipoxygenase (LOX). Eicosanoids such as prostanoids (prostaglandins and thromboxanes), and leukotrienes are potent modulators of inflammatory and immune responses playing a critical role on platelet aggregation, cellular growth and differentiation. The COX action results in prostaglandins and thromboxanes, while the LOX produces leukotrienes. Eicosanoids are derived from fatty acids with 20 carbon atoms such as AA and EPA (Larsson, Kumlin et al. 2004; Berquin, Edwards et al. 2008), a step where there is also another competition among PUFA where there is a greater affinity for the ω-3 PUFA. Thus,

EPA is preferentially metabolized and consequently eicosanoids from series 3 (prostaglandins and thrombaxanes) and 5 (leukotrienes) will be produced. The proportion of PUFA in the cell membranes is the main factor regulating which eicosanoids will be produced, and this proportion reflects the amount of PUFA present in the diet (Rose & Connolly 1999; Moreira, Sabarense et al. 2009; Russo 2009).

Increase in ω-6 fatty acids in the western diet contributes to greater production of eicosanoids from AA rather than from EPA, which are associated with an decreased rate of apoptosis and a increased tumoral growth, and are also more reactive compared to pro-inflammatory eicosanoids derived from EPA (Roynette, Calder et al. 2004); eicosanoids from AA are biologically active even in small amounts. Lee et al. (1984) demonstrated that leukotriene B_5 has only 5 to 10% of the activity of leukotriene B_4 (from AA). If AA eicosanoids are formed in greater quantities, especially with a diet rich in ω-6 fatty acids, it shifts the physiological state to a prothrombotic, proaggregation and pro-inflammatory condition (Simopoulos 2008), while the increase in ω-3 intake could result in the reduction of the pro-inflammatory state (West, Clark et al. 2010).

As eicosanoid production influences carcinogenesis, the isoform of the enzyme producing them also play a role. There are two isoforms of COX: COX-1, which is constitutively expressed in many tissues, and COX-2, which is the inducible form. COX-2 expression occurs in most tissues, but after stimulus its expression can increase considerably (Zha, Yegnasubramanian et al. 2004). Elevated levels of COX-2 have been observed in colon tumors in humans and animals by chemically induced carcinogenesis (Shao, Sheng et al. 1999; Ceccarelli, Piazzi et al. 2005). This increase in COX-2 indicates an increase in the capacity to produce prostaglandin E_2 (PGE_2) in the presence of AA. The PGE_2 can promote carcinogenesis through the inhibition of apoptosis, induction of cell proliferation and angiogenesis (Leahy, Ornberg et al. 2002; Pai, Nakamura et al. 2003). Singh et al. (1997) induced CRC in rats and found elevation of COX-2 expression in the group treated with corn oil compared to the group receiving fish oil, indicating that ω-6 rich oils participates in this mechanism.

A significant correlation was found between inducible nitric oxide synthase (iNOS) enzyme and COX-2 expression. The PGE_2 production also correlated with iNOS activity (Cianchi, Cortesini et al. 2004). The iNOS is responsible for the synthesis of nitric oxide (NO) from L-arginine. The NO mediates intra and extracellular processes; however, it is potentially toxic since it can react with oxygen or superoxides, damaging tissues and DNA. The activation of iNOS and the subsequent excess of NO production can also inhibit the activity of enzymes related to DNA repair and modulate caspases and p53 activity (Jaiswal, LaRusso et al. 2000). A significant correlation was found between iNOS and COX-2 expression. The PGE_2 production also correlated with iNOS activity (Cianchi, Cortesini et al. 2004). Another correlation found was between iNOS and angiogenesis in patients with CRC (Cianchi, Cortesini et al. 2003). Narayanan et al. (2003) showed that DHA can reduce iNOS expression in vitro. Reduction of the endogenous level of ω-6 PUFA is beneficial since lower expression of the iNOS enzyme and increased expression of the transforming growth factor beta (TGF-β) in transgenic animal model chemically submitted to CRC was observed (Nkondjock, Shatenstein et al. 2003). Reduction of TGF- β expression has been shown to significantly increase chemically-induced colon carcinogenesis (Tang, Böttinger et al. 1998). On a molecular level, TGF-β was shown to inhibit transformation through inhibition of the Akt pathway in intestinal epithelial cells (Cao, Deng et al. 2006). Therefore, ω-6 fatty acids again appear to exert a negative influence on induction of enzymes related to carcinogenesis and reduction of protective molecules.

The ω-6 and ω-3 PUFAs can also influence colorectal carcinogenesis by affecting expression of genes associated with vital functions of the cell, enzymes activities and signal transduction molecules. Cellular proliferation (mitosis) and growth are important aspects of tumors development when they occur uncontrolled. In this situation, the induced cell death through apoptosis is activated, which is recognized as an anti-carcinogenesis mechanism. When this mechanism fails, tumors can develop (Arlt, Müerköster et al. 2010).

The activated *Ras* protein regulates mitosis, and its increased expression is related to tumor development (Mangues, Seidman et al. 1992). Diets containing greater proportions of ω-6 (corn oil) increases the expression of *Ras* protein by 13% in the colonic mucosa of rats treated with azoxymethane in comparison to animals receiving a diet rich in ω-3 (fish oil) (Davidson, Lupton et al. 1999). Cellular growth can be promoted by insulin-like growth factor (IGF) through autocrine and paracrine actions, and IGF binding proteins (IGFBP) modulate IGF actions. Increases in IGF-II expression was demonstrated in patients with CRC (Freier, Weiss et al. 1999), while treatment of Caco-2 cells with EPA or DHA reduced IGF-II secretion and increased IGFBP-6 secretion, inhibiting cellular proliferation. On the other hand, LA can increase the level of IGF-II without altering IGFBP-6 secretion and stimulate Caco-2 cell proliferation (Kim, J. et al. 2000). It has also been suggested that hyperinsulinemia and insulin resistance can play a significant role in CRC etiology (Trevisan, Liu et al. 2001; Ma, Giovannucci et al. 2004). Insulin acts as growth factor for colonic cells because it enhances IGF-I activity, cell proliferation and differentiation, and inhibits apoptosis (Biddinger & Ludwig 2005; Giovannucci 2007). Smith et al. (2010) demonstrated that reduction of the ω-6:ω-3 ratio can increase glucose tolerance and improve insulin sensitivity in healthy animals.

Meanwhile, the expression of some proteins related to apoptosis can be beneficial. The type of oil, and consequently its fatty acid profile, can exert different influences on protein expression. Cancerous cells usually overexpress *Bcl-2* proteins involved in apoptosis inhibition (Bronner, Culin et al. 1995), so that reduced expression could be beneficial. In cultured human cancer cells (Caco 2 and HT-29) supplemented with olive oil, fish oil, oleic fatty acid and LA, it was verified that *Bcl-2* expression was reduced in cells supplemented with EPA and DHA (fish oil group) (Llor, Pons et al. 2003). Moreover, Cheng et al. (2003) found an elevation in *Bax* protein expression, associated with the increase in apoptotic cell activity in individuals with CRC who consumed greater quantities of fish oil. Signaling molecules such as the transcriptional factor NFκβ have been shown to play a role in carcinogenesis once it regulates the expression of genes involved in migration, proliferation and apoptosis (Dolcet, Llobet et al. 2005). Reduction of the endogenous level of ω-6 PUFA led to lower NFκβ activity in transgenic animal models chemically submitted to CRC (Nowak, Weylandt et al. 2007). Ravasco et al. (2009) also observed that patients with elevated intake of ω-6 PUFA, saturated fat, animal protein, refined carbohydrates and alcohol – a typical western diet – present greater expression of this transcriptional factor. However, patients with higher ω-3, fibers, vitamin E, isoflavonoid, β-carotene and selenium intake showed reduced NFκβ expression.

Another mechanism that links apoptosis, PUFA and CRC development refers to the peroxisome proliferator-activated receptor (PPAR). DHA and EPA (in lesser extent) can inhibit PPAR-δ which is associated with the increase in cell proliferation and apoptosis suppression (Lee & Hwang 2002; Lund 2006; Wang & DuBois 2008). On the other hand, Xu et al. (2006) demonstrated that AA can act as an activator of PPAR-δ. Thus, sources of ω-6

fatty acids appear to be disadvantageous for cancer prevention while ω-3 fatty acids are beneficial.

The PUFA have also demonstrated to influence the activity of the enzyme ornithine decarboxylase (ODC), which is a limiting enzyme in polyamines synthesis (putrescin, spermidin and spermine). These polyamines are involved in cellular growth, renewal and metabolism, and have been found in high concentrations in colorectal tumors of rats treated with dimethylhydrazine and humans due to increased ODC activity (Lamuraglia, Lacaine et al. 1986; Shigesawa, Onoda et al. 1998). Bastram et al. (1993) observed a reduction in ODC activity in the colon of healthy individuals consuming fish oil compared with corn oil (rich in LA). Rao and Reddy (1993) encountered results reinforcing the role of ω-3 fatty acids from fish oil in suppressing ODC activity in animal models of carcinogenesis in comparison to ω-6 fatty acids from corn oil.

It can be highlighted that the suppressive effects of EPA and DHA on CRC tumors are consistently verified, while ALA effects are still controversial (Daniel, McCullough et al. 2009). This inconsistency can be related to the small conversion of ALA to EPA, which requires further investigations (Berquin, Edwards et al. 2008). In relation to the adverse effects from ω-3 PUFA intake, it is important to highlight that these fatty acids present a greater number of double bonds (unsaturations) which makes cell membranes more susceptible to lipid peroxidation. Thus, it is recommended to increase the intake of antioxidant vitamins to compensate for the increase in PUFA intake, mainly ω-3, in order to stabilize the double bonds (Institute of Medicine 2000).

However, it is not correct to consider that all mediators formed from AA are pro-inflammatory while those formed from EPA are always less potent. The action of ω-6 and ω-3 fatty acids and their derivatives on inflammatory processes can actually involve more complex mechanisms than those already recognized and requires more studies to clarify their mechanisms and effects (Calder 2009).

6. PUFA intake recommendations

The ω-3 long chain fatty acids (LCFA), EPA and DHA, have received special attention as a recommended nutrient for secondary prevention of CVD and for the prevention and treatment of disorders with an inflammatory component (including type 2 diabetes, irritable bowel syndrome, macular degeneration, rheumatoid arthritis, asthma, several cancers, and psychiatric disorders) since these illnesses have shown to be a problem around the world (Hibbeln et al, 2006). The high ω-6:ω-3 ratio is unfavorable, especially in health conditions characterized by aggravation of the inflammatory response (Garófolo & Petrilli 2006). There are some studies associating PUFAs and CRC in humans suggesting a possible protective role of ω-3 in this disease (Table 2).

Establishing healthy ω-6 and ω-3 intakes is difficult since there are various factors influencing optimal intake. Hibbeln et al. (2006) discussed these aspects in an ecological study design. Some of their considerations are very important to be discussed in this chapter to aid in answering whether soybean oil has a positive or negative health effects in comparison with other oils.

1. A tissue composition of 60% ω-3 LCFA may prevent 98.6% of the worldwide risk of cardiovascular mortality potentially attributable to ω-3 LCFA deficiency. However, the ω-3 LCFA intake required to achieve 60% ω-3 fatty acids in tissue varied 13-fold among nations depending on essential fatty acid availability. In general, as concurrent LA

availability increased, the estimated requirement for ω-3 LCFA intake increased, although intake of ALA and AA also played a role. For Americans consuming a 2000-kcal/d diet, 2178 mg/d (0.98% of energy) may achieve 50% ω-3 LCFA in tissue, whereas 3667 mg/d is necessary to reach 60% ω-3 LCFA.

2. Available intake of ω-3 LCFA to provide 0.34% of energy was sufficient to reduce risk for 98% of the mortality and morbidity in all illness models.
3. Lowering LA intake can likely decrease an individual's need for ω-3 LCFAs by one-tenth.
4. Concurrent dietary intakes of LA, AA, ALA, EPA and DHA should be considered in predicting final tissue proportions of ω-3 LCFAs. Thus, a healthy dietary intake of ω-3 LCFA must be dependent on concurrent intakes of LA, AA and ALA. A healthy dietary allowance of 3.5 g EPA plus DHA/d, which is based on the current per capita available intake of ω-6 fatty acids and ALA in the US, could be reduced to one-tenth of that amount if the intake of ω-6 fatty acids, in particular LA, can be lowered to 2% of the total energy.

Based on a critical analysis of available literature, Wijendran and Hayes (2004) suggest for health adults an adequate intake of about 6% of total calories from LA, 0.75% from ALA, and 0.25%–0.5% for EPA plus DHA. This corresponds to an ω-6:ω-3 ratio of 6:1. According these authors, intake of PUFA expressed in terms of mass (% kcal or g/d) is a better approach to dietary ω-6 and ω-3 fatty acid balance than a simple ratio.

Therefore, choosing the best oil to cook or to add in food products should consider its lipid profile and how much of it is used.

Reference	Study Design	Comparative Indicator	Results
	Linoleic Acid (LA, ω-6)		
Tuyns et al. (1987)	Case-control 818 cases/2851 controls	Food Intake	Inverse association between LA and cancer
Bar et al. (1998)	Cross-sectional 17 cases /12 controls	Fatty acids in plasma	Significant ↓ LA in cancer patients
Terry et al. (2001)	Cohort – Sweden 61463 women	Food Intake	No relationship
Astorg (2005)	Meta Analysis Epidemiological research	Food Intake	No relationship
Hall et al. (2007)	Case-Control (Physicians' Health Study). 178 case men/282 control men	Blood Levels	No relationship
Theodoratou et al. (2007)	Prospective case-control study 1455 cases/ 1455 controls	Food Intake	No relationship
Murff et al. (2009)	Cohort Study (Study Shanghai Women's Health) 73242 woman	Food Intake	No relationship

Reference	Study Design	Comparative Indicator	Results
	α-Linolenic Acid (ALA , ω-3)		
Fernández-Bañares et al. (1996)	Cross-sectional 49 CRC cases /12 controls	Mucosal fatty acids	Significant ↓ ALA in intestine mucosal of cancer patients
Bar et al. (1998)	Cross-sectional 17 CRC cases /12 controls	Fatty acids	Significant ↓ ALA in plasma of cases
Nkondjock et al. (2003)	Case-control 402 cases/668 controls	Food Intake	Significant inverse association between ALA and CRC
Terry et al. (2001)	Cohort (1987-1990) 61463 women	Food Intake	No relationship
Astorg (2005)	Meta Analysis Epidemiological Research	Food Intake	No relationship
Theodoratou et al. (2007)	Prospective case-control study 1455 cases/1455 controls	Food Intake	No relationship
Murff et al. (2009)	Cohort Study (Study Shanghai Women's Health) 73242 women	Food Intake	No relationship
	Eicosapentaenoic acid plusDocosahexanoic Acid (EPA/DHA , ω-3)		
Jiang et al. (2010)	Randomized clinical trial Intravenous infusion of soybean oil or fish oil 203 cases	Inflammatory response and lenght of hospital stay	Significant ↓ in fish oil
Hall et al. (2007)	Case-Control (Physicians' Health Study) 178 case/282 control (men)	Blood Levels	EPA and DHA were inversely associated with CRC risk
Theodoratou et al. (2007)	Prospective case-control.1455 cases/ 1455 controls	Food Intake	Significant ↓ in the risk were associated with ↑ EPA and DHA intake
Kin et al. (2010)	Case-control 929 cases/943 controls	Food Intake	Inverse association between EPA, DHA and CRC
Gee et al. (1999)	Case-control 14 cases/14 controls	Colorectal cytokinetics	No relationship
Terry et al.	Cohort-Sweden (1987-1990),	Food Intake	No Relationship

Reference	Study Design	Comparative Indicator	Results
(2001)	61463 women		
Astorg (2005)	Meta Analysis Epidemiological research	Food Intake	No relationship
Murff et al. (2009)	Cohort (Study Shanghai Women's Health), 73242 women	Food Intake	No Relationship
Oh et al.(2005)	Cohort (Nurses' Health Study) 34451women	Food Intake	No relationship
	Eicosapentaenoic acid (EPA, ω-3)		
Fernández-Bañares et al.(1996)	Cross-sectiiional 49 cases CCR/12 controls	Mucosal Fatty acids	Significant ↓ in intestine mucosal EPA content in cancer patients
Courtney et al.(1996)	Case-control 14 cases/14 controls	Apoptosis and crypt cell proliferation	Significantly ↓ in cancer patients
Hall et al.(2008)	Prospective Study (Physicians' Health Study), 500 men	Food Intake	EPA significantly ↓ the risk for CCR
West et al.(2010)	Clinical Trial 55 patients	The number and size of polyps	Protective effect of EPA in colon cancer patients
	Docosahexanoic Acid (DHA , ω-3)		
Kuriki et al.(2006)	Case-control 74 cases/221 controls	Erythrocyte fatty acids	Inverse association between erythrocyte compositions and DHA
Hall et al.(2007)	Case-Control (Physicians' Health Study), 178 case/282control	Blood Levels	DHA were inversely associated with CRC risk

Table 2. Human studies examining the effect of ω-6 and ω-3 PUFAs on colorectal cancer risk

7. Future perspectives

Sufficient evidence was shown to portray the shift in concern on the role of dietary fat in carcinogenesis: from the amount of fat consumed to the type of fat (fatty acids profile), that is, ω-3 *vs* ω-6 PUFAs. Most evidence for benefits apply to the ω-3 LCFAs. Considering this, two problems are indicated: western diets are typically low in EPA and DHA because consumption of the primary source of these fatty acids from oily fish (such as salmon, mackerel, albacoretuna, and sardines) is low (Harris, Lemke et al. 2008) and it is problematic

to recommend to patients or the community to increase fish intake due to price and availability of oily fish (James, Ursin et al. 2003). Because conversion of dietary ALA into EPA is limited and a fish/fish oil based diet is not easily adopted, dietetic solutions to this limitation should provide great perspectives for the future.

Some vegetables oils are good sources of ω-3, such as canola and linseed oils, but in the ALA form. However, conversion of ALA to EPA is inefficient in adult humans and increased intake of ALA does not reproduce the health benefits of ω-3 LCFAs. Initially, it is generally assumed that LA reduces EPA synthesis because of the competition between ALA and LA for common desaturation and elongation enzymes. Hence, one of the dietetic solutions thought to favor the conversion of ALA into the ω-3 LCFA would be the increase of ALA intake or decrease of LA intake. Goyens et al. (2006) designed a nutritional intervention trial to determine whether *in vivo* conversion of dietary ALA is influenced by the absolute amounts of LA or ALA in the diet or by the ALA-to-LA ratio. They concluded that ALA and LA content in the diet, and not its ratio, determines the conversion of ALA into EPA/DHA, suggesting that the increase in DHA is directly related to ALA content in the diet, while EPA formation is favored by reduced LA levels in the diet (Goyens, Spilker et al. 2006).

Besides reducing dietary intakes of the 18-carbon precursor, LA lowers the available tissue composition of AA. According to Hibbeln et al. (2006), it is likely that the success and failure of different clinical trials using similar doses of ω-3 LCFAs may have been influenced by differing background intakes of the ω-6 LA and AA fatty acids. The biological availability and activity of ω-6 LCFAs, in particular AA, is inversely related to ω-3 LCFAs in tissue. Greater compositions of EPA and DHA in membranes competitively lower the availability of AA for the production of inflammatory eicosanoids.

A useful land-based dietary resource for increasing tissue concentrations of ω-3 LCFAs in humans would be of great economical interest to provide a wide range of dietary alternatives to fish and encapsulated fish oil supplements to increase tissue concentrations of ω-3 LCFAs without altering common dietary habits. Stearidonic acid (SDA, 18:4 ω-3) rich oils could provide this potential land solution, since it has also been studied as a precursor for ω-3 LCFAs in humans. SDA is an intermediate metabolite between ALA and EPA in the ω-3 biosynthetic pathways. It has been shown that ALA in the diet is converted to EPA, with only a fraction of the efficiency of SDA in healthy subjects (James, Ursin et al. 2003). Further, Δ-6 activity has been shown to decline with age (Bourre, Piciotti et al., 1990) and with a significant number of clinical conditions, such as cancer, making utilization of dietary ALA even less efficient (Damude & Kinney 2008).

Recently, soybean plants have been genetically modified to produce oil containing a substantial amount of SDA (15-30% w/w of total fatty acids) with value in both food and feed applications (Ursin 2003). Some studies are consistent with the premise that Δ6 desaturation is the rate-limiting step in the conversion of ALA to EPA and that SDA has a biochemical advantage over ALA in elevating the levels of others ω-3 LCFAs in tissues. The efficiency of ALA conversion into EPA was shown to be 0.09%, while SDA conversion was 16.6% (relative to EPA). Hence, another strategy for raising tissue EPA levels with a plant-based ω-3 is to consume foods containing SDA (Harris, Lemke et al. 2008). James et al. (2003) showed greater enrichment of EPA in plasma and lipid cells if subjects supplemented their diet with ethyl ester of SDA than if supplemented with the same amount of the ethyl ester of

ALA. Therefore, SDA appears to be superior to ALA for increasing ω-3 LCFAs status in humans and thus sources of SDA might offer some human health benefits. Supplementation of SDA led to an elevated increase in EPA compared to ALA supplementation, but no increase in DHA. SDA also demonstrated to be superior to ALA for lowering the level of COX-2 protein and mRNA expression in breast cancer cells. Thus, the authors suggested that dietary SDA may be more beneficial than ALA in reducing the risk of breast cancer by effectively antagonizing PGE_2 production from AA (Horia & Watkins 2005).

It should be highlighted that the effectiveness of SDA as a nutritional fatty acid is dependent on the ability of humans and animals to convert SDA to EPA and DHA. Thus, the beneficial effects of EPA and DHA have incentivated the development of soybean seeds that accumulate oils with EPA and DHA. It is envisioned that these oils can be produced at a lower cost in soybean seeds compared with fish and algae, the current commercial sources of EPA and DHA-containing oils. Additionally, soybean offers a more sustainable production platform than fish. It is likely that soybean oil enriched in EPA and DHA is used in the manufacture of food and feed products, including salad oils, infant formulas, and feed rations for farm fish (Clemente & Cahoon 2009). Thus, in relation to soy, the future strides in making a better bean.

8. Conclusion

Cancer, as said before, is a multifactorial disease. To blame fat as the only factor behind colorectal carcinogenesis would be irresponsibility, but it plays an important role in etiology for which public health campaigns and food manufacturers can discuss possible changes to benefit all.

Human studies evaluating dietary pattern to estimate risk factors are frequently difficult to interpret since individuals with healthier dietary patterns also present other healthier behaviour. On the other hand, experimental studies have contributed to comprehension of the relation between PUFAs and CRC and demonstrate that lower cancer progression is influenced by ω-3 intake, mainly LCFA, while a higher progression is related to elevated ω-6 intake. Thus, the ideal dietetic solution for a westernized dietary pattern would be the combination of an increase in ω-3 LCFA and reduction of ω-6 PUFA. More discussions regarding what unit to use to recommend fatty acid intake (% of calories, g/d or ω-6:ω-3 ratio) should take into consideration the results of controlled studies.

If we consider that overconsumption of oils rich in ω-6 can favour a pro-inflammatory state and predispose to the development of chronic diseases, such as CRC, sunflower oil, followed by soybean and corn oils are not the best type of oil to be used for cooking or added to food products. On the other hand, if we look to the ω-3 level, soybean oil is similar to canola oil and superior than corn, sunflower and olive oil. The ω-3 content of flaxseed and fish oil is so high that makes them inadequate for cooking or as food ingredient due to higher susceptibility to oxidation and formation of undesirable flavor. Considering this and the ω-6:ω-3 ratio in the others more usable oils, soybean oil would be the best choice for cooking after canola oil, even better if we take into account soybean oil prices in comparison to others oils.

Research investments have been made in order to improve fatty acid profile in the soybean and consequently in its oil, bringing new perspectives for this discussion. Genetic modifications allow that products provide a more balanced fatty acid profile as an alternative to the traditional soybean and these studies are under investigation, showing

good potential with increased levels of SDA. This modification seems to overcome the main limitation of vegetable oils, which is the fact that although they are good sources of ω-3 in the ALA form, the conversion to pharmacologically active compounds (EPA and DHA) is low. Besides, there is also a reduction of LA in the SDA rich soybean oil, which may help to reduce LA intake and consequently reduce the ω-3 fatty acid needs. Although this genetically modified (OGM) soybean brings good perspectives in a clinical application point of view, the disadvantages or risks of using OGM´s from other viewing angles should be also considered.

9. Acknowledgment

A special thanks to Evan Michael Visser for the revision of the English version of this Chapter.

10. References

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17

Anti-Diarrhoeal Aspects of Fermented Soya Beans

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1. Introduction

Generally, fermentation can be described as the modification of biological material using microorganisms with the purpose to obtain a desirable product. Both plant and animal ingredients can be fermented and also the microorganisms used can belong to diverse groups, namely bacteria, yeasts and filamentous fungi (moulds) (Nout et al., 2005).
The origin of fermented foods goes back many thousands of years and fermentation is one of the oldest ways of food processing. Popular fermented products, such as beer, bread, wine and sausages, have been around for centuries (Campbell-Platt, 2004; Nout *et al.*, 2005). Other examples of popular fermented food products are yoghurt, cheese, coffee, tea, alcoholic drinks, soy sauce, sauerkraut and tempe.
Fermentation can have diverse roles: (a) enrichment of the diet, through development of a diversity of flavour and texture of food, (b) preservation through alcoholic, lactic acid, acetic acid and alkaline fermentation, (c) nutritional enrichment of the food with vitamins, essential amino acids or fatty acids, (d) detoxification and removal of anti-nutritional factors, and (e) decreased cooking times (Steinkraus, 1996).
During the fermentation processes, enzymes that are synthesized by the microorganisms are important, since they carry out reactions, which contribute to the development of specific textures, tastes and aromas.
Many fermented products especially fermented soya bean products, find their origin in Asia. Fermented soya bean foods are attractive products and enjoy an increasing interest because of their nutritional and health benefits. One of these products is tempe. Tempe is a collective name for legumes, cereals and other biological materials, fermented by a mould. This technique has its origin in Indonesia. Soya beans are the most commonly used substrate for the tempe production. During fermentation, the soya beans are bound together by the mycelium of the mould into a compact cake in which the macronutrients are enzymatically degraded by the fungal enzymes.
In this chapter, firstly a description will be given of the product tempe. Processing, composition and fermentation characteristics will be described. Also different health effects of the product will be discussed with a special focus on the anti-diarrhoeal aspects of tempe. The second part of the chapter will focus on the anti-diarrhoeal aspects in more detail. The mode of action and the chemical characteristics of the bioactive component will be

discussed. Finally we will discuss the possibilities for application of the product in food or feed industry.

2. Tempe

2.1 Definition

Tempe is a collective name for cooked and fermented beans, cereals or food-processing by-products, penetrated and bound together by the mycelium of a living mould. Yellow-seeded soya beans are the most common and preferred raw material to produce tempe (Nout & Kiers, 2005).

Fig. 1. Tempe, as sold in the Netherlands.

Tempe originates from Indonesia, the authentic Indonesian spelling is "tempe", whereas the spelling "tempeh" is also used in literature. In this thesis the authentic Indonesian spelling will be used. Tempe is pronounced as TEM-pay (Shurtleff & Aoyagi, 2001). In Indonesia, tempe is consumed as a protein-rich meat substitute by all economic groups. Outside Indonesia, tempe gains interest as a major protein source other than meat (Astuti, 2000; Nout & Kiers, 2005; Steinkraus, 1996).
Figure 1 shows soya bean tempe manufactured and sold in the Netherlands. The mould grows not only on the surface of the bean cake, but throughout the bean mass, knitting the soya beans into a compact cake.

2.2 The origin and history of tempe

Tempe is unique among major traditional soya foods, because it is the only fermented soya food product that did not originate in China or Japan (Shurtleff & Aoyagi, 2007). Tempe originates from Central and East Java in today's Indonesia. The earliest references in Indonesian literature are from the early 1800s, but tempe is believed to have evolved long before that time.

As Indonesia has been a Dutch colony for centuries since the late 1600s, some early research findings were published by Dutch scientists. In 1875 the term tempe was defined in the Javanese-Dutch dictionary as "fermented soybeans or press-cake baked or fried in flat pressed cakes. It is well liked as a side dish with rice". In 1895 the Dutch microbiologist and chemist Prinsen Geerlings made the first attempt to identify the tempe mould (Shurtleff & Aoyagi, 2007). Up till now many publications have dealt with microbiological, biochemical and nutritional changes during the tempe fermentation. Also different books (chapters) and reviews about tempe have been published (Ko & Hesseltine, 1979; Nout & Kiers, 2005; Nout & Rombouts, 1990; Shurtleff & Aoyagi, 2001; Steinkraus, 1996; Tibbott, 2004). For many decades tempe has been regarded as a meat alternative for poor communities because of its high protein content. As a result of the low-cost technology available for processing this food, its low price, and its nutritional value, tempe is a traditional food consumed by indigenous Indonesians (Karyadi & Lukito, 1996).
Nowadays, tempe obtains its popularity from its non-meat protein-rich nature, nutritional and health functionality. Figure 2 shows the main reasons why tempe is an ideal food for use in developing countries as a source of tasty and inexpensive high-quality protein (Shurtleff & Aoyagi, 2001).

1. Production requires only simple low-level technology with low costs.
2. The only ingredients are soya beans or other raw material (i.e. legumes, grains) including waste products, water and a starter.
3. The warm or tropical climates characteristic of so many developing countries greatly facilitate the tempe fermentation.
4. The fermentation is unusually simple and short (24 to 48 hours) as compared with several months for many other fermented foods.
5. Tempe has a taste and texture, appearance and aroma that are well suited to use in local cuisines.
6. Tempe is an ideal meat substitute, healthy, tasty and easy to digest.

Fig. 2. Characteristics of tempe as an ideal food in developing countries (Adapted from: Shurtleff and Aoyagi 2001).

2.3 Production of tempe

Yellow-seeded soya beans are the most common and preferred raw material for tempe. Nevertheless, other substrates such as barley (Eklund-Jonsson *et al.*, 2006; Feng *et al.*, 2007), chick pea (Ashenafi & Busse, 1991), cowpea (Egounlety, 2001; Kiers et al., 2000), groundbean (Egounlety, 2001), horse bean (Ashenafi & Busse, 1991), lima bean (Ko & Hesseltine, 1979), pea (Ashenafi & Busse, 1991), oats (Eklund-Jonsson *et al.*, 2006), sorghum (Mugula & Lyimo, 2000) and wheat (Hachmeister & Fung, 1993) have been reported to be suitable substrates. Some substrates can only be processed in combination with soya beans. Also mixtures of legumes with non-legumes, and other plant materials, such as apricot seeds or maize or

food-processing by products, can be used in the tempe fermentation (Feng, 2006; Nout & Kiers, 2005).

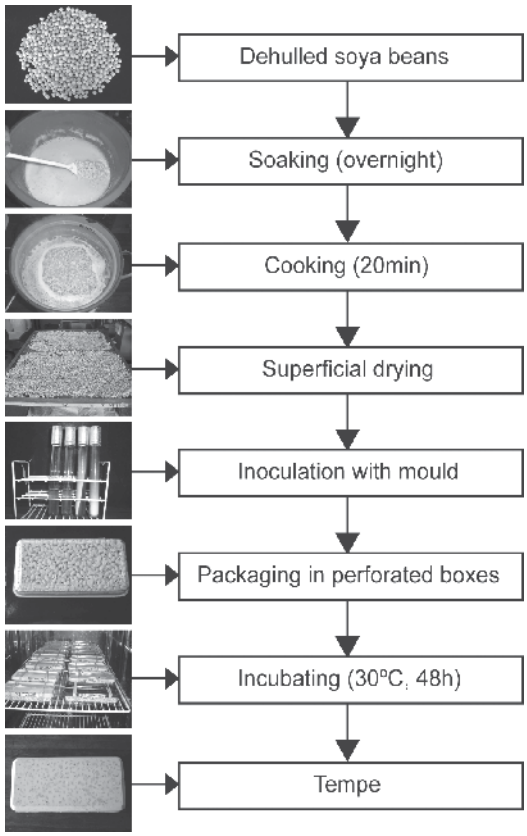

Fig. 3. Production process of soya bean tempe.

The main process operations that differ according to the use of various substrates used in tempe fermentation are the selection of optimum pre-treatments such as de-hulling, optimum soaking conditions or duration of boiling. The process of tempe manufacture from soya beans is shown in figure 3. The process starts with the de-hulling of the soya beans, which can be done manually by rubbing the seed coats from soaked soya beans, or by mechanical abrasion of dry beans. In the Netherlands, tempe manufacturers purchase dry de-hulled soya beans, ready for use. The soya beans are soaked for 6-24 h, in order to: (1) increase the moisture content of the beans, (2) to enable microbial activity in the soaking water, (3) render the beans edible and (4) to extract naturally occurring antimicrobial substances (saponins) and bitter components.

Some manufacturers add lactic or acetic acid or naturally acidified soaking water "backslop" (Nout *et al.*, 1987) at the start of the soaking, to control microbial spoilage.

Subsequently, the soaking water is discarded and the beans are cooked for 20-30 minutes in fresh water. After cooking, the cooking water is discarded and the beans are spread out on perforated trays to remove free water, steam-off and achieve a rapid cooling. When working at a large scale, basket centrifuges are used to remove the cooking water. The cooled soya

beans are inoculated using a tempe starter containing sporangiospores of mainly *Rhizopus* spp., and sometimes *Mucor* spp. with a concentration of about 10^4 colony-forming units (CFU) g^{-1} of cooked beans. Traditionally, the beans are packed in punctured banana leaves allowing a limited supply of air to the beans (figure 4).

Fig. 4. Fresh tempe at the market, Jakarta, Indonesia (Photo taken by: Sakurai Midori).

Nowadays, flexible plastic bags, tubing (sausage casings) or hard plastic boxes with adequate perforation openings, to allow aerobic growth of the mould, are in use. The inoculated and packed beans are incubated for 1-2 days at 25-30°C. Due to the restricted air supply, the formation of fungal sporangiospores is restricted, resulting in an attractive creamy, white fresh tempe cake. Fresh tempe is not eaten raw, but first cooked or fried and used in a variety of dishes. The traditional and modern tempe processing has been reviewed extensively (Nout & Kiers, 2005; Nout & Rombouts, 1990; Shurtleff & Aoyagi, 2001; Steinkraus, 1985; Steinkraus, 1996).

2.4 Microbiological composition of tempe

The microflora in tempe is complex, as tempe is a result of a mixed culture fermentation by moulds, yeasts, lactic acid bacteria and various other bacteria. The major genus of importance is the mould *Rhizopus* with different species such as *R. microsporus, R. oligosporus* and *R. oryzae* (Nout & Kiers, 2005).

Lactic acid bacteria play a role in the acidification of the soya beans during soaking, thereby preventing the growth of spoilage microorganisms (Ashenafi & Busse, 1991; Nout *et al.*, 1987) and thus improving the shelf life of tempe. During fermentation, lactic acid bacteria grow up to 10^9 CFU g^{-1} in final tempe products.

The microbial quality of 110 samples of commercial tempe in the Netherlands was studied and it was shown that most had an aerobic plate count exceeding 10^7 CFU g^{-1}, with high numbers of Enterobacteriaceae and lactic acid bacteria. Yeast levels higher than 10^5 CFU g^{-1} were found in 69% of the samples and some also contained *Staphylococcus aureus*, *Bacillus cereus* or *Escherichia coli* (Samson *et al.*, 1987). Also Ashenafi (1994) found high numbers of enterobacteria, enterococci and staphylococci, whereas Mulyowidarso *et al.* (1990) found high numbers of *Bacillus* species in tempe. The contribution of bacteria and yeasts to the

properties of tempe is only partly understood, but they can play a role in flavour development and substrate modification, and in the safety of the product (Nout & Rombouts, 1990).

2.5 Biochemical changes occurring during fermentation

During fermentation of soya beans several biochemical changes take place, which enhance the nutritional and sensory quality of the tempe. This is mainly due to the activity of the fungal enzymes. The mould, *Rhizopus* spp. produces a variety of carbohydrases, lipases and proteases, which degrade the macronutrients into substances of lower molecular mass, with a higher water-solubility. Also vitamins, phytochemicals and anti-oxidative constituents are formed (Astuti, 2000; Nout & Kiers, 2005).

Table 1 shows a compilation of published data concerning the composition of cooked soya and tempe. Different varieties of soya beans and other processing parameters can influence the composition. During fermentation only small changes in total crude protein, crude lipid and total carbohydrates were reported.

	Cooked soya (100g)		Tempe fresh (100g)				
Reference	2	3	1	2	3	4	5
Energy (kJ)	590	624	657	808	691	603	
Moisture (g)	69		60	60		72	64
Protein (g)	12	14.3	20	19	15.7	12	18
Crude lipids (g)	6	7.7	8	11	6.4	8	4
Carbohydrates (by difference) (g)	11	8.5	10	9	14.1	6	13

1. Shurtleff & Aoyagi, (2001)
2. USDA, (2009)
3. USB, (2010)
4. Voedingscentrum (2006)
5. Voedingswaardetabel (2004)

Table 1. Composition of cooked soya beans and tempe.

Whereas the change in total nitrogen content during fermentation is negligible, an increase of free amino acids takes place, due to hydrolysis of the proteins. The major soya proteins are glycinin and β-conglycinin. β-Conglycinin is more sensitive to protease activity than glycinin, which phenomenon can be related to its chemical structure (De Reu et al., 1995; Nowak, 1992). The degree of hydrolysis strongly depends on the fungal strain and the fermentation conditions.

The concentration of fatty acids present in triglycerides decreased during fermentation, whereas the free fatty acids increased in the final product, but *Rhizopus* is also using part of the fatty acids as a carbon source (De Reu *et al.*, 1994). The production of only small amounts of glycerol indicates that triglycerides were primarily hydrolysed to mono- and diglycerides and free fatty acids (Ruiz-Terán & Owens, 1996).

Carbohydrates in soya beans comprise mainly cell wall polysaccharides and the small sugars fructose, raffinose and stachyose. These small sugars are removed during soaking, cooking and fermentation of the soya beans (Egounlety & Aworh, 2003; Mulyowidarso *et al.*, 1991). The insoluble cell wall polysaccharides, such as pectin, cellulose and hemicellulose

are (partly) degraded during fermentation by the enzymes of the mould which leads to their enhanced water-solubility (Kiers et al., 2000). The major monosaccharide constituents in soya bean cell walls are galactose, glucose, arabinose and galacturonic acid (Huisman *et al.*, 1998). The major carbohydrases of *R. oligosprorus* in tempe were reported to include polygalacturonases, cellulases, xylanases and arabinanases (Sarrette *et al.*, 1992). Soya bean pectin consists of two types of backbones, namely a polygalacturonic acid (1,4)-backbone and a rhamnogalacturonan backbone. The rhamnogalacturonan backbone is substituted with polymers like arabinans, galactans or arabinogalactans. During fermentation of soya beans the pectin fraction and its arabinogalactan side chains are predominantly solubilised (De Reu *et al.*, 1997).

The anti-nutritional factors (ANF), such as trypsin inhibitors and lectins, are mainly leached out or inactivated during soaking, cooking and fermentation. The decrease of phytic acid is very important, because it binds to minerals, thereby lowering the mineral bioavailability (Astuti, 2000; Egounlety & Aworh, 2003; Nout & Kiers, 2005; Prinyawiwatkul *et al.*, 1996; Tawali *et al.*, 1998). The levels of some vitamins of the B group, especially riboflavin, niacin, vitamin B6 and vitamin B12, increase during fermentation, because of fungal and bacterial metabolic activities (Bisping *et al.*, 1993; Denter *et al.*, 1998; Keuth & Bisping, 1993).

Soya beans and soya products contain three isoflavones, genistein, daidzein and glycitein and also various saponins. The concentrations of isoflavones and saponins vary according to soya bean varieties, growing location, cultivation year and degree of maturity (Hubert *et al.*, 2005). Processing of soya beans can result in losses of some isoflavones, especially during soaking and cooking. Fermentation was reported not to cause a significant loss of isoflavones, but aglycons are released from the glucosides by the action of β-glucosidase (Murphy *et al.*, 1999; Wang & Murphy, 1996). Research on saponins during processing of soya also showed enzymatic deglycosylation and some losses during cooking and soaking, but data are still limited (Hubert *et al.*, 2005; Sinha *et al.*, 2005).

2.6 Health aspects of tempe

The effects of soya beans on the health of man and animals have been the subject of several studies. Indeed a number of beneficial effects were reported. Despite the large number of studies that have been performed, many conflicting data have been found, especially in relation to the prevention of chronic diseases (Balk et al., 2005; Messina et al., 2002). Soya is associated with beneficial health effects on cardiovascular diseases, menopausal symptoms, endocrine function, cancer, bone health, reproductive health, kidney diseases, cognitive function and glucose metabolism. Many of the health aspects are related to the phytochemicals present, such as isoflavones and saponins. Only one health claim for use on food labels was approved by the U.S. Food and Drug Administration in 1999. This claim is: ”The inclusion of 25 g soya protein per day in a diet low in saturated fat and cholesterol, may reduce the risk of heart disease” (FDA, 1999). Carefully controlled efficacy studies may still be useful to pin down the relative effects of various components of soya (Balk *et al.*, 2005).

Whereas soya beans are the main ingredient of tempe and the health effects of soya beans can also be associated with tempe, tempe is associated with certain specific health effects.

After fermentation, the absorption and digestibility of the soya beans increase which can have beneficial physiological effects in case of malfunction of the gastrointestinal digestive system (Kiers et al., 2000). The high digestibility of tempe was already observed during

World War II when prisoners suffering from dysentery were able to digest tempe much better than soya beans (Steinkraus, 1996; Tibbott, 2004).
Karyadi and Lukito (1996) described studies performed in Indonesia on the hypolipidemic properties of tempe. In a number of clinical intervention trials, total cholesterol and low-density lipoprotein cholesterol were significantly reduced in persons treated with tempe, whereas HDL cholesterol was raised (Astuti, 2000; Karyadi & Lukito, 1996; Karyadi & Lukito, 2000). Soya beans contain natural antioxidants and during fermentation the anti-oxidative capacity increases (Berghofer *et al.*, 1998; Chen-Tien *et al.*, 2009). Furthermore, several studies demonstrated an anti-diarrhoeal effect of tempe. The next paragraph will deal with the latter aspect in more detail.

3. Tempe and diarhoea

3.1 Antibacterial effects of tempe

In the early 1960s, tempe was reported to contain an antibacterial substance, acting especially against a number of Gram-positive bacteria i.e *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus* and *Clostridium* spp. (Kobayasi et al., 1992; Wang et al., 1972; Wang et al., 1969). These studies suggest the presence of a component from the tempe or the *Rhizopus* spp, that inhibits the growth of these bacteria.
Recently we investigated the antibacterial activity of tempe extracts towards a range of bacterial strains as shown in table 1. A distinct antibacterial effect was observed against *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, whereas growth of *Lactobacillus plantarum*, isolated from tempe, was not affected. Also, *Bacillus* strains were inhibited by the tempe extract and a low antibacterial activity was observed against *Listeria* strains. This antibacterial effect towards *Listeria* was stronger when higher concentrations of tempe extract were used. No antibacterial activity of tempe against *E. coli* or *Salmonella* was observed. Tempe extracts appear to be antibacterial mainly to certain Gram-positive bacteria. Other studies have also shown antibacterial activity by tempe or a mould extract against Gram-positive bacteria (Kiers et al., 2002; Kobayasi et al., 1992; Wang et al., 1972; Wang et al., 1969).

Strain	Growth delay (h)
Listeria innocua	1.6
Listeria monocytogenes	1.0
Lactobacillus bulgaricus	>22[2]
Streptococcus thermophilus	>22
Lactobacillus plantarum (LU 857)	-2.0
Lactobacillus plantarum (LU 852)	-2.3
Escherichia coli K88 (ID 1000)	-0.3
Salmonella enteritidis (97-198)	0.0
Bacillus cereus (ATCC 14579)	6.3
Bacillus subtilis	>12.5

[1] Tempe extract (1% w/v) was prepared by solubilizing 10 g l^{-1} dry soluble material in BHI.
[2] > Means that the growth delay is longer than the total measurement period.

Table 2. Antibacterial activity expressed as growth delay (h) of various strains exposed to tempe extracts (1% w/v)[1] compared to growth in BHI control.

Further research on the antibacterial effect towards *Bacillus* strains was described in Roubos–van den Hil et al. (2010a). In this article it is shown that during the fermentation of soya beans with *Rhizopus*, components are released with high antibacterial activity against *B. cereus* cells and spores. Optimum conditions for maximum antibacterial effect were established and the antibacterial spectrum to several *Bacillus* strains was determined. All *Bacillus* strains tested were strongly affected by the antibacterial action as expressed by growth delay compared to growth in control media without tempe. Vegetative cells of *Bacillus* were inactivated during the first 15 minutes of exposure to tempe extracts. After several hours a re-growth of bacteria was observed, which could be due to either a degradation or shortage of the antibacterial component or to a surviving sub-population. *B. cereus* spores were inactivated immediately after germination as shown by optical density and microscopic observations. This inactivation of the germinated spores appears to be caused by permeabilization of the cytoplastic membrane as shown by fluorescence microscopy.

Recently, the antibacterial effects of the well-known antibacterial component nisin against *Bacillus anthracis* spores were investigated (Gut *et al.*, 2008). While the results of that study also pointed to disruption of the membrane integrity, the tempe antibacterial component is nevertheless different from nisin, based on the sensitivity of nisin to heat and low pH, and its inactivation by different proteases. Furthermore, the antibacterial activity was not found in pure *Rhizopus* strains, cultured on agar plates. This suggests that the antibacterial component(s) are degradation products of soya proteins, and that the fermentation starter microorganisms play a mediating role. The observed antibacterial component(s) can possibly play a role in food preservation and pathogen control.

3.2 *In vivo* effects of tempe on diarrhoea

The role of tempe, as part of the diet and the effect on the development of diarrhoeal infection in animals (table 3a) and humans (table 3b) was investigated by several researchers. Most of this work has been done in Indonesia and not all is available in English literature, but all studies published in English are presented in table 3.

Table 3a shows the research on the effects of tempe on diarrhoea in piglets (Kiers *et al.*, 2003) and rabbits (Karmini *et al.*, 1997). Both were infected with an *E. coli* strain and both showed a lower severity of diarrhoea, when fed with tempe. Table 3b shows four studies of the effect of tempe addition to a human diet, on the severity of diarrhoea. All studies gave similar results, namely a shorter duration of diarrhoea, when tempe was consumed.

Kiers (2001) observed that during the fermentation of soya beans a major degradation of macronutrients resulted in increased nutrient availability for pigs. This was shown by higher absorption of nutrients and better weight gain in early weaned piglets, fed with tempe, compared with soya beans.

The fermented soya beans are especially of interest in patients suffering from intestinal digestive defects. The combination of the high digestibility and nutritional value makes the food important for individuals suffering from malnutrition and/or acute diarrhoea for whom the need of easily digestible rehabilitation foods is high. The anti-diarrhoeal properties of the product make it even more attractive in the prevention and management of malnutrition (Nout & Kiers, 2005). In countries with malnutrition, tempe-based weaning food can be helpful. Research showed that there is a potential for using tempe-based formulas in weaning diets (Osundahunsi & Aworh, 2002).

References	Target group	Treatment	Microorganism	Exposure time	Main results
Karmini *et al.*, (1997)	6-week old male rabbits (n=84)	Tempeh based formulated food (TF), soya bean-based formulated food (SF), milk-based formulated food (MF) and formulated food without protein (FO)	EPEC O125:K70(B) H19 on 4 consecutive days	2 weeks	Onset of diarrhoea: TF 5.07, SF 4.0, MF 3.64 PF 2.36 days. Diarrhoea occurred in 36% of rabbits in the TF group and in 50-64% in the other groups
Kiers *et al.*, (2003)	4-week old piglets (n=96)	Cooked soya (CS), tempe (T), *Bacillus*-fermented soya beans (BT), de-hulled full-fat toasted soya beans (TS)	ETEC O149:K91:K88[ac] on day 1 of the experiment	4 weeks	Diarrhoea incidence: CS 37, T 33, BT 38, TS 46%, diarrhoea severity: CS 1.9 T 1.7, BT 1.8, TS 2.3, days with diarrhoea CS 5.0, T 4.3, BT 4.8, TS 6.2 days

Table 3a. Effects of tempe on *E. coli* infection in animals.

References	Target group	Treatment	Dosis	Main results
Kalavi *et al.*, (1996)	Protein-energy malnourished children 6-60 months (n=117)	Milk-yellow maize porridge (MYMP) (n=61) and tempe-yellow maize porridge (TYMP) (n=56)	*Ad libitum* during 1 month	Duration of diarrhoea (days) MYMP 4.6, TYMP 0.7
Mahmud *et al.*, (1985)	Children <5 years (n=111) with chronic diarrhoea	Tempeh-based formula (n=79) or milk based infant formula (n=32)	Supplementary during diarrhoeal episode	Duration of diarrhoea (days) tempeh-based food mixture 2.39, milk-based formula 2.94
Partawiharddja, (1990) in Karyadi and Lukito, (1996)	Children aged 6-24 months with acute diarrhoea (n=304)	Formulated food without tempeh (A1) (n=75), tempeh formulated food (A2) (n=81), tempeh powder (A3) (n=75) and homemade food (A4) (n=73)	Supplementary during diarrhoeal episode	Duration of diarrhoea (days) A1 6.36, A2 4.83, A3 5.13 , A4 5.83 days
Soenarto *et al.*, (1997)	Children aged 6-24 months with acute diarrhoea (n=214)	Traditionally produced tempe-based formula (TT) (n=72), industrially produced tempeh (IT) (n=72), soya bean powder formulated foods (IS) (n=68)	Starting in hospital and continued at home for up to 90 days after hospitalization; supplementary 2 sachets of formula daily	Duration of diarrhoea (days) TT 3.4, IT 3.5, IS 3.9

Table 3b. Effects of tempe on human diarrhoea patients.

3.3 The influence of tempe on the adhesion of ETEC to intestinal cells

Enterotoxigenic *Escherichia coli* is a global cause of severe, watery diarrhoea in the offspring of some animal species such as newborn (suckling) calves and suckling and weaned pigs (Nagy & Fekete, 1999). In humans, ETEC is recognised as one of the most frequent causes of childhood diarrhoea in developing countries. Also, it is an important causative agent of traveller's diarrhoea (Bhan, 2000; Dalton et al., 1999). Many similarities can be found in the pathogenisis by ETEC infections of animals and humans; this provides opportunities to understand human ETEC infections by the use of animal models (Nagy & Fekete, 2005).

Some of the *in vivo* studies described in paragraph 3.2 showed a specific effect on *E. coli* induced diarrhoea, but in paragraph 3.1 we discussed the antibacterial effect of tempe not to be antibacterial towards *E. coli* bacteria. The anti-diarrhoeal effect is probably not a result of a growth inhibiting effect of the bacteria. Another important step in the infection by ETEC bacteria is the adhesion to and colonization of the intestinal epithelial cells. If this adhesion could be disrupted by food products, the bacteria would not be able to colonize and could pass the intestine without causing infection (Nagy & Fekete, 2005; Nataro & Kaper, 1998).

Indeed, i*n vitro* research showed a strong inhibition of the adhesion of Enterotoxigenic *Escherichia coli* (ETEC) to piglet intestinal cells after addition of tempe extracts (Kiers *et al*., 2002). Further research indicated that adhesion inhibition to piglet brush border cells was caused by components present in aqueous extracts of all stages of tempe production. The highest activity was observed in the fermented products irrespective of the duration of fermentation. The extract of fermented soya beans also showed *in vitro* inhibition of adhesion to human intestinal epithelial Caco-2 cells (Roubos-van den Hil et al., 2009).

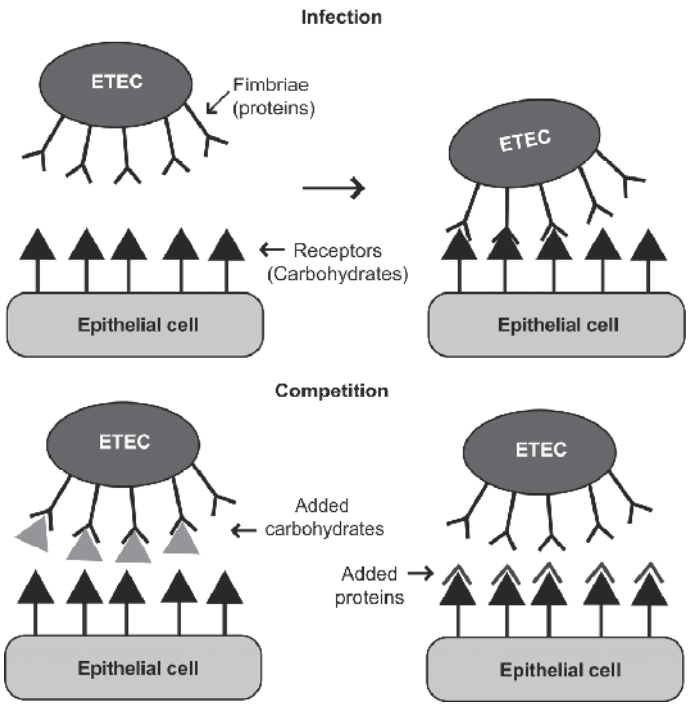

Fig. 5. Infection of intestinal tissue cells by ETEC bacteria and the mechanisms of inhibition of adhesion by specific food components.

The question remains how a fermented soya extract can prevent intestinal cells for adhesion. It is known that the adhesion of ETEC bacteria to intestinal epithelial cells can be mediated specifically by fimbriae. These (proteinaceous) structures bind to receptors (binding sites) at the intestinal epithelial cell surface. Competition of this adhesion can be mediated by carbohydrates and proteins as depicted in figure 5. Carbohydrates, which are structurally similar to the binding sites of the epithelial cells, can adhere to the bacteria (Ofek et al., 2003; Pieters, 2007; Sharon, 2006). For example, human breast milk contains many oligosaccharides that act as "anti-adhesins" (Bode, 2006). Also proteinaceous component(s) can interact with this specific adhesion by binding to the receptors at the intestinal epithelial cells. Blomberg *et al*. (1993) showed that proteinaceous components released from lactic acid bacteria could decrease the adhesion of ETEC to piglet ileal mucus. Bifidobacteria are also known to excrete a proteinaceous component that prevents the adhesion of ETEC to intestinal epithelial cells (Fujiwara *et al.*, 2001). Moreover, bacteria living in the mucus layer can prevent the attachment of pathogenic microbes by occupying available binding sites (Pluske *et al.*, 2002).

Another mechanism which has been shown to inhibit adhesion of ETEC to intestinal cells is by modification of the fimbriae with proteolytic enzymes. It was shown that the adhesion of ETEC was inhibited, possibly by degradation of the proteinaceous fimbriae. For example, bromelain, a proteolytic enzyme from pineapple stems, reduced ETEC adhesion to intestinal cells significantly (Chandler & Mynott, 1998). However, Roubos-van den Hil et al. (2010c) showed that after heating, during which the proteolytic enzymes were inactivated, the tempe extracts remain bioactive. Thus, proteolytic enzymes could not be responsible for the adhesion inhibition of tempe extracts.

Treatment No.[1]	Sample mixture[2]		Washing[3]	Addition (after 30 min)[4]	Adhesion (%)[5]
1 (pos. control)	BB	ETEC +	None	PBS	100 ± 2.7[a]
2 (neg. control)	BB	ETEC -	None	PBS	3.4 ± 1.6[b]
3	BB	ETEC +	None	TE	87.9 ± 4.4[a]
4	BB	TE	None	ETEC +	4.1 ± 2.1[b]
5	BB	TE	PBS (once)	ETEC +	61.6 ± 6.7[c]
6	ETEC +	TE	None	BB	7.4 ± 2.3[b]
7	ETEC +	TE	PBS (once)	BB	2.7 ± 1.9[b]
8	ETEC +	TE	PBS (twice)	BB	2.0 ± 1.1[b]
9	ETEC +	PBS	PBS (twice)	BB	72.3 ± 2.3[c]

1 Treatment number corresponds to the sample mixture mentioned in columns 2 and 3.
2 Initial mix of two components: BB: 50 µl brush border cells, ETEC +: 50 µl ETEC strain ID 1000, ETEC -: 50 µl ETEC strain ID1084, TE: 50 µl Tempe extract 1 g l-1 of 72 h fermented tempe, PBS: 50 µl Phosphate buffered saline.
3 Washing by centrifugation of the sample mixture followed by suspension in PBS.
4 Addition of the third component.
5 Adhesion expressed as % of the positive control (treatment 1) without tempe addition ± SEM. Significant differences are indicated by different superscripts alphabets.

Table 4. Adhesion of ETEC to brush border cells, as affected by composition and timing of reaction mixture (Roubos-van den Hil et al., 2009).

Further research about the interactions between intestinal epithelial cells, ETEC and tempe extracts was performed. Pre-treatment of ETEC with tempe extracts resulted in strongly reduced adhesion (table 4). Washing of the bacteria, which removed non-bound tempe extract did not restore the adhesion, which suggested a strong interaction between ETEC and component(s) in the tempe extracts, which was not lost by washing steps.
Incubation of intestinal epithelial cells with tempe extracts prior to the ETEC addition reduced adhesion strongly. However, this reduced adhesion was restored by washing (removal of tempe) the epithelial cells before ETEC addition. These results point towards the interaction between ETEC and the tempe extracts as the mechanism of adhesion-inhibition
In addition, an adhesion of ETEC to tempe extracts was measured with tempe extracts bound to high binding polystyrene plates. More adhesion of ETEC bacteria was shown on wells coated with tempe extracts, which also provides evidence for the adhesion between ETEC and tempe extracts. Thus, the inhibition of adhesion is mediated by an interaction with the bacteria, which suggests the bioactive component to contain carbohydrates (Roubos-van den Hil et al., 2009).

3.4 Chemical characteristics of the anti-adhesion component in tempe

The anti-adhesion activity of fermented soya beans is expected to be mediated by a specific component or specific structure. Roubos-van den Hil et al. (2010b) investigated the effect of various fermentation substrates on the inhibition of ETEC adhesion to intestinal epithelial cells. During fermentation of various legumes (soya bean, cowpea, green pea and red bean), the bioactivity increased to a similar extent as was measured in fermented soya beans. Interestingly, the cereal (wheat, oat and barley) derived tempe products showed no bioactivity at all. It was concluded that the mould mycelial biomass itself is not responsible for the bioactivity, since the mould mycelia showed equally good growth in the cereal- and legume-derived tempe's. The bioactive component is specific for legumes and is released or formed by enzymatic breakdown during fermentation.
Tempe is traditionally fermented with moulds, mainly *Rhizopus* spp., but tempe also contains bacteria, i.e., lactic acid bacteria (LAB), *Bacillus* spp., and yeasts (Nout & Rombouts, 1990; Samson *et al.*, 1987), of which less is known in relation to their functions in tempe. Research was done on the fermentation of soya beans with several pure cultures of microorganisms, isolated from tempe or other fermented products, to detect bioactivity. The *Bacillus* spp., moulds and some yeast were able to degrade macronutrients of the soya beans and showed bioactivity by inhibiting the adhesion of ETEC to intestinal cells. However, LAB fermentation of soya beans only resulted in lactic acid formation but not in degradation of soya macromolecules, and did not result in bioactivity. Thus, the capability to release or form bioactive components from the soya beans is not specific for *Rhizopus* spp. Several strains that can degrade macromolecules could be used to elicit bioactivity in legumes (Roubos-van den Hil et al., 2010b).
Further chemical characterization experiments were performed by Roubos van den Hil et al. (2010c). Tempe extracts were defatted whereupon the extracts remained bioactive. After ultrafiltration, the bioactive component was recovered in the >30 kDa part and size exclusion experiments showed the bioactive component to be intermediate in size. This is in agreement with (Kiers et al., 2003, 2007), who found the bioactive component to be larger than 5 kDa. Furthermore, the bioactivity of the tempe extracts was not influenced by two broad spectrum proteolytic enzymes and heating at 100°C. These results, combined with

those found by Roubos-van den Hil et al., (2009), indicating that the inhibition of adhesion is caused by an interaction between ETEC and tempe extracts, suggest that the bioactive component in tempe is of carbohydrate nature.
Tempe contains cell wall polysaccharides from both fungal and soya bean origin. Since bioactivity is not caused by the biomass of the mould, we focused on the cell wall polysaccharides of soya. These cell wall polysaccharides are (partly) degraded during fermentation by the enzymes of the mould, which leads to enhanced solubility (Kiers *et al.*, 2000). Pectin and its arabinogalactan chains are predominantly solubilized during fermentation (De Reu *et al.*, 1997). Since the bioactivity of the fermented extracts was measured in water-soluble extracts (Roubos-van den Hil et al., 2009), an increase of solubility could also be responsible for the higher bioactivity of the fermented extracts (enriched in these carbohydrates) compared with normal soya bean soluble extracts.
Galactose, glucose, arabinose and galacturonic acid are the major monosaccharide constituents in soya cell walls (Huisman *et al.*, 1998). After defatting, ultrafiltration, protease treatment and heating, the bioactive tempe extract was found to be rich in arabinose, galactose and galacturonic acid (Roubos van den Hil et al., 2010c), which corresponds to the observation of the predominant degradation of pectin and arabinogalactan during fermentation of soya beans (De Reu *et al.*, 1997).
Roubos-van den Hil et al (2010c) treated the bioactive tempe extract with different polysaccharide degrading enzymes, to obtain more detailed information about the bioactive component. Thereafter the monosaccharide composition was determined and arabinose was shown to be an important component of the bioactive fraction. After enzymatic degradation of the arabinose containing polysaccharides, the bioactivity was lost, whereas the enzymatic removal of galactose and galacturonic acid from the polysaccharides did not specifically cause a loss of activity. Further purification experiments also showed an increase of arabinose in molar proportion in the purified active fractions.

	Fuc[1]	Rha	Ara	Gal	Glc	Man	Xyl	GalA	GlcA
Soya	2.3	1.8	19.5	24.4	32.5	4.1	4.6	9.6	1.4
Organic Soya	3.1	1.6	31.8	23.3	21.0	4.9	5.9	7.4	1.0
Cowpea	nd	nd	30.0	14.4	40.5	2.4	7.7	4.6	0.4
Green pea	nd	0.5	35.5	10.2	42.4	1.4	3.6	6.1	0.3
Red bean	nd	1.0	22.3	23.5	44.4	0.5	3.0	5.0	0.5
Wheat	nd	nd	9.3	4.1	68.4	1.1	16.1	0.9	0.2
Oat	nd	nd	3.5	2.5	86.3	1.2	5.5	0.7	0.3
Barley	nd	nd	2.6	0.6	88.2	1.2	7.0	0.2	0.2

nd, not determined
1 Fuc (fucose), Rha (rhamnose), Ara (arabinose), Gal (galactose), Glc (glucose), Man (mannose), Xyl (xylose), GalA (galacturonic acid), GlcA (glucuronic acid).

Table 5. Monosaccharide composition (mol %) of leguminous and cereal grains fermented with *Rhizopus microsporus* (LU 573) after hydrolysis.

The monosaccharide composition of leguminous and cereal grains was determined (table 5). These results show a higher molar proportion of arabinose in the leguminous materials compared to the cereal grains. This is in agreement with the bioactivity, which was only present in the leguminous materials. These results all support our conclusion that the bioactive component has to contain at least arabinose.

Arabinose is an important constituent of pectic cell wall polysaccharides of soya beans. It is especially present in the rather long arabinan and (arabino)galactan side chains of rhamno-galacturonans (Huisman, 2000). We hypothesize that some structural epitopes of such arabinans or arabinogalactans are responsible for the bioactivity in tempe. During fermentation these structures become more soluble and thus more accessible to the intestinal cells.
The bioactivity was found only in leguminous substrates and increased during fermentation as a degradation product of the enzyme activity. Fractions containing material >30 kDa are the most active, which suggests that only a partial degradation of cell wall polysaccharides is needed to obtain the bioactive component.
During fermentation the macromolecules are degraded by the enzymes of the microorganism (Nout & Kiers, 2005), but macromolecules can also be degraded by addition of enzymes, which possibly can give the same health effects. Carbohydrases could offer potential for the use in piglet diets to improve nutrient utilization and disease prevention, especially for the degradation of non starch polysaccharides (NSP) of the cell walls of animal feeds. After degradation of NSP by carbohydrases, the hydrolysis products may influence enteric bacterial infections in piglets (Kiarie et al., 2007; Meng et al., 2005; Pluske et al., 2002). Indeed it was previously reported that the addition of carbohydrases to piglet diets reduced the frequency and severity of non-specific diarrhoea (Partridge, 2001). Kiarie *et al.,* (2008) investigated the effect of NSP hydrolysis products of soya bean meal against ETEC infected piglets *in situ* with intestinal segments. It was shown that NSP hydrolysis products of soya bean meal were beneficial in maintaining fluid balance during ETEC infection (Kiarie *et al.*, 2008). These results can be compared with the results in this chapter that discuss the effect of hydrolysis that takes place by the enzymes of the moulds.

3.5 Anti-adhesion bioactivity in a broader perspective

Most of the results described in this chapter were performed with *in vitro* studies with a specific target ETEC strain as a model to investigate adhesion inhibition. The question arose whether the observed adhesion inhibition observed could also be detected with other diarrhoea causing strains in piglets. Moreover, could tempe also be used to prevent diarrhoea in humans?
Roubos- van den Hil et al. (2010b) described the adhesion of different ETEC strains isolated from piglets with diarrhoea to brush border cells, and the inhibition of adhesion by tempe extracts. Tempe extracts decreased the adhesion of most ETEC tested on piglet brush border cells. Consequently tempe extracts could prevent intestinal cells being colonized by different strains of ETEC causing diarrhoea in piglets.
Furthermore, several strains with different serotypes of *E. coli* were collected and tested for their adhesion inhibition to piglet brush border cells and Caco-2 human intestinal epithelial cells. Especially the different human ETEC strains with known colonization factors (CS) were of interest, because of the possible interaction of the tempe extracts with these specific colonization factors, since ETEC is also an important causative organism in childhood diarrhoea (Bhan, 2000; Qadri *et al.*, 2005).
Figure 6 shows the adhesion of these *E. coli* strains to piglet brush border cells and their adhesion in the presence of tempe extracts. Three strains showed adhesion to the brush border cells in the same order as the positive control. This positive control was used in earlier brush border experiments as a reference strain. Whereas three strains showed some

adhesion to the brush borders cells, the other fourteen strains did not show any adhesion to the piglet brush border cells. After addition of tempe extracts, none of the strains showed inhibition of adhesion. This suggests that the tempe extract was not bioactive against the colonization of these strains in the piglet intestinal cells. The strains tested were of human origin from different categories of diarrhoeagenic bacteria, but for all of them adhesion is an important step in pathogenisis. Since the brush borders are from piglets and not from humans, it is possible that the specific adhesion-ligand interaction can not be formed. This is indeed indicated by the low number of *E. coli* strains that are capable to adhere to piglet brush border cells.

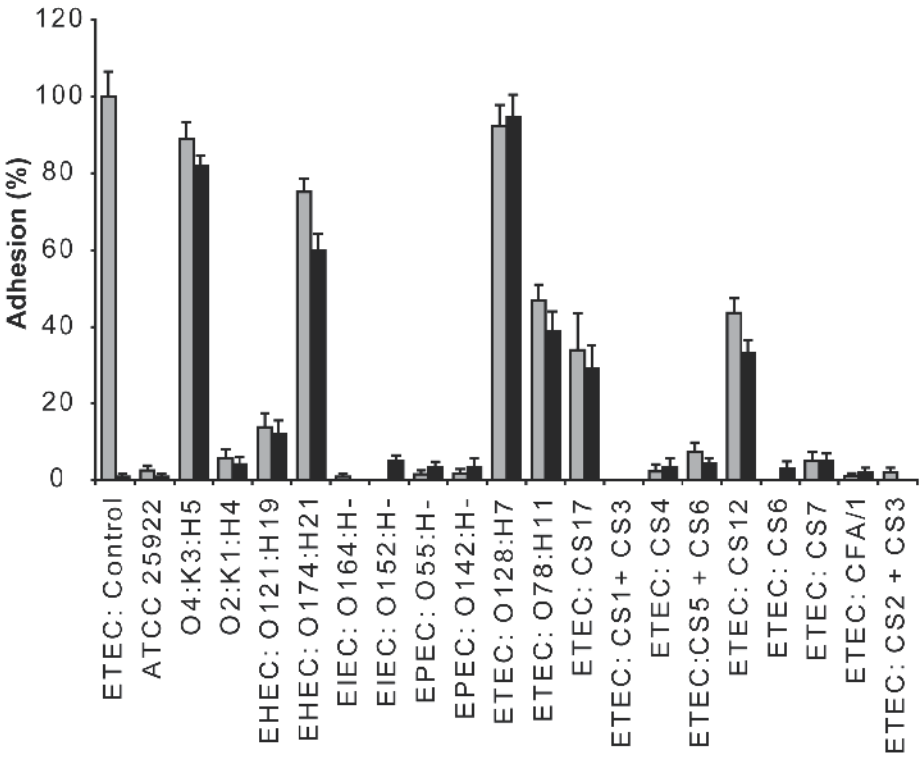

Fig. 6. Adhesion of different human *E. coli* strains to piglet intestinal brush border cells (gray bars) and the adhesion of these strains with addition of tempe extracts (black bars). The strains were obtained from the following sources: ETEC: control: piglet K88-positive O149:K91 (ID 1000), ID-Lelystad, Lelystad, The Netherlands; ATCC25922, O4:K3:H5 and O2:K1:H4 from VWA, Zutphen, The Netherlands; EHEC: O121:H19 (1120700042), EHEC: O174:H21 (1120700050), EIEC: O164:H- (ECOL396), EIEC: O152:H- (ECOL384), EPEC: O55:H- (ECOL280), EPEC O142:H- (ECOL372), ETEC: O128:H7 (ECOL522) and ETEC: O78:H11 (ECOL402) from RIVM, Utrecht, The Netherlands; ETEC CS17 (E20738A), ETEC CS1+CS3 (E1392-75), ETEC CS4 (E11881/9), ETEC CS5 + CS6 (VM75688), ETEC CS12 (350C1A), ETEC CS6 (E11881/14), ETEC CS7 (E29101A), ETEC CFA/I (258909-3), ETEC CS2 + CS3 (278485-2) from University of Gothenburg, Gothenburg, Sweden. Bars represent mean values of 12 measurements, expressed as % adhesion compared to the control without tempe addition. Error bars represent SEM.

Subsequently, some diarrhoea causing *E. coli* strains were selected and tested for their adhesion inhibition to the human Caco-2 intestinal epithelial cells. All tested strains showed adhesion to the Caco-2 cells (data not shown) and the inhibition of this adhesion is shown in figure 7.

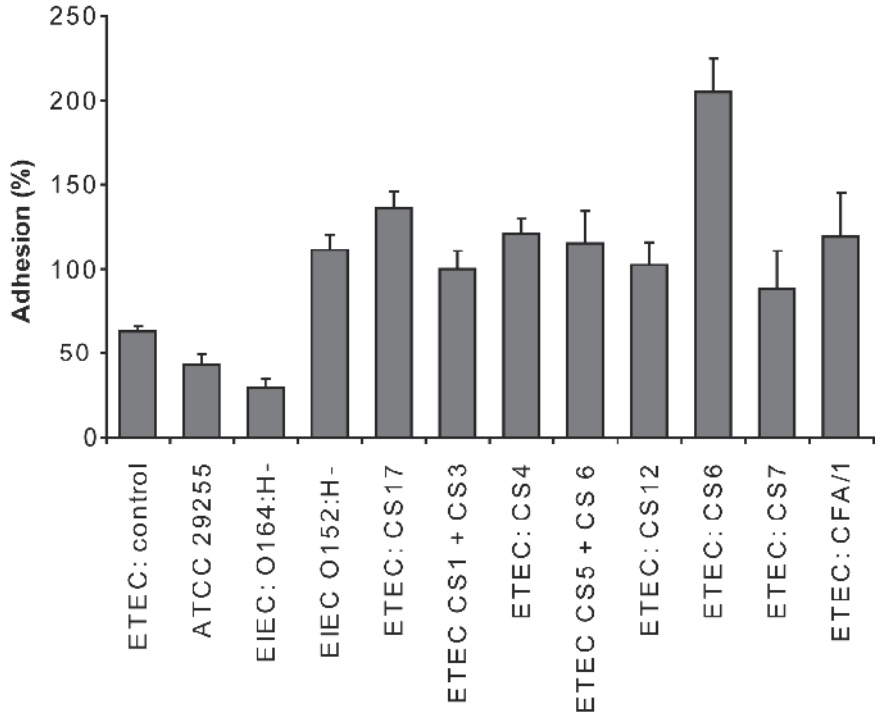

Fig. 7. Adhesion of different *E. coli* strains to Caco-2 intestinal epithelial cells. Bars represent mean values, expressed as % adhesion compared to the adhesion of the strain without tempe addition. Error bars represent SEM. The strains were collected from the following sources, with in brackets the number of replicates tested: ETEC: control: (n=22) piglet K88-positive O149:K91 (ID 1000) from ID-Lelystad, Lelystad, The Netherlands; ATCC25922 (n=6), from VWA, Zutphen, The Netherlands; EIEC: O164:H- (n=6) (ECOL396), EIEC: O152:H- (n=3) (ECOL384) from RIVM, Utrecht, The Netherlands; ETEC CS17 (n=3) (E20738A), ETEC CS1 + CS3 (n=6) (E1392-75), ETEC CS4 (n=3) (E11881/9), ETEC CS5 + CS6 (n=3) (VM75688), ETEC CS12 (n=6) (350C1A), ETEC CS6 (n=3) (E11881/14), ETEC CS7 (n=2) (E29101A) and ETEC CFA/I (n=3) (258909-3) from University of Gothenburg, Gothenburg, Sweden.

The piglet positive control, strain ATCC 29255 and EIEC: O164:H- showed an inhibition of adhesion up to 70%. The different human ETEC strains with known colonization factors (CS) did not show any inhibition of adhesion by the tempe extracts. One of them (ETEC: CS6) even showed a higher adhesion value after addition of the tempe extract.
Nevertheless, we found three (two and the control) *E. coli* strains of which the adhesion to intestinal Caco-2 cells was inhibited by tempe extract. However, presently no further conclusions about the specific interactions between *E. coli* and tempe within humans can be drawn.

The anti-diarrhoeal effect of tempe was demonstrated (see table 3) in several human studies with children suffering from diarrhoea (Kalavi *et al.*, 1996; Karyadi & Lukito, 1996; Mahmud *et al.*, 1985; Soenarto *et al.*, 1997). Since the organisms causing diarrhoea in these studies were not known, a specific study to explain the effect of tempe on these bacteria was not possible. The nutritional status also has a potential impact on diarrhoeal episodes. The interactions between diarrhoea and malnutrition as a cause or an effect are well recognised (Gadewar & Fasano, 2005; Gracey, 1996). Tempe might have potential in breaking this vicious cycle of malnutrition and diarrhoea, since it is nutritious, easily digestible and absorbable and might also protect intestines by specific interaction with the adhesion op pathogens to the intestinal cells in humans.

4. Conclusions and future perspectives

4.1 Conclusions

The fact that diarrhoea is a major health problem worldwide in children as well as in farm animals, underlines the importance of the search for anti-diarrhoeal agents and investigation of their mode of action.

Tempe was found to be bioactive in two ways towards diarrhoea-associated bacteria. On the one hand, tempe (and soya beans to a lesser extent) inhibit the adhesion of ETEC to intestinal cells, which can be of interest in the recovery and prevention of diarrhoea in piglets as well as in humans. On the other hand, tempe is antibacterial against *B. cereus* cells and spores, which can be of interest in food preservation and pathogen control.

The anti-adhesion activity is caused by an interaction between ETEC and tempe extracts, which results in a loss of adhesion capability of ETEC to the intestinal cells. This bioactivity is found in tempe derived from leguminous seeds, whereas tempe derived from cereals is inactive. The bioactive component(s) are released or formed during fermentation by enzymatic degradation of leguminous matter. Fermentation with several other microorganisms also resulted in the formation of bioactive component(s).

Furthermore, the bioactive component(s) are of carbohydrate nature, and contain arabinose as an important monosaccharide constituent. The bioactive component(s) are supposed to originate from arabinan or arabinogalactan chains of the pectic cell wall polysaccharides of legumes.

The antibacterial activity of tempe is caused by a proteinaceous component, which is liberated during the fermentation of soya beans. The inactivation of bacterial cells and spores appears to be caused by permeabilization of the cytoplastic membrane.

4.2 Future perspectives

Further characterization of the anti-adhesion component is needed to fully understand the mechanism of action. A well purified and characterized bioactive component could be tested in animal and human studies to verify the *in vitro* results in *in vivo* situations. Further research is required to exploit potential application of the bioactive principle in food or feed matrices. Another important issue to be researched is the possibility to liberate the arabinose containing medium-weight polysaccharides through the addition of specific enzymes in stead of fermentation.

In addition, a characterization of the antibacterial component in tempe by chemical analyses will be required to assess its potential in the industry. A full characterization could open new possibilities for producing the bioactive component and application in food and feed preservation and pathogen control.

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18

Antioxidant and Hypocholesterolemic Effects of Soy Foods and Cardiovascular Disease

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1. Introduction

Cardiovascular disease (CVD) is widespread in the modern world and represents one of the main causes of mortality (Levi et al., 2009). Increased blood cholesterol content (Hozawa, 2011) and oxidative modification of low density lipoproteins (Vogiatzi et al., 2009) represent risk factors for the development of CVD. The right nutrition is considered effective in the prevention and treatment of CVD (Rudkowska & Jones, 2007). The results of epidemiological, clinical and experimental studies show that soy foods decrease blood cholesterol in individuals with hyperlipidemia, as well as mortality rates from CVD both in Asians and Caucasians (Anderson et al., 1995; Borodin et al., 2009; Messina & Messina, 2003). Soy beans contain antioxidants that exert an antioxidant effect when people consume soy foods (Bertipaglia de Santana et al., 2008). This effect may be also beneficial for CVD patients. This review focuses on the discussion of modern data on antioxidant and hypocholesterolemic effects of soy foods, as well as on the potential role of soy foods in the prevention and treatment of CVD in Russia.

2. Antioxidant effect of soy foods

The antioxidants of soy beans are represented by isoflavones, tocopherols, ascorbic acid and some other compounds (Barnes, 2010; Borodin et al, 2001). When soy beans are processed into different foods the particular antioxidants content of the produced foods may change depending on the processing procedure (Xu et al., 2010) and storage conditions (Rau De Almeida Callou, 2010) and differ from the initial antioxidant content in the soy beans (Anderson & Wolf, 1995). However, antioxidants of soy beans are also present in soy foods. So, the consumption of soy foods is followed by the antioxidant effect (Bertipaglia de Santana et al., 2008; Borodin et al., 2001). Among the antioxidants of soy beans mainly it is

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isoflavones that had attracted the attention of researches in explaining the health effects of consumption of soy foods (Barnes, 2010; Messina, 2010). Much less attention has been paid to the tocopherols and other soy antioxidants (Borodin et al., 2001; Mikoluc et al., 2009).

2.1 Antioxidants of soy beans and some soy foods

Table 1 shows the results of our measurements of some antioxidants content in the species of soy beans, planted in the Far East of Russia, and some locally produced soy foods.

Soy beans and soy foods	Isoflavones*		Tocopherols		β-carotenes µg/g, µg/mL	Ascorbic acid µg/g, µg/mL
	µg/g, µg/mL	mmoles/mole of triglycerides	µg/g, µg/mL	mmoles/mole of triglycerides		
Soy beans	580-3800	10-70	380	3.80	19	70
Full fat soy flour	580-3800	10-70	370	3.37	13	97
Lipoxygenase soy flour	580-3800	10-70	310	2.92	15	101
Semi-fat soy flour	370-2500	10-70	265	3.99	10	110
Soy milk	30-175	9-50	21	3.47	Trace	15
Tofu	-	-	124	4.17	Trace	51.7

Table 1. Isoflavone, tocoferol, β-carotene and ascorbic acid content of soy beans and some soy foods. Tocoferol and β-carotene content was measured in the lipid extracts from soy beans and soy foods by colorimetric methods and ascorbic acid content was measured by titration of acidic extracts with 2,6-dichlorphenolindofenol. * - data from Anderson R. & Wolf W. (Anderson & Wolf, 1995).

The major antioxidants in soy beans are isoflavones and tocopherols. Total isoflavone content of soy beans (580-3800 µg/g of raw weight or 10-70 mmoles/mole of triglycerides) is 1.5-10 times higher than such of tocopherol content (380 µg/g of raw weight or 3.8 mmoles/mole of triglycerides). In different types of soy flour the content of these antioxidants is nearly the same as in soy beans. Isoflavone and tocoopherol content of soy milk and tofu is considerably less if expressed in µg/g or µg/mL of product. However, it is very similar to that of soy beans if expressed in mmoles/mole of triglycerides. Soy bean oil contains 3-4 molecules of tocoferols per 1000 molecules of triglycerides. Despite the fact that isoflavones are polar compounds, which are not soluble in oils, the formal calculation of the total isoflavone content in soy beans and soy foods as mmoles/mole of triglycerides shows that 10-70 molecules of isoflavones correspond to 1000 molecules of triglycerides. β-carotene content of soy beans and soy flour is 20-30 times lower than tocopherol content and 200-300 times lower than isoflavone content. Ascorbic acid content in raw soy beans (70 µg/g) and soy foods (15-110 µg/g) is low and soy foods do not play any substantial role as a source of this vitamin for humans.

Due to the high amounts of antioxidants soy beans and soy foods are resistant to oxidative modification. In the analyzed samples of soy beans and soy foods the primary products of lipid peroxidation, namely, dien conjugates and lipid hydroperoxides have been determined but their content was rather low – from 2 to 7 conjugated double bonds and less than 1 hydroperoxide group per 1000 molecules of triglycerides (table 2).

Soy beans and soy foods	Dien conjugates		Lipid hydroperoxides	
	nmoles/g, nmoles/mL	mmoles/mole of triglycerides	nmoles/g, nmoles/mL	mmoles/mole of triglycerides
Soy beans	1690	7.28	160	0.69
Full fat soy flour	940	3.68	130	0.51
Lipoxygenase soy flour	870	3.53	97	0.39
Partly defatted soy flour	690	4.46	110	0.71
Soy milk	26	1.77	14	0.95
Tofu	122	1.77	34	0.97

Table 2. Dien conjugate and lipid hydroperoxide content of soy beans and some soy foods. Dien conjugate and lipid hydroperoxide content was measured in the lipid extracts from soy beans and soy foods by the absorbance at 233 nm and by the ability to oxidize Fe^{2+} ions, respectively (Borodin et al, 2001).

2.2 Antioxidant effect of soy milk in vitro

It is easy to demonstrate the antioxidant effect of soy milk in vitro by its ability to inhibit the process of lipid peroxidation in a system containing fast oxidizing substrate, for example in liver microsomes (table 3). The rate of lipid peroxidation in liver microsomes consists of 2.52±0.31 nmoles of malonic dialdehyde (MDA) min^{-1} mg^{-1} of phospholipids in the presence

Type of milk	Ascorbate-dependent lipid peroxidation		NADPH-dependent lipid peroxidation
	Oxidizeability (nmoles of MDA min^{-1} mL^{-1})	AOA (%)	Oxidizeability (nmoles of MDA min^{-1} mL^{-1})
Liver microsomes*	2.52±0.31	-	1.5±0.18
Soy milk	0.48±0,18	57.3±9.3	2.80±0,09
Caw milk	0.28±0,17	10.9±2.5 ($p<0,05$)**	2.63±0,17

Table 3. Oxidizeability and antioxidant activity of soy and cow milk at lipid peroxidation in liver microsomes. I mL of the incubation mixture contained 0.8 mM ascorbate (or 1mM NADPH); 0.2 mM sodium pyrophosphate; 50 mM tris-HCl buffer, pH 7.4.; 1 mg of microsome protein and 0.1 mL of milk. The time of incubation was 5 min for ascorbate-dependent lipid peroxidation and 20 min for NADPH-dependent lipid peroxidation. Lipid peroxidation was initiated by the addition of Fe^{+2} ions in final concentration 2 μM. The reaction was stopped by the addition of 30% trichloracetic acid in final concentration 1.5 mM. MDA – malonic dialdehyde. AOA – antioxidant activity. AOA was calculated by the ability of soy and caw milk to inhibit MDA accumulation in the incubation medium at the initiation of lipid peroxidation in liver microsomes (Borodin et al., 2001). MDA content in the incubation medium was determined by the color reaction with thiobarbituric acid (TBA-test) (Borodin et al., 1993). Values are means ± SEM. * - oxidizeability of microsomes was expressed as nmoles of MDA min-1 mg-1 of microsome phospholipid; **- p values corresponds to the differences between the AOA of soy and cow milk.

of ascorbic acid and Fe^{2+}ions being significantly higher than oxidizeability of soy- and caw milk (0.48±0.18 and 0.28±0.17 nmoles of MDA min^{-1} mL^{-1}, respectively). In contrast to this in the presence of NADPH and Fe^{2+} ions soy milk and caw milk oxidize faster (2.80±0,09 and 2.63±0,17 nmoles of MDA min^{-1} mL^{-1}) than liver microsomes (1.5±0.18 nmoles of MDA min^{-1} mg^{-1} of phospholipids). The difference between the oxidizeability of soy milk and caw milk in the presence of ascorbic acid and Fe^{2+} ions is not statistically significant. In the presence of NADPH and Fe^{2+} ions both types of milk oxidize at the same rate. It is interesting to note that in the presence of NADPH soy milk oxidize 5-times faster and caw milk 10-times faster than in the presence of ascorbate. The antioxidant effect of soy milk on ascorbate-dependent lipid peroxidation in liver microsomes is 5-fold higher than the effect of cow milk. To calculate the antioxidant effect of soy and caw milk on NADPH-dependent lipid peroxidation in liver microsomes seems impossible because the rate of the oxidation of microsomes is slower than the rate of oxidation of milk. The results obtained testify to the high antioxidant properties of soy milk, probably due to high isoflavone and tocopherol content. Because of this soy milk oxidizes rather slowly despite the high content of polyunsaturated fatty acids in its lipids (table 4).

2.3 Fatty acid composition of soy- and caw-milk

The lipids of caw- and soy milks are characterized by the following marked differences in fatty acid composition, which may be important for their dietary value for CVD patients (table 4). 1) total content of saturated fatty acid in caw milk (63.8%) is 3,7 times higher than in soy milk (17.8%); 2) total monousaturated fatty acids content in caw milk is also higher and consists of 30,4% versus 21,5% in soy milk; 3) total content of polyunsaturated fatty acids (PUFA) in caw milk (61.2%) is 10,6 times higher than in the caw milk (5,7%); 4) the content of omega-3 PUFA, which is considered favorable for the prophylaxis of CVD (Saravanan et al., 2010), is 4.2 times higher in soy milk than in caw milk (8.53% versus 2.03%, respectively; 5) the fatty acid composition of the lipids in soy milk is less diversified than in cow milk. Short chain fatty acids (C<10) are not represented in the lipids of soy milk and represent minor components of caw milk; 6) the principal fatty acids of soy milk are polyunsaturated linoleic acid (18:2n-6) (52%), monounsaturated oleic acid (18:1n-9) (18%) and saturated palmic acid (16:0) (12%) while the main fatty acids of caw milk are saturated acids – palmitate (16:0) (25%) and stearate (18:0) (14%) and monounsaturated oleic acid (18:1n-9) (21%); 7) the specific minor fatty acids of soy milk are 18:3n3, 10:1n-9 and 22:0 and caw milk 4:0, 8:0, 10:0, 12:1, 14:1, 15:0-iso, 15:0-antyiso, 16:0-iso, 16:1n-9, 16:2, 17:0-iso, 17:1, 18:1-trans (table 4). High content of PUFA in soy milk and especially of omega-3 family PUFA, represents an important dietary advantage of this milk compared to caw milk for the prevention and treatment of CVD. The striking feature of soy milk in comparison with cow milk is lower triglyceride and higher tocoherol and phospholipid content. As a result, the ratio phospholipids/triglycerides in soy milk is 3 times higher than in caw milk (Borodin et al., 2001), which represents another advantage of soy milk for CVD patients.

2.4 Antioxidant effect of consumption of soy milk in vivo

The results presented above show that soy milk contains complex of antioxidants and reveals antioxidant effect both in vitro and in vivo. The consumption of soy milk is followed by an increase in the α-tocoferol content in the blood (Borodin et al., 2001, 2003). It was therefore of interest to determine to what extent the antioxidant effect of soy milk may be attributed to α-tocoferol? To answer this question we performed the following study.

Fatty acids	Cow-milk	Fatty acids	Soy-milk
4:0	3.28		
6:0	2.02		
8:0	0.48		
10:0	2.22		
12:0	2.63	12:0	0.11
12:1	0.10	14:0	0.22
14:0-iso	0.19	15:0	0.06
14:0	9.24	16:0	11.80
14:1	0.69	16:1n-7	1.92
15:0-iso	0.38	17:0	0.11
15:0-antiiso	0.68	18:0	4.34
15:0	1.20	18:1n-9	18.05
16:0-iso	0.33	18:1n-7	1.32
16:0	25.37	18:2n-6	51.88
16:1n-9	1.21	18:2n-4	0.82
16:1n-7	0.40	18:3n-6	0.73
17:0-iso	0.56	18:3n-3	7.80
16:2	0.52	20:0	0.32
17:0	0.65	20:1n-9	0.20
17:1	0.28	22:0	0.32
18:0	14.18		
18:1n-9	21.14		
18:1n-7	4.51		
18:1-trans	2.07		
18:2n-6	2.60		
18:2n-4	0.60		
19:0	0.23		
18:3n-3	1.05		
18:4n-3	0.98		
20:0	0.21		

Table 4. Fatty acid composition of lipids of soy- and cow-milk (% from total fatty acids). Fatty acid composition of soy- and cow-milk was measured by the gas-liquid chromatography of methyl esters of fatty acids.

26 healthy young volunteers aged 17-19 years (16 females and 10 males) were recruited on a voluntary basis. The participants were informed in detail about the purpose, advantages and disadvantages of the study, and their rights and duties concerning their lifestyle. Informed consent from all participants was obtained in writing. The protocol was approved by the Amur State Medical Academy Ethics Committee according to the Helsinki Declaration on human studies. The subjects were assigned to two groups with similar age and gender. Participants in the first group received one glass of soy milk daily for 2 weeks. Participants in the control group received 5 mg of tocopherol acetate (pharmaceutical preparation of vitamin E) daily in the form of an oil solution. Exactly that amount of α-tocoferol is contained in one glass of soy-milk. The subjects were instructed to consume similar amounts of food from day to day and do not consume any vitamins or drugs within the study period. At the beginning and at the end of the study the samples of blood were taken and then the biochemical indices reflecting the state of lipid peroxidation were measured in blood serum. Results are presented in the table 5.

Soy milk was well accepted by all subjects and there were no any complaints. At the beginning there were no significant differences in the values of the biochemical indices between the groups. At the end of the study the increase in the α-tocopherol content in the control group from 9.2.±0,71 to 10.8±0,74 mg/L was not significant ($p>0.1$). The 30% increase in the α-tocopherol content in the group receiving soy milk from 7.8±0.44 mg/L at the beginning up to 10.6± 0.77 mg/L was statistically significant ($p<0.005$). The increase in α-tocopherol content in serum was characteristic for 11 participants of the group receiving soy-mil and only for 7 participants of the group receiving tocopherol acetate. Consumption of soy milk or tocopherol acetate for 2 weeks was followed by a 2-fold or 1,7-fold decrease of serum lipid hydroperoxide content, respectively ($p<0.05$). However, the decrease of the dien conjugate content from 31.2±5.0 μmoles/L to 22.5±2.1 μmoles/L was characteristic only for the experimental group but the changes were statistically insignificant ($p>0.1$). The consumption of soy milk or tocopherol acetate does not influence the content of TBA-reactive substances and oxidizeability of serum. Thus, the consumption of both soy milk and tocopherol acetate for 2 weeks by young healthy people resulted in an antioxidant effect, manifested by a decrease in the lipid hydroperoxide content and an increase in the α-tocopherol content in blood serum, but the strength of the effect was higher in the group of persons receiving soy milk. The stronger antioxidant effect of soy milk was correlated with higher and statistically significant increase in the α-tocopherol content in the serum of the participants. The results appear strange because both groups of participants received the same amounts of tocoferol - 5 mg daily. The only difference was the form of tocopherol. In soy beans and soy foods tocopherols are present in a free form, while the paharmaceutical preparation of tocopherol (vitamin E) is tocopherol acetate. In contrast to free tocopherols, tocopherol esters can not express an antioxidant effect because phenol hydroxyl, which is important for this effect, is blocked by fatty acid. This may be the reason for the weaker antioxidant effect of tocopherol acetate. The colorimetric method of determination of tocoferols is based on their ability to reduce Fe^{3+} ions. Tocoferol acetate is not able to reduce Fe^{3+} ions and therefore may not be determined by this method. To exert an antioxidant effect in humans and to be determined by the colorimetric method tocopherol acetate whould have to be first hydrolyzed by esterase. The other explanation for this finding would be worse intestinal uptake of tocoferol acetate when it is administered in the form of oil solution.

Indices	Groups studied			
	Treated with tocopherol acetate (n=13)		Treated with soy milk (n=13)	
	Before the treatment (1)	After the treatment (2)	Before the treatment (3)	After the treatment (4)
α-Tocoferol (mg/L)	9.2±0.71	10.8±0.74 $p_{1,2}>0.1$	7.8.±0.41 $p_{1-3}>0.1$	10.6±0.77 $p_{3,4}<0.005$ $p_{2-4}>0.1$
Dien conjugates (μmoles/L)	23.3±1.42	20.9±2.58 $p_{1,2}>0.1$	31.2±5.0 $p_{1-3}>0.1$	22.5±2.09 $p_{3,4}>0.1$ $p_{2-4}>0.1$
Lipid hydroperoxides (μmoles /L)	5.82±1.09	3.10±0.51 $p_{1,2}<0.05$	7.47±1.51 $p_{1-3}>0.1$	3.73±0.80 $p_{3,4}<0.05$ $p_{2-4}>0.1$
TBA-reactive substances (μmoles/L)	5.62±0.86	5.96±0.71 $p_{1,2}>0.1$	5.89±0.71 $p_{1-3}>0.1$	6.63±0.74 $p_{3,4}>0.1$ $p_{2-4}>0.1$
Oxidizeability of serum (nmoles of MDA min^{-1} mL^{-1})	1.17±0.037	1.22±0.042 $p_{1,2}>0.1$	1.20±0.047 $p_{1-3}>0.1$	1.26±0.056 $p_{3,4}>0.1$ $p_{2-4}>0.1$

Table 5. The influence of soy milk and tocoferol acetate on the content of α-tocopherol and lipid peroxidation products and oxidizeability of serum of young healthy persons. The methods of the measurements are indicated above in the legend to the tables 1-3. Values are means ± SEM. p values correspond to the differences between the values of the index in the indicated groups of persons (t-test for independent samples).

3. Hypocholesterolemic effects of soy foods

People, consuming soy foods, have lower blood cholesterol (Anderson et al., 1995; Devell et al., 2006; Zhan & Ho, 2005) and mortality rates from cardiovascular diseases (CVD) (Beaglehole, 1990; Messina & Messina, 2003). It was shown both in Asians (Ho et al. 2000; Nagata et al., 1998) and Westerners (Rosell et al., 2004; Teixeira et al., 2000; Tonstad et al., 2002). Both soy protein and combination of soy protein with isoflavones are effective in reducing blood cholesterol (Dewell et al., 2006). In 1999 the Food and Drug Administration of the USA recommend daily consumption of 25 g of soy protein to control cholesterol content of blood (Food and Drug Administration, 1999). However, consumption of 25 g of soy protein a day may be not enough to low blood cholesterol level and recent meta-analysis showed that favorable results were observed only in the studies with high amounts of soybean protein (up to 60 g a day) (Reynolds et al., 2006) and there were no effects when the participants consumed 25 g of soy protein isolate (SPI) daily for 6 weeks or less (West et al., 2006). These results led some AHA experts to question the effectiveness of soy protein in decreasing blood cholesterol (Sacks et al., 2006). From above it is possible to conclude that that the daily dose of soy protein should be at least 30 g and the duration of study should be more than 6 weeks to achieve favorable changes in lipid concentrations in blood. For longer studies, the problem of acceptability of test foods is very important. If the taste or smell of

foods was not acceptable, participants often failed to follow the study program. It is very common in interventional studies with SPI that a significant proportion of participants do not finish the study. The poor acceptability of SPI to the subjects could have been the reason for the relatively short periods of the previous studies with SPI. The problem of acceptability of the test foods is particularly important for people who are not familiar with soy foods, for example for Russians.

3.1 Test foods with soy- and milk protein for interventional studies

Test foods for interventional studies with soy protein should meet both scientific demands and consumer's choice, but it is rather difficult to meet both requirements because they are contradictory. Two types of proteins are usually used in such studies: SPI for the experimental group of participants and milk protein casein for the control group. For scientific reasons, it is better to use pure proteins probably in the form of a drink because other ingredients, such as fats and carbohydrates (especially in high amounts) may substantially increase the energy content of the foods and influence the blood cholesterol level. However, water solubility of SPI and casein is low and drinks with these proteins have a rather bad taste and smell so they may not be consumed by the participants over a long period. Most previous studies did not include a sufficient description of the formulas for the test foods enriched with SPI and casein. The authors simply wrote: "The test proteins were incorporated into a variety of baked products and ready-to-mix beverages" (Teixeira et al., 2000).

We felt it was impossible to prepare drinks with SPI and casein with relatively good taste and smell. To investigate the effect of SPI on blood cholesterol in Russian adults with moderate hyperlipidemia we developed SPI-enriched cookies in cooperation with food specialists (composition per 1 kg of cookies: wheat flour - 333g, SPI - 333g, margarine - 183g, sorbitol - 233g, egg - 33g, salt- 6,7g, baking soda - 3,3g, ammonia carbonate - 1,7g, energy value - 340 kkal in 100g). Thus, the protein content of a cookie was high while the energy content was rather low (340 kcal/100g) due to the use of sorbitol instead of sucrose. The daily total amount of cookies made up 30g in terms of protein content and was divided into 2 or 3 servings throughout the day. SPI (FUJIPRO) was obtained from JILIN FUJI PROTEIN CO., LTD. China. Preparation of cookies with casein was difficult because of its low solubility in water and formation of glue-like structures. So, we used skimmed card (SMP – skimmed curd milk protein, protein content 18%, fat - 0.6% and carbohydrates - 1.5%) as a source of milk protein for the control group instead of casein powder. The composition of SMP-enriched cookies was identical to the composition of SPI-enriched cookies with the exception of test protein. Skimmed curd was purchased from an authorized local provider. Picture 1 shows images of SPI and SMP-cookies.

3.2 The influence of the two-month consumption of 30 g a day of SPI or SMP on blood lipids in Russians with moderate hyperlipidemia*

*The results of this study had been published previously in the J. Nutr. Sci. Vitaminol. (Borodin et al, 2009).

The aim of this study was to investigate the ability of SPI to decrease blood lipids in Russians, whose dietary habits and life-styles predispose them to low longevity, as a result of a high mortality rate from CVDs (Kharchenko et al., 1997). A cardiologist had elected 14 male and 39 female Russians aged 32-67 years with elevated blood lipids and overweight on a voluntary basis and suggested them to become participants of the study. They were informed in detail about the purpose, the advantages and disadvantages of the study, and their rights and duties concerning their lifestyle. We instructed the subjects to follow their usual life and nutrition

styles, to minimize differences in energy intake from day to day, to maintain their body weight, and to avoid the use of lipid-lowering drugs throughout the study. The physical characteristics, nutrient intake, and physical activities of subjects were documented. The blood samples were taken after a lead-in period of 2-3 weeks. Samples of serum were frozen and analyses of lipids, GPT and GOT were performed. From the total number of subjects thirty (9 males and 21 females) aged 32 - 64 years were selected on the basis of ability to follow the protocol. The inclusion criteria were overweight (BMI 25-34 kg/m^2), fasting serum total cholesterol 240-330 mg/dL, non-HDL-cholesterol 150-280 mg/dL, HDL-cholesterol 40-70 mg/dL, and triglycerides 100-280 mg/dL. The exclusion criteria were the presence of endocrine, liver, renal and gastrointestinal diseases. The protocol was approved by the Amur State Medical Academy Ethics Committee in accordance with the Helsinki Declaration on human studies. Informed consent from all participants was obtained in writing.

Picture 1. SPI enriched cookies (left) and SMP-enriched cookies (right).

The 30 subjects with hyperlipidemia were randomly assigned to two groups, consisting of 15 persons each. These groups of subjects received either SPI cookies or SMP cookies for 2 months. After a month washout period, the subjects received the opposite test food for another 2 months. So, we used a crossover design in this study, which has apparent advantages for studies with small groups of persons. Twenty-eight participants (19 females and 9 males) could complete the trial. Fasting blood samples were drawn before and after one moth and two months of the dietary treatments. Serum samples were frozen and analyzed for lipids, total protein and glucose concentrations, GPT and GOT activities by the conventional laboratory diagnostic methods. A nutrition survey was done every month, including the wash-out period, for 3 non-consecutive days (2 week days and 1 weekend day) by the 24-hour recall method.

The baseline characteristics of the subjects are shown in the table 6.

Subjects were characterized by overweigh with average BMI 29.0±3.87. There were some differences in energy intake and physical activity between male and females participants. The values of serum total cholesterol, non-HDL-cholesterol, HDL-cholesterol, and triglycerides were typical for moderate hyperlipidemia. Both the SPI cookies and SMP cookies were well accepted by the majority of the subjects and twenty-eight subjects successfully completed the trial.

Index	Males	Females
Number of participants	9	21
Physiological indices		
Age (years)	51±2	50±2
Weight (kg)	87.2±3.8	77.6±2.4
Height (cm)	173±2	164±1
BMI	29.6±1.2	28.7±0.9
Energy consumption (kcal per day)	2022±218	1409±93
Nutrients intake (g per day):		
Proteins	107,0±6,7	78,9±5,9
Fats	80,0±7,4	52,5±2,9
Carbohydrates	272,0±20,0	152,7±8,9
Steps per day	9397±1373	8569±642
Biochemical indices		
Total Cholesterol (mg/dL)	301±26	247±7
HDL-Cholesterol (mg/dL)	54±3	63±4
Non HDL-Cholesterol (mg/dL)	247±25	214±9
Triglycerides (mg/dL)	255±33	183±30
Glucose (mg/dL)	80±9	94±8
Total protein (g/L)	78±2	77±1
GOT (U/L)	20±1	20±2
GPT (U/L)	16±2	18±3

Table 6. Characteristics of the participants at baseline (from Borodin et al., 2009). Values are means ± SEM except the number of participants.

The participants consumed similar amounts of food from day to day within the study period. There were no significant differences in BMI, energy, protein, fat and carbohydrate intakes, and physical activity throughout the trial (table 7).

The changes in serum lipid, glucose and total protein concentrations and GPT and GOT activities before, within and after the consumption of the test foods are shown in the table 8.

In agreement with the results of the previous studies (Reynolds et al., 2006), in our study one-month SPI consumption was not enough to decrease blood lipids and only triglycerides in blood serum decreased by 10%, while serum cholesterol did not change. In contrast to this, the consumption of SPI for 2 month was followed by the significant changes in serum

lipids, namely, by the reduction of total-cholesterol by 17 mg/dL, of non-HDL-cholesterol by 22 mg/dL, and of triglycerides by 31 mg/dL, and by the increase of HDL-cholesterol by 5,1 mg/dL (table 8). The observed effect of consumption of 30 g a day of SPI should be considered as strong, bearing in mind the results of the recent meta-analysis of the effect of soy protein supplementation on serum lipids, which shows that the overall pooled net effect of soy protein supplementation on serum lipids was -5.26 mg/dL for total-cholesterol, -4.25 mg/dL for LDL cholesterol, 0.77 mg/dL for HDL cholesterol and -6.26 mg/dL for triglycerides (Reynolds et al., 2006). The important results of our study are the clear increase in HDL-cholesterol concentration and clear decrease in non–HDL concentration (table 8). In previous studies, even those with a high SPI administration, only a few studies revealed significant changes in the HDL-cholesterol concentration (Sacks et al., 2006). The established ability of SPI to decrease serum triglycerides and glucose indicates that soy foods may be useful in the prophylaxis and treatment of metabolic syndrome. The consumption of the same amount of SMP did not induce any changes in serum lipids and glucose concentrations.

Indices	Groups	Initial Values	After 1 months	After 2 months
BMI	SPI	28.7±0.7	28.5±0.7	28.8±0.7
	SMP	29.2±0.7	29.0±0.7	28.9±0.7
Energy intake (kcal/day)	SPI	1713±110	1644±115	1670±121
	SMP	1574±92	1612±97	1555±138
Nutrients intake (g/day):				
Protein	SPI	86.9±4,8	89.9±4.8	88.1±4.6
	SMP	82.4±3.0	88.4±3.0	81.7±3.5
Fat	SPI	62.1±3.3	63.1±3.3	67.4±3.8
	SMP	56.6±2.1	58.6±2.1	57.0±3.0
Carbohydrate	SPI	195±7	190±10	182±12
	SMP	185±9	182±9	172±10
Activity* (steps/day)	SPI	8757±955	8629±845	8710±640
	SMP	8910±805	8727±910	9157±702

Table 7. BMI, energy and nutrient intakes and physical activity of the participants at baseline and after 1 and 2 months of the dietary treatments (from Borodin et al., 2009).
* - Physical activity of the subjects was monitored by the measurement of the number of steps per day with a help of pedometer (Omron HJ-005-E, China). Values are means ± SEM. Total number of participants in each pooled group is 28.

Indices	Groups	Initial Values	After 1 month	After 2 months
Total Cholesterol (mg/dl)	SPI	280±7	281±10	263±9**
	SMP	277±9	282±10	272±9
HDL-Cholesterol (mg/dL)	SPI	57.4±2.5	60.2 ±2.6	62.5±2.9***
	SMP	59.3±3.3	60.1±2.4	56.6±2.6
Non HDL-Cholesterol (mg/dL)	SPI	223±7	221±10	201±8.8***
	SMP	219±10	222±10	215±9
Triglycerides (mg/dL)	SPI	204±23	183.3±2*	173±16*
	SMP	192±17	193±20	201±17
Glucose (mg/dL)	SPI	85.8±4.7	83.4±3.0	79.0±3*
	SMP	87.8±5.5	86.1±4.8	88.6±3.6
Total protein (g/L)	SPI	78.0±1.0	78.3±0.8	78.9±0.8
	SMP	76.2±0.8	78.0±0.7	77.6±1.1
GOT (U/L)	SPI	22.1±2.4	20.8±1.7	22.2±2.1
	SMP	17.2±1.4	20.7±1.4	18.9±1.7
GPT (U/L)	SPI	22.6±3.1	18.3±2.6	21.1±1.9
	SMP	16.5±1.7	16.4±1.7	18.9±1.9

Table 8. Serum lipid and glucose concentrations and GOT and GPT activities at the beginning and after 1 and 2 months of the dietary treatments (from Borodin et al., 2009). Values are means ± SEM. Total number of participants in each pooled group is 28. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.005$. P values correspond to the deference between the initial values of the index and values after 1 or 2 month (t-test for dependent samples)

We see at least three reasons of the observed favorable effects of consumption of SPI in this study. The first reason may be the relatively long administration of the test foods and use of crossover design. In nutrition interventional studies proper care of the subjects is very important and because of this it is not easy to increase the number of participants. Inter-individual variances are usually large and therefore a large number of subjects is required to have significant effects in a parallel-group study, while a relatively small number of participants is sufficient for a study with crossover design because the inter-individual differences are thereby minimized. A review paper by AHA shows that most studies with crossover design were less than 6 weeks (Sacks et al., 2006). In our study, the results at 1 month were not effective but those at 2 months were effective, suggesting that only long studies may be able to decrease blood lipids of hyperlipidemic Russians at 30 g SPI a day. The second reason for the favorable results may be the good acceptability to the subjects of the test diets. Sustainability is always required for these types of studies to show the expected results. The reason for the relatively short periods of the previous studies

mentioned above might have been the poor acceptability and poor sustainability of SPI to the subjects. SPI at 30 g a day may be easy for Asians to accept but not for Westerners and Russians. For this reason, we developed well-accepted test-foods with a high SPI content. The intakes of energy, proteins, fats and carbohydrates were similar at all periods of study: in the initial period, during the first and second months and at the washout period, which may also mean that the cookies were well accepted. The third reason for the favorable results may be the level of hyperlipidemia in our subjects. As reviewed by Anderson et al. (1) the cholesterol-lowering effect of SPI was observed more clearly in subjects with high serum Total- and LDL-cholesterol concentrations than in those with normal and low lipid concentrations. Our inclusion criteria were overweight (BMI 25-34 kg/m^2), fasting serum total cholesterol 240-330 mg/dL, non-HDL-cholesterol 150-280 mg/dL; HDL-Cholesterol 40-70 mg/dL, triglycerides 100-280 mg/dL.

Conclusion

Consumption of the low-energy SPI-enriched cookies (30 g of SPI a day) for 2 months with a crossover design confirmed the favorable effects of SPI on serum lipids in Russians with moderate hyperlipidemia.

4. CVD, soy and soy foods in Russia

In the 90-th of the last century the situation with the health of Russian people drastically deteriorated and CVD had became the main reason of mortality. Above we discussed the antioxidant and hypocholesterolemic effects of soy foods and our personal results, showing the effectiveness of soy foods in the decreasing of blood cholesterol in Russians with moderate hyperlipidemia. In the last part we will focus attention on the epidemiology of CVD in modern Russia, dietary habits and lifestyle of Russians, which predispose high mortality rate from CVD, and on the possibility of use of soy foods for the prevention and treatment of CVD in Russia.

4.1 Epidemiology of CVD in Russia

An epidemiologic feature of the present Russia is the high mortality rate from CVD. According to the WHO Statistical Information System Mortality Database, the mortality rate per 100,000 from CVD in Russian adults under 65 years increased from 141-161 in 1986-1991 to 233-251 in 2001-2005 (WHO Statistical Information System Mortality Database). The rates were similar to those in the USA (241.8 and 244.4 for males and females, respectively) and England (263 and 235) and higher than those in Japan (136 and 138) and France (172 and 176) (WHO Statistical Information System Mortality Database). The average lifespans of Russians in late 1990s were only 58 years for males and 72 for females, while they were 79 and 86 for Japanese, 75 and 80 for Americans, and 77 and 81 for French individuals (UN Demographic Yearbook 2004). The dietary habits and lifestyle of Russians, namely high consumption of saturated fats (Solodkaia et al., 1998) and abuse of alcohol (Leon et al., 2007), associated with excessive body mass (Konstantinov et al., 2002), predispose individuals to short longevity as a result of a high mortality rate, especially from CVDs (Kharchenko et al., 1997), which is characteristic for the present-day Russians. Therefore, soy foods may be helpful for Russians in the prevention of CVD. Due to climate conditions, Russia is able to produce abundant soybeans but the consumption of soy foods by Russians is very limited

partly because of the low quality of the soy foods produced. Soy foods in Russia are used very infrequently in the treatment and prophylaxis of CVD in contrast to the Asian and European countries and USA.

4.2 Soy planting and soy foods in Russia

Initially, soybean was brought to Russia from northeastern China. Currently, soybeen is planted in the Far East of Russia (the Amur region and Prymorie), the Altai region and in the Kuban territory near the Black Sea. (picture 2).

Picture 2. Soy planting in Russia. The regions farming soya are colored in turquoise (blue/green).

Soybean is also planted in some other regions but the crop is small. The annual total crops of soyben in Russia makes up nearly a million metric tons which is very little in comparison with the main producers of soyben in the world. The All Russia scientific research institute of soya is based in the administrative center of Amur region Blagoveshchensk (http://www.amur.ru/~seray/index.html). The main task of this institute is the creation of the new species of soya, adapted to the local climatic condition, and the farming techniques of planting soya in these conditions. In Russia, soya traditionally processed into oil, flour and feeds. Soy flour is often associated with meat products – sausage, cutlet etc. One of the main obstacles in the promotion of soy foods in Russia is the image of soy products that, in the opinion of Russians, is not as foods for human but as feed for animals. The other obstacle is association of soya with genetically modified foods. Our 15-year long experience of promotion of soy foods in Russia as healthy foods shows that strong informational support from medical specialists is necessary to create a positive image of these foods in

Russian people. This informational support should be constant and prolonged. Our experience of promotion of soy foods in Russia with a help of specialists from Japan (Fuji Oil co.) shows that when Russian people have a chance to taste modern soy foods produced in Japan, they highly praised these foods and express desire to consume them (picture 3).

Picture 3. Promotion campaign of the Fuji Oil Co (Osaka, Japan) in Russia (2005-2007 years) The visit to the milk plant "Alev" (up left) and the conference with the mass media, medical specialist and cookery experts (up right) in Ulianovsk region; soy field in Amur region (bottom left); meeting with the participants of the study in the biochemistry department of the Amur State Medical Academy –(bottom right).

The campaign of promotion of soy foods had been initiated in Russia in the second part of the 1990s. Many enterprises producing oriental soy foods like soy milk and bean curd– tofu as well as textured soy protein – have flourished. However, the vast majority of these enterprises used just simple equipment and the taste of those products was not good for Russians. As a result, the society lost interest in soy foods. To revive this interest, it is necessary to begin production of good quality soy foods in Russia and request for help from medical specialists in explaining the healthy properties of soy foods to Russian people as was done in European countries and the USA.

5. Acknowledgment

The authors of the publication are indebted to Dr. Steve O. Balyakin, Senior Medical Director, Allergan, Inc., Irvine, CA, USA, for providing valuable advice.

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Important Minor Soybens Proteins: Soybean Allergens and Enzymes Inhibitors

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1. Introduction

1.1 Overall significance

Unfavourable organism response to food can be divided into toxic and non-toxic reaction with abnormal clinic response of the organism. As allergy is called such an organism response mediated always by the immune system in contrast to food intolerance. Food allergy can be further divided into reaction of the organism bound to IgE and without IgE reaction. Hypersensitiveness of the immune system can be classified into types I - IV. Type I is reaction of mast cells with bonded IgE antibodies for the presence of antigen (Gray and Chan, 2003). The prevalence of allergic reaction varies geographically and may depend of the frequency of consumption, the age of introduction into the diet and the genome (Öetles, 2005).

1.2 Physiological function

In case of food allergies mediated by IgE it is possible, especially according to the clinical symptoms, to define two forms of allergic reactions. Food allergic reactions can have a different manifestation in localization, in time horizon as well as in the importance of symptoms, which represent a wide scale of evidence from the most simple up to anaphylactic shock. The first form appears already shortly after birth and in the early infancy. The sensibilization is elicited by a reaction in the gastrointestinal tract and is most frequently manifested as atopic dermatitis. The second form appears in particular at a later age as a respiratory allergy, in case of which are often the sensitising agents also inhalation allergens (cross reactive allergens) (Metcalfe et al.2003).
From the acute symptoms is often present nausea, emesis and diarrhoea, and in serious cases even bloody diarrhoea. The pathological-anatomical picture of the allergic reaction is characterized by inflammatory damage of intestinal mucosa with the affection of the respiratory tract, particularly by bronchoconstriction (Gray and Chan, 2003).

1.3 Nomenclature and legislation

The official list of allergens was created by the World Health Organization and International Union of Immunological Societies. A substance is classified as an allergen if it causes an

allergic reaction with the prevalence of IgE reactivity above 5%. According to the percentage of activating IgE the allergens are further divided into major and minor. The name of the allergen is created from the abbreviation of the Latin name of the species from which the substance originates and an Arabic number (Mills and Tatham, 2003).

1.4 Occurrence and properties

Allergens represent a wide group of substances, antigenic molecules present in food. Most food allergens are proteins . Epitopes of food allergens have various tertiary and quaternary structures. However, their defining is not easy, as their conformation can be during food technologies modified (Mills and Tatham, 2003). Allergens of vegetal food mostly belong to compounds which are to protect the plant organism particularly against pathogenic microorganisms; they are so-called pathogen-related (PR) proteins. Other allergens are inhibitors of proteases, alpha-amylases, profilins, seed storage proteins, proteases and lectins. A number of these compounds are located in seeds (Mills and Tatham, 2003).
Soya and soya protein is increasingly used as raw material and food. Following this fact there is an increasing potential for atopic subjects to become sensitised. Soya belongs among five most significant kinds of food inducing type I allergy in children (Breiteneder and Ebner, 2000).

1.5 Structure

Soya allergens are low-molecular weight proteins or peptides. The cause of the protein allergenicity is still little explored. The allergen activity is proportionate to its resistance to food technology, namely to digestion in the gastrointestinal tract. A fundamental role plays the conformation of protein epitope. In some cases has a significant role also posttranslational modifications such as glycosylation, phosphorylation or termination modification and most food allergens are really glykoproteins. Soya contains several allergens. All of them belong to the glycoproteins group. Inducing dose for allergic patients is from 0.0013 to 500 mg (Ballmer-Weber et al., 2007; Becker et al., 2004; Mills and Tatham, 2003).
The overview of soya allergens is summarized in chart 1.

Allergen	Biochemical Name	MW(SDS-PAGE)	Subunits
Gly m 1	Hydrophobic protein	7	
Gly m 2	Defensin	8	
Gly m 3	Profilin	14	
Gly m 4	PR-10 protein	17	
Gly m 5	Beta-conglycinin (vicilin, 7S globulin)	subunits	α, α', β , β
Gly m 6	Glycinin (legumin, 11S globulin)	subunits	G1,G2,G3,G4,G5,G6

Chart 1. Soybean allergens (http://www.allergen.org/index.php)

Gly m 1- Plant lipid transfer proteins

Soya, besides a range of food of vegetal origin, contains an allergen from the group of so-called plant lipid transfer proteins, whose name is derived from the capability to transfer phospholipids from liposomes to mitochondria. This whole protein family plays a role in the plant protection by its antifungal and antibacterial activity. These allergens feature a high content of aminoacid cysteine in the molecule and with this related presence of 4 disulphide bridges. In soybean hulls it was identified as the main protein responsible for several asthma outbreaks in Spain. It is a low-molecular protein Gly m 1, which consists of two isoforms 1A and 1B, both of which are allergenic (Breiteneder and Ebner, 2000). Of 32 patients who suffered from asthma attacks during unloading of soybean at the seaports, 90% showed serum IgE binding to an 8 kD shell protein on immunoblot. This protein was later identified as gly m 1 (medline 95326747) (Baltes, 2000, http://www.allergen.org/index.php).

Gly m 2 - ***Defensin***

Purified Gly m 2 was recognized by serum IgE of patients suffering from soybean asthma on immunoblot. (http://www.allergen.org/index.php).

Profilin Gly m 3

Profilin is another potential soya allergen. Profilins regulate polymerisation of actin into filaments through the formation of profilactin. It is a thermolabile protein sensitive to digestive enzymes. (Lucas, Cochrane, Warner, & Hourihane, 2008; Scadding, 2008). The allergenic potential of this protein is reduced in the course of technological processing of soya. The principle of the reduction is a change of the binding epitope during heating, enzymatic hydrolysis, and fermentation (Amnuaycheewa and de Mejia, 2010). Of 12 subjects with soy bean allergy, 8 (67%) showed IgE binding to Gly m 3 on immunoblot (Baltes, 2000, http://www.allergen.org/index.php).

Gly m 5 - β – ***Conglycinin*** **(vicilin, 7S globulin)**

β – Conglycinin belongs together with glycinin among the most important soya allergens. It is a seed storage protein. The structure of β – Conglycinin is composed of trimer, consisting of subunits α, α′, β glycinin in various combinations (Baltes, 2000, http://www.allergen. org/index.php).

Gly m 6 - ***Glycinin*** **(legumin, 11S globulin)**

Glycinin is also a soya bean storage protein. It occurs in the form of hexamer or dimer (Snyder, 2003) and it is allergenetic even after the heat treatment of soya beans. It can be the cause of allergies from soya lecithin (Baltes, 2000).

Kunitz trypsin inhibitor

Kunitz trypsin inhibitor is a minor allergen, but it is at the same time an important antinutritional substance. However, the Kunitz soybean trypsin inhibitor was also reported to induce food anaphylaxis. (Breiteneder and Ebner, 2000).

Lectins

Lectins are known as plant agglutinin that binds to specific sequences of sugar determinants on glycoproteins. It occurs in seeds, particularly in leguminous plants. Some lectins react unspecifically with saccharide units of IgE, induce histamine-release and thus induce allergy-like symptoms (Breiteneder and Ebner, 2000).

A great problem of food are so-called hidden allergens. In case of food it means some functional ingredients added in the food. Soya can be added to bread and bakery products, sauces and soups, desserts and sweets and meat products for their moisture sorption and emulsifying properties. (Mills and Tatham, 2003).

1.6 Determination in food

Detection of allergenic components can be used to estimation in raw material and finished products.

Testing for food allergens should become part of the food industry's Good Laboratory Practise (GLP) and Hazard Analysis and Critical Control Points (HACCP) plans. Testing is a requirement for routine government inspections and other regulatory actions (Williams et al., 2005).

1.6.1 Immunochemicals methods

There are numerus immunoassay formats, all of them based on the binding of an antibody to the target analyte. Immunoassays have been widely used to analyze complex of food proteins because of their specifity, sensitivity, and the ability to analyze complex food samples without the need for preassay purification steps.

Immunochemical methods can be qualitative, semiquantitative, or quantitative. For qualitative assays, only one cut-off value is needed and samples are compared to negative and positive controls. Two-level positive controls are required for semiquantitative assays. In the case of quantitative assays, sample values are compared to serial calibrators as well as a negative control (Williams et al., 2005).

Enzyme-Liked Immunosorbent Assay (ELISA)

Sandwich format ELISA is the most common immunoassay for the detection of food allergens like almond, peanut, hazelnut, casein, egg, soy and others.

The method uses antibodies bound to a solid support – the alergen is detected by a second antibody, which is conjugated to an enzyme. There is direct, indirect or enhanced (biotin-streptavidin system) assay formats, further divided into competitive or noncompetitive (Williams et al., 2005).

- **Lateral Flow (Strip test)** is the variation of the dipstick ELISA, but analyte is first recognized by the second or detector antibody, which is coupled to a color reactant and is embedded on one edge of a nitrocellulose strip. This method is used for determination of soybean, gliadin and peach.
- **Biosensors.** Surface Plasmon Resonance (SPR) immuno(bio)sensors are being developed for food allergen detection, it measures changes of the refractive index value. One of the most important features of the technology is the real-time monitoring of the samples. Quantitative methods have been developed for peanut, soy, ovomucoid and others (Williams et al., 2005).

1.6.2 DNA-based methods

DNA-based methods do not analyze the protein directly, but detect instead the gene which encodes for that protein.

Polymerase Chain Reaction (PCR)

PCR allows the selective amplification of a specific DNA sequence. The two outstanding features of this technice are its specifity and sensitivity. PCR has been widely used to

characterize, clone, and produce recombinant food allergens using a DNA copy of the alergen-encoding mRNA. PCR assays are qualitative or quantitative. For qualitative analyses, conventional or traditional PCR uses a thermal cycler to amplify target DNA with the resulting amplicons visualized by agarose gel electrophoresis and ethidium bromide staining (Williams et al., 2005).

Real-time PCR (RT PCR)

RT PCR allows quantifying the initial template concentration and the elimination of detection as a separate step, the product is detected during each cycle.
At this time, there are only several methods developer for the detection of food allergens by PCR, most being qualitative. There are test for the detection of peanuts, soy and hazelnut allergens (Williams et al., 2005).

PCR-ELISA

PCR-ELISA is similar to traditional PCR methods in that specific DNA sequences are amplified using pairs of primers and DNA polymerase. R-Biopharm has commercialized a SureFood ELISA-PCR for almond, peanut, soy and hazelnut (Williams et al., 2005).

2. Enzymes inhibitors

2.1 Overall significance

The enzymes inhibitors belong among significant antinutritional substances of some food. Many of these substances can be found in vegetabilia. They have different physico-chemical properties, such as isoelectric point, different activity and thermal stability and different specificity towards the inhibited enzymes. The enzymes inhibitors mostly belong to the group of hydrolase. Some of them have already been mentioned before, taking into account that they can be also potential allergens. In case of legumes they are in principle divided into protease inhibitors and amylase inhibitors.
Protease inhibitors occurring in legumes seeds belong to two families:

- Kunitz and Bowman-Birk inhibitor: both types are present in soya.
- Amylase inhibitors (salivary and pancreatic α-amylase). This type of inhibitors is not accounted in soya.

2.2 Occurrence and properties and physiological function

The effect of the inhibitors in relation to influencing the consumer's health consists in inhibition of intestinal protein digestion. To other manifestations belong inflammatory diseases of the pancreas after feeding animals with soybean powder leading up to hypertrophy and hyperplasia of the pancreas with the hypersecretion of digestive enzymes. (Guillamona et al. 2008) The increased secretion of the pancreatic juice leads to higher secretion of the fecal nitrogen and possible deficiency of some aminoacids, the sulphur aminoacid in particular. (Belitz, Grosh, 1992; Guillamona et al., 2008). Many proteinase inhibitors show the same active centre as is the part of the peptide enzymes binding, whose function they inhibit. Such an example is the Kunitz and Bowman-Birk soya inhibitor, which belong to the low-molecular fraction of soya proteins. The Kunitz inhibitor affects against the enzymes of trypsin and chymotrypsin and the Bowman-Birk inhibitor only against trypsin. It is interesting that the Kunitz inhibitor is inactivated by the gastric juice, but is it

not in case of the Bowman-Birk inhibitor. As regards the stability of these inhibitors, the Bowman-Birk inhibitor is protein that is by its tertiary structure predetermined to higher stability towards denaturation. In a molecule it contains 7 disulphide bridges and a circular arrangement of reactive sites on inhibition and by that it gains higher thermal stability, stability towards acids as well as proteolysis by pepsin. It was demonstrated that the daily discarded amount of trypsin and chymotrypsin is completely inactivated by the intake of 100 g soya beans (Guillamona et al., 2008).

The positive or negative impact of the protein inhibitors is given by their received amount, but also by the kind of soya cultivar. For instance, in case of the Bowman-Birk inhibitor is described a great amount of isoforms with different inhibitory properties. The Kunitz and Bowman-Birk inhibitor isoforms are divided in five sub-groups, on the basis of different representation of aminoacids, molecular weight and imunological cross reactivity. This knowledge has led to breeding of soya cultivars with low up to zero activity of the Kunitz and Bowman-Birk inhibitor. (Guillamona et al., 2008).

The Kunitz and Bowman-Birk soya inhibitor increase secretion of cholecystokinin (CCK) by intestinal mucosa. In case of Bowman-Birk inhibitor was, though, in vitro discovered a positive function, namely by demonstrating the carcinogenesis reduction in animals. (Guillamona et al., 2008) however, this situation is not imminent, as the inhibitors are deactivated by heat treatment.

2.3 Possibilities of inactivation

A number of studies deal with the possibility of inhibitors inactivation. In principle, the protease inhibitors are thermolabile, but a lot of heat treatment factors enter, which influence the inactivation. An example is soaking. Heat treatment of soya beans at 100 degrees Celsius for 9 minutes destroys 87% of inhibitors (Belitz, Grosh, 1992).

Reduction of the trypsin-inhibitory effect of soya proteins is possible by the means of chemical or physical processes. From the physical technological steps is effective high temperature, however, under these conditions emerges during the Maillard reaction a risk of degradation of important, especially basic essential or semi-essential aminoacids. Chemical methods represent chemical modification of the reactive spots of the trypsin inhibitors, but there is still a remaining problem of possible residuals of used chemicals. A favourable alternative represents ultrasound treatment. This process can be used with the result that the reduction happens not only in case of trypsin inhibitors, but it also leads to decreasing some enzymes (Huang et al. 2008). A great advantage is maintaining the nutritional value of the product. From other technological interventions are effective e.g. extrusions, which was proved on animal models by Marsman et al. (1995). In a feeding experiment was observed the influence of extruded soya flour and was found out that by the extrusion process these inhibitors are reduced by 25-41 %. Huang et al. (2008) studied the impact of the ultrasound treatments on Kunitz and Bowman–Birk soybean trypsin inhibitors (KTI and BBTI). They concluded that ultrasound inactivates KTI by inducing a reduction in the disulfide bonds and thus the changes in the secondary structure of both proteins. The Kunitz inhibitor was more sensitive, which can be explained by a number of disulphide bonds, in contrast to BBTI (7 bonds), the molecule of the Kunitz inhibitor contains just 2 disulphide bonds (Huang et al., 2008).

Another possible technological step leading to reduction of the soya allergens activity is fermentation, as was demonstrated in a work by Song et al. (2008), who proved, using *L. plantarum*, a reduction by 96% to 99%.

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Soybean Allergens: Presence, Detection and Methods for Mitigation

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1. Introduction

As a major food ingredient, soybean provides numerous health benefits, for example, individuals who consume soybean-rich diets exhibit lower incidence of high plasma cholesterol, cancer (including bowel and kidney), diabetes mellitus, and obesity (Carroll and Kurowska, 1995; Friedman and Brandon, 2001; Duranti et al., 2004; Ali et al., 2004; Omini and Aluko, 2005; Kim et al., 2006). However, soybean is also among the "big 8" most allergenic foods, and the only possible solution currently to prevent allergenic reactions is total avoidance of the allergen-containing foods. Because soybean is ubiquitous in vegetarian and many meat based food products, avoidance has become increasingly difficult, and its prevalence will inevitably continue to rise. Due to the innumerable health benefits and economic importance of soy commercial products, there is a mounting need to remove the allergenic components contained in soybean proteins to a threshold deemed to be safe. The estimated threshold level for common food allergy is usually low and a small amount of the allergen may be enough to trigger an allergenic reaction (Poms et al., 2004). The threshold for soybean allergen is estimated to range from 88 mg to several grams of soy protein (Bindslev-Jensen et al., 2002; Fiocchi et al., 2003).

Soybean allergy is of particular importance because soybean is widely used in processed foods and represents a particularly insidious source of hidden allergens. Since finding foods that do not contain soy is difficult and total elimination of food allergens is practically impossible to attain, investigations on the hypoallergenization of soy ingredients and products are imperative. Current requirements by the labeling regulations also make it imperative to identify a processing technology that is capable of reducing or eliminating the allergens from soy containing products. Elimination or reduction of allergens in allergenic foods has been attempted for years by various investigators, which has included, among various strategies, the use of genetic engineering, thermal processing, enzyme treatment, ultrafiltration, chemical agents, microwave, irradiation, high pressure processing, pulsed ultraviolet light, power ultrasound and pulsed electric field. The reduction or elimination of allergens from soybean proteins by different processing technologies offers unique insight to the structure and biological interaction of the antigenic proteins. With allergens reduced, the industry can further profit from the economical attributes of soybean and promote the

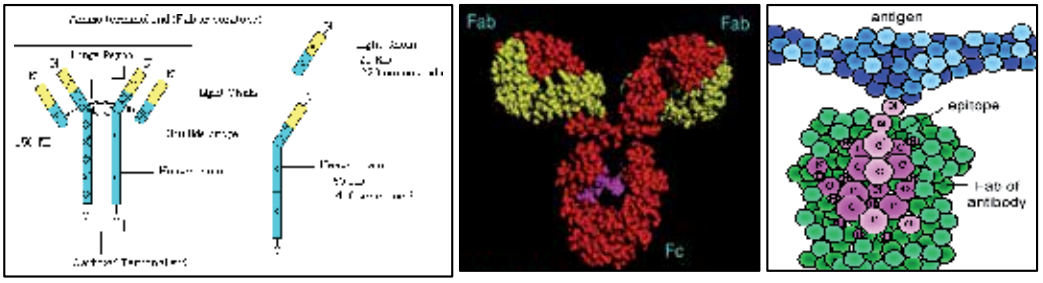
soy products more rigorously along the health route. The consumers will be less concerned about allergenicity of the product and can enjoy the health benefits of soybean without the threat of an allergic reaction.
Nevertheless, the use of conventional processing method, alone or in combination, has found the complexity of eliminating or reducing the allergenicity of foods and also affecting their functional or organoleptic properties. Processing methods, such as heat treatment and enzymatic fragmentation, can reduce soy allergen reactivity in food products, but a major obstacle is the risk to alter unique functional attributes of soy proteins. Furthermore, not enough emphasis has been given to understanding the way the insidious protein interacts with the IgE binding sites, while such understanding is important in guiding a proper modification of these sites of the allergic protein. Emerging alternative food processing technologies may reduce protein immunogenicity by inducing changes in protein conformations, which mask, inactivate or destroy IgE binding sites, known as epitopes. Novel processing methods, such as pulsed ultraviolet light (PUV) and high pressure processing (HPP), alone or combined with biochemical treatments, or extrusion combined with inclusion of bioactive compounds, hold a great promise for the development of hypoallergenic food products with unique functional properties.
The objective of this chapter is to provide an overview of the major aspects of soy allergen research, which includes the presence of soy allergens (i.e., major allergenic proteins identified and their mechanism of reaction), methods for soy allergen detection and characterization, and thermal and non-thermal methods to mitigate soybean allergens and their status of development.

2. Food allergy

Over the past 30 years the incidences of allergies have doubled not only in industrial countries, but in developing countries as well (Pearce and Douwes, 2006). This represents an intriguing problem from a medical, epidemiological, immunological, genetic and evolutionary view. Allergic disorders are typically characterized as an abnormal or hyperactive immune response in reaction to exposure to environmental agents or allergens (Galli et al., 2008). High levels of immunoglobulin E (IgE) are a defining characteristic of such a response (Galli et al., 2008).
Immunoglobulin E is an evolutionary conserved member of the immunoglobulin (Ig) family, and the titer of IgE is very low (nano- to micrograms per ml range) in plasma of normal healthy individuals. The IgE is most prominent in epithelia and mucosa where it is bound to specific receptors on highly potent effector cells like eosinophilic granulocytes and mast cells. Bound to these cells, IgE has a long half-life (weeks to months), while free in plasma, the half-life is very short (~6 h). This suggests that IgE plays a role in local immune defense mechanisms (Gould et al., 2003). Since life-threatening, systemic reactions like anaphylactic shock may happen, the potential hazards of high systemic IgE titers are underlined.
Food allergy is a relatively rare and violent reaction of the immune system towards food proteins. It is defined as an immunologically based adverse reaction in response to dietary antigens (Beyer and Teuber, 2004). The allergen provokes an initial IgE antibody response followed by a secondary IgE antibody response, which signals an allergic reaction (Babu et al., 2001). Food proteins bind to the allergen-specific IgE molecules residing in the mast cells and basophils, causing them to release inflammatory mediators, including histamine (Beyer

and Teuber, 2004). Food allergy affects 3% of the adult population and up to 6-8% of infants. Allergenic conditions directly affect millions of people worldwide (Sampson, 1997; Goodwin, 2004), and it is believed that one in 25 Americans is susceptible to food allergies (Westphal et al., 2004; Sicherer et al., 2004). The estimated prevalence of soybean allergies is about 0.5% of the total U.S. population (Sampson, 2002; Stephan et al., 2004; Sicherer and Sampson, 2006).

Small regions of allergenic proteins known as epitopes, composed by 5-7 amino acids or 3-4 sugar residues cause the IgE-mediated allergy by reacting with an antigen (Taylor and Hefle, 2001). Specifically, the crystallizable fragment (Fc region) of IgE binds strongly to high affinity receptors on mast cells and basophils, and together with the antigen, mediates the release of inflammatory agents from these cells (Figure 1A and 1B). Then, the allergen provokes an initial IgE antibody response followed by a secondary IgE antibody response, which signals an allergic reaction in the organism (Babu et al., 2001). The level of IgE is found in extraordinarily low concentrations in the serum of humans, varying from 20 to 500 ng/ml. Therefore, IgE is important since its biological activities are greatly amplified by binding to receptors on mast cells and basophils (Tizard, 1995). The hinge region is replaced by a constant domain so that each heavy chain contains four constant domains, and as a result, IgE has a molecular weight of 190 kDa. An IgE molecule contains two identical light chains (23 kDa) and two identical heavy chains (72 kDa). Between the two antigen binding fragments (Fab region), the molecule's surface has a depression that forms the antigen-binding site (Figure 1C). Individual protein allergens can have several recognition sites (epitopes) per allergenic protein.



A. B. C.

Fig. 1. A. Structure of an immunoglobulin, B. The tridimentional molecular model of an IgE molecule showing the antigen binding site (Fab) (heavy chain in red, light chain in yellow, and carbohydrate residues in purple). C. Diagram illustrating antigen bound to fab of the antibody (reprinted with permission).

According to Lehrer et al. (1996, 2002), the epitopes are not only fully characterized by their primary structure, but also by their tertiary structure conformations.

Food allergens and their epitopes are able to resist the effects of digestion and enzymatic reactions (Taylor et al. 1987) and individual allergen systems are affected differently by the processing methods. This is because food allergens are complex mixtures of potentially immunoreactive proteins. According to Wilson et al. (2005), soybean allergens comprise proteins with molecular masses from 7 to 71 kDa.

3. Soy allergens

In humans, 34 IgE reactive proteins have been identified and characterized as related to soybean allergy (FARRP, 2008; Xiang et al., 2008). Among the numerous allergenic proteins present in soybean, the strongest immunodominant allergenic protein identified is Gly m Bd 30K, or P34 (Ogawa et al., 1993, 2000), recognized by 65 percent of soybean sensitive individuals with atopic dematitis. Gly m Bd 30K is known as a soybean oil-body associated glycoprotein that consists of 257 amino acids residues. The P34 is associated with α, α′ and β subunits of the globulin β-conglycinin by bi-sulfide linkage (Ogawa et al., 1993) and has a 30% homology to Der p 1, a major allergen of house dust mite, classified under the papain super family.

Ogawa et al. (1993, 2000) identified three major soy allergens: Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K. Gly m Bd 60K is an α subunit of 7S β-conglycinin, well known as a major storage protein. β-conglycinin is a primarily constituent of 11S glycinin globulin fraction and includes three subunits: α (~67 kDa), α′ (~71 kDa), and β (~50 kDa) (Rihs et al., 1999). Gly m. Bd 28K is a vicillin-like glycoprotein of 473 amino acids (Xiang et al., 2004), initially isolated from soybean meal as a 28 kDa glycosylated protein. Gly m Bd 28K (Hiemori et al., 2000) constitutes a minor component fractionated into 11S glycinin globulin fraction in soybean seed flour that has been recognized by soybean sensitive patients with about 25% of incidence (Ogawa et al., 2000). Moreover, Gly m Bd 28 K protein shares sequence homology with proteins in pumpkins and carrots (Rihs et al., 1999). Studies revealed a slightly stronger IgE-binding region present in the C-terminal domain of Bd 28K (Xiang et al., 2004) than the N-terminal half of this protein (Ogawa et al., 2000). An important IgE binding region was found in the C-terminal 23 kDa polypeptide, which contains an Asn-N linked moiety with the same sugar composition as that of P34 (Hiemori et al., 2000).

Additional to the list of major soy allergens, Cordle (2004) includes soy hydrophobic protein (Gly m 1a), soy hull protein (Gly m 2), soy profiling (Gly m 3) (Klein-Tebbe et al., 2002), glycinin (320-360 kDa), β-conglycinin (140-180 kDa) and Kunitz tripsin inhibitor (20 kDa) as major soy allergenic proteins. The acidic subunit of glycinin G1 (Beardslee et al., 2000) and the basic subunit of glycinin G2 (Helm and Burks, 2000) have been classified as important allergens in patients affected by soybean allergens. Glycinin is a hexameric pure protein present in the 7S globulin fraction and each of its subunits contains an acidic and a basic polypeptide linked by a disulfide bond (Marayama et al., 2003).

Soybean processing may inactivate some antinutritional factors through heat treatment or extrusion; however, those processes may also reduce availability of amino acids, especially when the soybean product is overcooked (Danielson and Crenshaw, 1991). Fermentation has proven to improve the nutritional value of soybean by increasing the bioavailability of nutrients and reducing antinutritional factors (Hotz and Gibson, 2007; Egounlety and Aworh, 2003). Moreover, it has been demonstrated that fermentation of soybean proteins reduces its immunoreactivity toward human IgE, probably through the removal of epitopes present in the native protein (Song et al., 2008a,b; Frias et al., 2008).

4. Basic mechanisms of allergen reactions

Epitopes are generally categorized as linear or conformational, where linear epitope involves a contiguous stretch of amino acids, and a conformational epitope involves noncontiguous amino acids forming a three-dimensional or structural motif. Individual patients may differ

significantly in their sensitivity toward an allergen; however, the basis of such differential sensitivities remains to be elucidated. More than one epitope or IgE binding site is required per fragment of an allergen to cause IgE cross-linking. Therefore a molecule with a single IgE binding site must be bound or cross-linked to another molecule with an IgE binding site in order to cause histamine release. Understanding molecular properties of the epitope(s) is therefore important in learning the nature of IgE–allergen interaction. In case of linear epitopes, amino acid residues that determine whether allergen would bind with IgE or not are known as critical amino acid residues. Any modification, deletion, or substitution of such critical amino acid residues may result in loss of IgE binding and may potentially result in reduction or elimination of allergenicity. If the epitope is conformational in nature, change in epitope conformation may permit modulation of allergic activity.

Food processing, under appropriate conditions, offers opportunities to alter the nature of epitopes. For example, epitope conformation may be modified as a result of protein denaturation treatments (e.g., various thermal processing treatments) leading to reduction or elimination, or in some cases, an increase, in IgE binding. Acid or enzyme hydrolysis of an allergenic protein may help delete critical amino acids of an epitope. Whether caused by protein denaturation or hydrolysis, loss of epitope and thus loss of IgE binding may help reduce or eliminate the bioactivity of an allergen. It should be emphasized here that processing, depending on the allergen and the processing method, may not affect the allergenic properties of all allergens.

Physicochemical changes will alter the way in which allergens are broken down during digestion and may modify the form in which they are taken up across the gut mucosal barrier and presented to the immune system. Certainly, the structure of the food matrix can have a great impact on the elicitation of allergic reactions and fat-rich matrices may affect the kinetics of allergen release, potentiating the severity of allergic reactions (Grimshaw et al., 2003). Understanding the impact of food processing and food structure on allergenic potential is central to managing allergen risks in the food chain. However, our current knowledge of the impact of food processing on allergen structure indicates that there are no clear rules regarding how different allergens respond to food processing.

5. Methods for soybean allergen detection

Reliable detection and quantification methods for food allergens are required to ensure compliance with food labeling regulations and minimize risks to highly sensitive consumers from undeclared allergens in the food supply (L'Hocine and Boye, 2007; Taylor et al., 2006). Detection of allergens in food products can, however, be very difficult, as they are often present in trace amounts and can be masked by the food matrix (Poms et al., 2004). This is particularly true for soybean, which is an insidious hidden allergen (e.g., pastries, bakery products, infant foods, sausages, processed meats, hamburgers, and dairy products).

Presently, various methods have been applied to detect soybean in food products. The majority of methods developed to detect soybeans in food products are based on immunochemical assays.

5.1 SDS-PAGE, immunoblotting and dot blotting

Several methods based on electrophoretic separation coupled with immunological detection have been developed primarily to identify soy proteins in meat products (Catsimpoolas and Leuthner, 1969). Sensitivities of these procedures are usually in the g/kg range. Janssen et al.

(1986) presented a more sensitive protocol to determine soy in meat products by SDS-PAGE/immunoblotting and dot blotting. This procedure achieved a detection limit of 0.02% (200mg/kg) soy in meat. Two-dimensional gel electrophoresis followed by immunoblotting and identification of IgE-reactive proteins are proteomic approaches to identify new allergens in foods. Protein profiling using two dimensional electrophoresis and allergens detection by IgE has become a powerful method for analyzing changes of allergen content in complex matrices during food processing (De Angelis et al., 2010).

5.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a powerful analysis tool for detection of specific proteins. It has the advantage of simultaneously testing a larger series of samples at a high level of sensitivity. A large number of investigators have used ELISA methods for the detection of soy allergens in food products (Koppelman and Hefle, 2006). Immunochemical methods are generally limited to the qualitative screening of raw or mildly processed products, since protein denaturation often alters the antigen-antibody interaction.

5.2.1 ELISAs for soy glycinin

Ravestein and Driedonks (1986) raised antibodies against the SDS-denatured acidic polypeptides of glycinin in rabbits. An ELISA constructed with these antibodies in an immunoblotting experiment decreased the detection limit enormously, but this was not considered relevant since soy is usually used in concentrations at 1% and higher. Meisel (1993) use IgY antibodies obtained from egg yolk of immunized chickens, an enzyme-linked immunoassay (ELISA) and an immunoblotting procedure has been developed for specific determination of soy protein. The procedure for antigen preparation provides a rapid method for the isolation of SDS-denatured glycinin A from polyacrylamide gels that could be used directly for immunization. ELISA with SDS-denatured soy protein isolate was linear in the range 0.5-256 µg/ml and covers the expected levels of soy protein in simulated milk and milk products. IgY antibodies used in immunostaining of SDS-PAGE blots allowed the detection of glycinin A at nanogram levels (Meisel, 1993). Plumb et al. (1994) raised monoclonal antibodies (Mabs) against beta-conglycinin. They found that heating glycinin at pH 7.6 caused the immunoreactivity to decrease to around 50% of the original value but it increased sharply above 92°C (Plumb et al., 1994). These results are in agreement with Demonte et al. (1997) but differ from the results of Iwabuchi and Yamauchi (1984), who found that the antigenic activity disappeared after heating. Huang et al. (1998) found that the epitope identified by Plumb et al. (1994) corresponded to residues 86-104 of the acidic polypeptides of glycinin AlaB1b and lies on the C-terminus of the proteolytic intermediate known as glycinin-T. The epitope seems to be continuous in nature. Iwabuchi and Yamauchi (1984) studied the effect of ionic strength (I) on thermal denaturation of soybean glycinin. Up to I=0.7, no effects of ionic strength were found. A reduction in immunogenicity also occurred when glycinin was taken to pH 2.0 and pH 11.0 and exposed to high temperatures (Iwabuchi and Shibasaki, 1981).

5.2.2 ELISAs for soy ß-conglycinin

Plumb et al. (1994) raised specific antibodies for the native α and α′ subunits of ß-conglycinin; ß-conglycinin immunoreactivity was increased as the protein was heated, reaching a maximum at the denaturation temperature of 65°C (Plumb et al., 1995). This

phenomenon is unusual as most thermally denatured proteins have low immunoreactivity when probed with antibodies raised against native protein. This was also found at pH 7.6 at different ionic strengths (Iwabuchi and Shibasaki, 1981). The epitope of the antibody used by these researchers corresponds to the residues 78-84 in the acidic extension present in the α′ subunit of ß-conglycinin, and seems to be continuous in nature (Huang et al., 1998). A linear epitope, in contrast to a conformational epitope, is expected to be more heat stable and may become more exposed after denaturation. This may explain the increased detectability of heated ß-conglycinin.

5.2.3 ELISAs for soy trypsin inhibitors

Brandon et al. (1989) used antibodies that bind the Bowman-Birk protease inhibitor. They identified an epitope that was destroyed by heat and developed an ELISA for specific recognition of native Bowman-Birk inhibitor (BBI) in the presence of denatured forms (Brandon et al., 1989). None of these tests were optimized for sensitivity. Brandon et al. (2004) developed ELISA approaches using monoclonal antibodies against the Kunitz (KTI) and the Bowman-Birk trypsin inhibitors. This assay was able to measure 1 and 3 ng/mL for BBI and KTI, respectively in processed food. An antibody was raised against Kunitz-type soybean typsin inhibitor that worked equally well against native and denatured protein (Barkholt et al., 1994).

5.2.4 ELISAs for other soy allergenic proteins

Porras et al. (1985) developed an ELISA for the detection of soy protein in soy lecithin, margarine and soy oil, with sensitivity between 100 and 200 mg/kg. Two soy allergens were measured by a sandwich ELISA (Gly m Bd 30K and Gly m Bd 28K, respectively) with monoclonal antibodies for both the immobilized and the capturing antibodies (Tsuji et al., 1995; Bando et al., 1998). These assays were developed for soy products and processed foods that contain soybean protein isolates. Soy protein was detected in various food products within the range 140–700 mg/kg, but could not be detected in fermented products. A competitive enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibody 6G4 was established to determine ß-conglycinin from soybean and soybean products and showed an IC_{50} value of 4.7 ng/mL with a detection limit of 2.0 ng/mL (You et al., 2008).

5.2.5 ELISA for total soy proteins

Several methods use antibodies raised not against just one particular protein but against the whole soy protein fraction (Song et al., 2008a,b). For example, Janssen et al. (1986) detected soy proteins in meat products up to 0.1% by gel electrophoresis, followed by blotting and dot blot. All major soy fractions were recognized by the antibody (Janssen et al., 1986).

Presently, the ELISA technique has been the preferred approach for allergens detection because of its high precision, simple handling and good potential for standardization; however, as with any type of analysis, there are inherent disadvantages related to food matrix interferences and effects of food processing on the targeted immunogens.

5.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is presently being developed for the detection and identification of the major food allergens. These techniques are based on the amplification of specific DNA

fragment by the polymerase chain reaction. DNA-based methods offer many advantages over protein-based methodologies, primarily that the target DNA is less degraded than the proteins from food matrices (Poms et al., 2004). In addition, the ELISA technique is more expensive and time consuming than PCR (Espiñeira et al., 2010).

Up to date, several assays have been developed and applied to evaluate the presence of genetically modified soy in food products. Duplex real-time PCR method was used to simultaneously detect traces of lupin and soya in processed food. Both lupin and soya at a level of 2.5 mg/kg food matrix could be detected in cookies (Galan et al., 2011). Koeppel et al. (2010) developed two novel quantitative multiplex real-time PCR systems. They simultaneously determine the DNA of peanut, hazelnut, celery, soy, egg, milk, almond and sesame, respectively. The specificity and sensitivity of test are in the range of 0.01% (Koeppel et al., 2010). Espiñeira et al. (2010) compared end-point and real-time PCR for the detection of soy protein in a wide range of foods. Real-time PCR method is better in specificity and sensitivity compared with the end-point PCR and is the simpler and more rapid process, with a higher potential for automation, therefore it is currently the most suitable screening method (Espiñeira et al., 2010).

Processing and ingredients can influence the detection limits. The lower detection limits were obtained for full-fat and defatted soybean flours than those for toasted soybean flour and soy fiber samples (Gryson et al., 2008). The detection limits for unprocessed and heat-processed pork meats are 0.01% and 0.06% (w/w) of soybean protein, respectively (Soares et al., 2010).

Comparisons of ELISA results with PCR results suggest a qualitative accordance, but a low correlation of quantitative results (Koeppel et al., 2010). The presence of the DNA is an indication of the presence of the allergenic food but not of the allergen itself. There is a lot of controversy over the employment of DNA in the analysis of allergens, since proteins are the allergenic components and processing may affect nucleic acids differently (Poms et al., 2004).

5.4 Mass spectroscopy

Mass spectroscopy is a very promising method to quantify the allergens in soybean. Ten allergens were quantified from 20 non-genetically modified commercial soybean varieties using parallel, label-free mass spectrometry approaches (Houston et al., 2011). Also, a multi-method was developed for the detection of seven allergens based on liquid chromatography and triple-quadrupole tandem mass spectrometry in multiple reaction modes. It is based on extraction of the allergenic proteins from a food matrix, followed by enzymatic digestion with trypsin. The chosen marker peptides were implemented into one method that is capable of the simultaneous detection of milk, egg, soy, hazelnut, peanut, walnut and almond. This method has been used to detect all seven allergenic commodities from incurred reference bread material, which was baked according to a standard recipe from the baking industry. Detected concentrations range from 10 to 1000μg/g, demonstrating that the mass spectrometric based method is a useful tool for allergen screening (Heick et al., 2011).

5.5 Commercially available methods

Since the 1990s, several tests to detect soy in food have become available commercially, most of which are ELISAs (Koppelman and Hefle, 2006). Table 1 gives an overview of the available methods and their most important characteristics. Tepnel BioSystems kit (ELISA

Technologies, FL, USA) is a quantitative ELISA (<0.5% detection limit) which specifically detects soy protein in raw and cooked/canned foods and meats/meat products. The ELISA Systems kit (ELISA Systems, Windsor, Australia) is based on a sandwich ELISA and determines the soy in the food sample with a sensitivity of 2.5 ppm. Recently, ELISA Systems, Neogen Corporation and Ceogen companies launched several soy protein screening assays. The detection limit is 2.5-10ppm, and those are well suited to allergen detection. Veratox for Soy Protein and Soy Flour Allergen (Neogen Corporation, MI, USA) is a sandwich ELISA used for the quantitative analysis of minimally processed soy and soy flour protein in processed food products such as cookies, crackers and cereal.

Analyte	Type of method	Kit manufacturer	Sensitivity	Web site
Soy protein	Inhibition ELISA	Tepnel	5000 ppm	www.tepnel.com
Soy protein	Sandwich ELISA	ELISA Systems	2.5 ppm	www.elisasystems.net
Soy protein	Sandwich ELISA	Neogen	2.5 ppm	www.neogen.com
Soy lectin gene	Real-time PCR	Congen	10 ppm	www.congen.de

Table 1. Commercially available methods to detect soy.

6. Soy allergen mitigation

A number of thermal and nonthermal techniques have been researched for reducing allergens in soybean and other foods. Food allergen reactivity due to thermal processing (treatment) (i.e., moist and dry heat) has been extensively studied. Heating can alter proteins by inducing denaturation (e.g., tertiary and/or secondary conformational changes), cross-linking, aggregation or rearrangements of disulfide bonds, which may cause changes in allergen reactivity (Mondoulet et al., 2005). In some cases, the changes are shown to decrease allergen reactivity (Beyer et al., 2001; Mondoulet et al., 2005), yet in other cases, the changes caused an increase in allergen bioactivity (Leszczynska et al., 2003; Simonato et al., 2001; Pasini et al., 2001), due possibly to exposing new IgE binding sites. Thermal treatments by moist heat include boiling, frying, extrusion, autoclaving and retorting, and those by dry heat include baking, roasting, and microwaving. In this chapter, for thermal treatments, only the effect of direct heating and extrusion on soybean allergens will be reviewed.

Nonthermal processing methods, which can help preserve original characteristics, organoleptic properties and nutritional benefits compared to thermal processing, have been used more in recent years to reduce allergen reactivity of different foods including soybean. These treatments include pulsed ultraviolet light, high hydrostatic pressure, irradiation, power ultrasound, and pulsed electric field, which will be reviewed in this chapter.

6.1 Thermal methods

6.1.1 Thermal processing

Thermal processing itself was not intended to reduce allergens when it was first developed. Rather, it was aimed to enhance food safety (pasteurization or sterilization), change of physical attributes or texture (drying, gelation), or induce flavor profile modification (baking) (Davis and Williams, 1998). However, unintentionally thermal processing also

affects protein structure. Some of the protein tertiary structure can be altered by temperatures as low as 50 °C and as temperature increases the extent of structural changes becomes greater (Lee, 1992). In general, linear epitopes are more difficult to be eliminated than conformational epitopes by thermal process or any other technique, because they are more likely to withstand structural change caused by processing. Conformational epitopes, on the other hand, are relatively easy to be removed by changing the tertiary or secondary protein structure, which can occur within reasonable temperature ranges of 50 – 125 °C (Lee, 1992). However, in some cases IgE binding activity of a soybean based material increases after thermal process. Gly m Bd 30K (P34) is an example of such cases. It was reported that IgE binding activity was significantly increased after retorting at a temperature of 121.1 °C (Yamanishi et al., 1995). It was hypothesized that the native epitopes may be refolded after the thermal process. On the contrary, it was also reported that Gly m Bd 30K (P34) was not present in a texturized soy protein after extrusion (Franck et al., 2002). Both retorting and texturization processes use elevated temperatures, but the thermal energy applied is lower for texturization than for retorting. So, it can be assumed that there are other factors present than just thermal energy that are responsible for the disappearance of the allergen.

Another issues related to thermal processing is the possibility to create new epitopes. During protein structural changes, there is a potential for residual structures to form new epitopes that were not present before the process. The process and product have to be carefully examined, although there are also evidences to support the incidences of no additional epitope development after various thermal processes (Franck et al., 2002). The modification of soybean allergen potency is quite obvious after thermal process, as the protein profiles have changed. Most likely the allergen reactivity will be reduced. However, there is a consensus among researchers that thermal processes alone may not be enough to produce hypoallergenic soy foods.

6.1.2 Extrusion

Extrusion is a widely used processing method which controls heat, shear and pressure to produce texturized proteins or expanded cereals or snacks in the food industry. In addition to heat controlled by temperature of extrusion barrel, mechanical energy controlled by screw configuration, screw speed, die size and shape, and barrel fill defines the extrusion process as a whole. Combination of these parameters may help change conformational epitopes and reduce allergen potency of soy products. It is believed that the extrusion process can be effective on reducing allergens using different extrusion equipment and process parameters (Ohishi et al., 1994; Saitoh et al., 2000; Franck et al., 2002). Saitoh et al. (2000) concluded that extrusion is an effective technique to produce low soy allergen products. A twin-screw extruder with 6 kneading blocks and temperature above 100 °C could reduce soy allergen level to 1%. Franck et al. (2002) also suggested that texturized soy protein might eliminate Gly m Bd 30k (P34), one of the major allergens in soy. Although the specific extrusion parameters were not provided, a typical texturization process is less intense compared to the process that Saitoh et al. (2000) has used and still managed to eliminate Gly m Bd 30k (P34). Ohishi et al. (1994) found that twin-screw extrusion using kneading blocks with temperature higher than 66 °C could reduce the allergen level to 0.1% of native soybean due to protein configuration change. In one of the authors' laboratories, three corn-soy blend samples, 51% soy flour, 21% soy flour, and 38% soy protein isolate (SPI), were extruded using a twin screw extruder with elevated temperatures of 140 °C. The results are shown in Fig. 2.

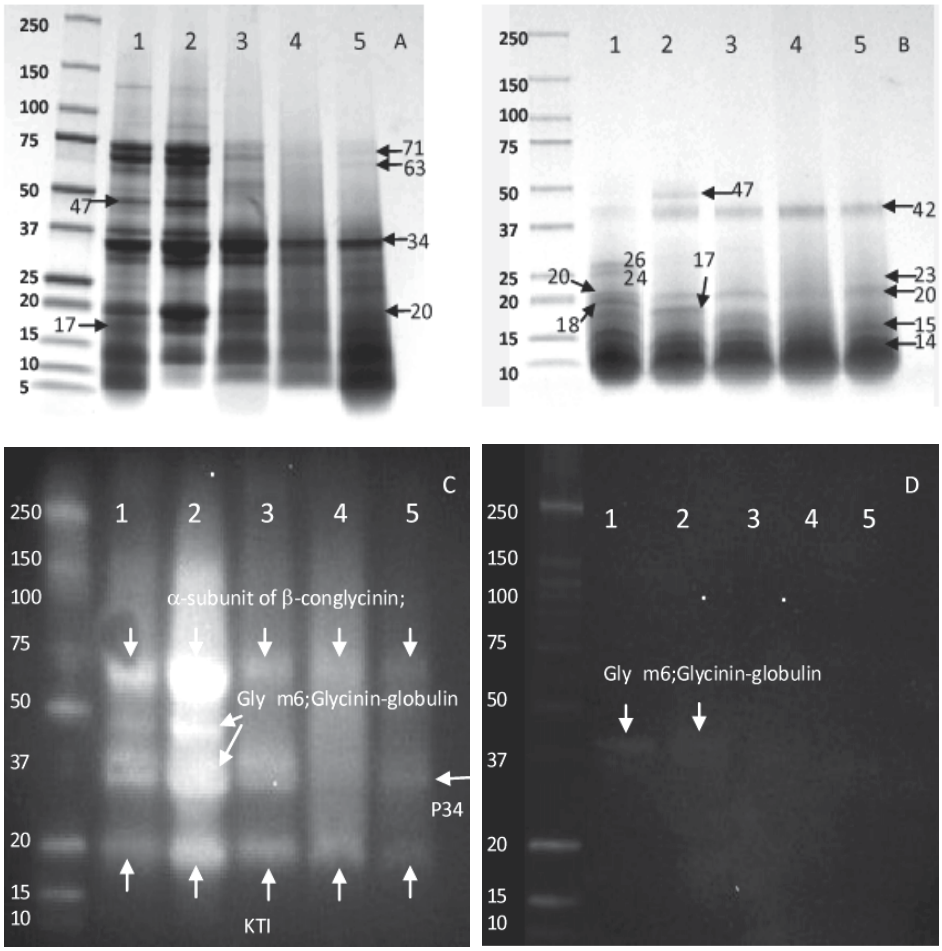


Fig. 2. SDS-PAGE profile of peptides in soybean products before (A) and after (B) hydrolysis with pepsin and Western blot band of peptides in soybean products before (C) and after (D) hydrolysis. Sample: 1: SPI; 2: soy flour; 3: 52% soy flour; 4: 21% soy flour; 5: 38% SPI.

The protein profiles changed due to different extruded samples from the soy flour and soy protein isolate (SPI). Although P34 band (Gly m Bd 30) was not completely removed, 38% SPI sample showed significant reduction of P34 compared to the other two samples. The results provide the same conclusions as in the previously mentioned studies, that is, the extrusion process is promising but not a complete solution. Additional steps such as pulsed ultraviolet light, fermentation or HPP may be required to achieve hypoallergenic soy products.

6.2 Nonthermal methods

6.2.1 Pulsed ultraviolet light

Pulsed ultraviolet light (PUV) consists of intense broad photonic spectra emitted within several nanoseconds from ionized inert gas (e.g., Xenon) under high voltage. The PUV radiation contains approximately 54% UV-C, 25% visible light, and 20% infrared light (Oms-Oliu et al., 2010; Shriver et al., 2011). The PUV light can be thousands of times more intense

than conventional, continuous mercury UV light (Dunn et al. 1995; Krishnamurthy et al., 2009).

Fig. 3 shows two types of PUV systems used for food applications: a batch unit and a continuous unit. In the batch system, foods are manually loaded to the sample rack in the treatment chamber, and the operator can adjust rack position (distance to lamp), treatment time and pulse frequency (typically 1-20 pulses/s). In the continuous system, a conveyor belt passes the food under one or multiple lamps in succession. Conveyor speed, distance to lamp (adjusted by a hydraulic mechanism), pulse frequency, treatment duration, and lamp tilt angle relative to conveyor belt direction can all be adjusted. The continuous system can also be used for batch treatment by setting the conveyor speed to zero.

Fig. 3. A. SteriPulse XL-3000 batch PUV system; B. LHS40 continuous PUV system, as manufactured by Xenon Corporation (Wilmington, MA).

The PUV treatment is a process of photophysical, photothermal and photochemical effects (Chung et al., 2008; Krishnamurthy et al., 2009; Yang et al., 2010; Shriver and Yang, 2011). Normally, PUV is regarded as nonthermal as long as the duration of exposure is short (e.g., seconds), as the temperature rise is insignificant; however, a prolonged PUV exposure (e.g., minutes) can incur significant temperature rise and moisture loss of the sample, where both the nonthermal (mostly due to the UV spectrum) and photothermal (mostly from the infrared spectrum) effects co-exist (Yang et al., 2011). Fiedorowicz et al. (2001) found that prolonged UV light treatment caused formation of insoluble complex in food, depolymerization of starch, peroxidation of unsaturated fatty acid, carbohydrate crosslinking, protein crosslinking, and protein fragmentation. Although proteins, including soybean allergens, have a peak absorbance at 280 nm wavelength (Gómez-López et al., 2005), light with shorter wavelength is more effective in changing protein molecules. The PUV treatment could not easily break the peptide bonds in protein. Photons absorbed by cystine had a higher chance of inactivating a protein than photons absorbed in the aromatic amino acids. The absorbed photons ionize the protein (Setlow, 2002). Aromatic amino acids (e.g., tyrosine and phenylalanine) can absorb UV radiation and recombine to form covalent cross-links in proteins (Gennadios et al., 1998).

The UV portion of PUV can form oxygen radicals, superoxide radicals, hydrogen peroxide and super oxide radicals. Oxygen radicals lead to generation of ozone. Super oxide radicals can induce protein cross-linking and protein fragmentation. Water molecules absorb UV photons and produce hydroxyl and hydrogen radicals (OH^- and H^+) which are powerful protein-modifying agents (Krishnamurthy et al., 2009; Davies et al., 1987). Hydroxyl radicals

are shown to induce alteration to the primary structure of bovine serum albumin (BSA). Oxygen radicals significantly alter the effects of hydroxyl radicals on protein primary structure. Protein aggregation by hydroxyl radical may involve intermolecular bityrosine formation (Davies et al., 1987). However, there may be other covalent modification involved in the process of PUV treatments.

It is believed that PUV's photothermal, photophysical and photochemical effects could alter allergen conformation (Krishnamurthy et al., 2007) or cause protein aggregation (Chung et al. 2008; Yang et al., 2010), resulting in the loss or modification of conformational epitopes. Furthermore, radicals may be induced during PUV treatment due to molecular ionization under its high-energy burst of lights. The visible and infrared waves in the PUV spectra are believed to be responsible for vibration and rotation of molecules, respectively (Krishnamurthy et al., 2009).

Apart from its microbial pasteurization or sterilization effects, PUV was applied by Chung et al. (2008) and Yang et al. (2011) to mitigate peanut allergens. The PUV radiation has also been found to significantly decrease the allergenic potency of soybean extracts (Yang et al., 2010), shrimp extracts (Shriver et al., 2010), egg extracts (Anugu et al., 2010), milk proteins (Anugu et al., 2009), and wheat extracts (Nooji et al., 2010).

For the peanut protein extracts and liquid peanut butter, PUV reduced the IgE binding by 6- to 7-fold compared to the control (Chung et al., 2008). It was found that PUV treatment caused protein aggregation of the major peanut allergens Ara h 1 and Ara h 3. It is believed protein conformation and IgE binding epitopes were altered accordingly. They found that insoluble proteins caused by PUV were insoluble in concentrated urea (2 M) or sodium chloride (1 M). In the study by Chung et al. (2008), the solubility of Ara h 2 (18-20 kDa), the most potent allergenic peanut protein (Chu et al., 2008; Dodo et al., 2005) was unaffected, but in a separate study by Yang et al. (2011) where raw peanut extracts, roasted peanut extracts and peanut butter slurry were treated with PUV under different conditions, Ara h 2 was significantly inactivated.

Specifically on soybean, Yang et al. (2010) noticed a pronounced reduction in soybean allergens (glycinin, 14-34 kDa and β-conglycinin, 50 kDa), when PUV was applied to soybean protein extracts for a duration up to 6 min with 3 pulses per second. SDS-PAGE (Fig. 4) shows a marked reduction in glycinin (14-34 kDa) and β-conglycinin (50 kDa). However, soybean proteins of higher molecular weights (e.g., 45-75 kDa) were slightly reduced, exhibiting considerably more resistance to PUV light treatment. Proteins of a larger molecular weight were formed in the region of 150-250 kDa, signifying that protein crosslinking or aggregation occurred during the PUV treatment. ELISA results (Fig. 5) show a marked decrease in IgE binding after the PUV treatment for 6 min.

Shriver et al. (2010) treated shrimp protein extracts with PUV for 4 min and noted a pronounced reduction in the potency of major shrimp allergen, tropomyosin, as probed through ELISA using pooled human sera from patients of shrimp allergy. Boiling treatment (100°C) of the samples was found to cause negligible allergen reduction. The researchers attributed shrimp allergen reduction under PUV conditions to conformational changes of tropomyosin, particularly intramolecular crosslinking.

Exposure of milk to PUV for 150 s was found by Anugu et al. (2009) to be effective in reducing its allergen levels, as evident from the undetectable level of allergenic casein and whey in SDS-PAGE after the treatment. ELISA results showed that IgE binding was reduced by 7.7 folds in whey protein and 7.4 folds in α-casein. Anugu et al. (2010) applied PUV to egg protein extracts and found PUV was effective in reducing egg allergens, for example, a 2-min PUV treatment caused all IgE binding to major allergens to be undetectable on

Western blot, except for ovalbumin that was still active in IgE binding to a certain degree. ELISA results showed that the total egg allergen reactivity was reduced by 9.5-fold at 2 min. The PUV exposure for 45 s with heat (boiling 100°C) and without heat caused the allergen reactivity of wheat gluten to be reduced (Nooji et al., 2010), as probed by SDS-PAGE and Western blotting. Although thermal treatment alone did not affect the allergen reactivity of wheat gluten, a combination of PUV and heat treatment enormously enhanced wheat allergen reduction compared to PUV alone.

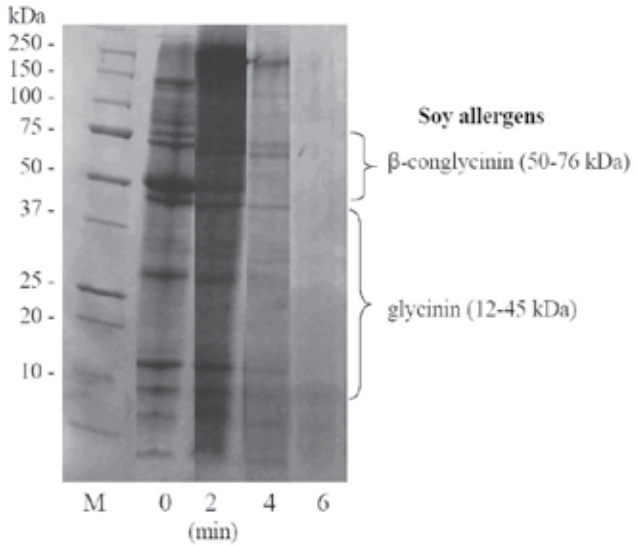


Fig. 4. SDS-PAGE of soybean protein extracts treated with PUV for 0 (control), 2, 4 and 6 m as indicated in figure. Reproduced with permission. M for protein marker.

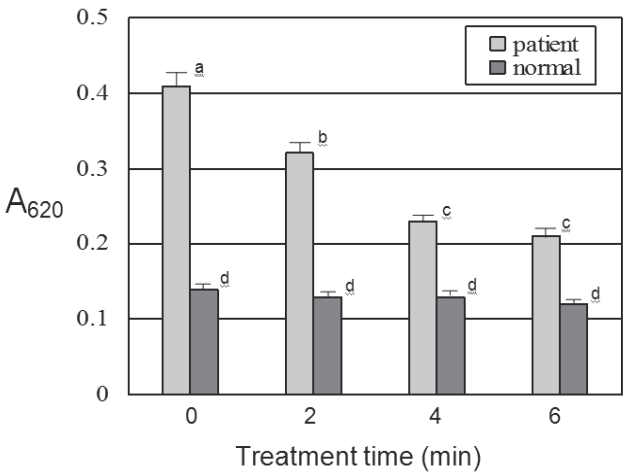


Fig. 5. The IgE binding capacity (expressed as the absorbance value A_{620} read at 620 nm) of PUV-treated and untreated raw soy extracts as probed by indirect ELISA. A620 Data represents mean of three replications. Data with same letters are not statistically different from each other ($p < 0.05$). Reprint with permission.

Although the foregoing treatments, including soybean extracts, with PUV were conducted in liquid form, the photothermal effect of PUV after an extended exposure also enables it to be applied directly to solid foods, e.g., whole almond (Li, 2011), for allergen mitigation.

6.2.2 High hydrostatic pressure

High hydrostatic pressure (HHP), also described as ultra high pressure (UHP) processing, subjects a food material, with or without packaging or supplemental heat, to pressures normally between 100 and 800 MPa for 10-30 min. During HHP treatment, the pressure is uniformly distributed in all directions (Spilimbergo et al., 2002), and it breaks non-covalent bonds (hydrogen, ionic, and hydrophobic bonds) that are present in compounds like proteins.

High hydrostatic pressure has been used to inactivate microorganisms and enzymes by protein alteration and denaturation (Mozhaev et al., 1996). Alterations of the secondary and tertiary structure of major proteins in meat and milk reportedly took place at pressures greater than 200 MPa, however, higher pressures (e.g., 300-400 MPa) are needed for egg and soy proteins to change structure (Messens et al., 1997).

Although more direct research is needed to examine the efficacy of HHP on the allergenic proteins of foods including the soybeans, HHP has been shown to induce conformational changes in proteins. Because of this, it is believed that HHP can change allergen potency by altering its epitope structures (Yamamoto et al., 2010). Kleber et al. (2007) found that the allergen reactivity of major milk allergen, β-lactoglobulin, was actually underscored by HPP from 200 to 600 MPa. With supplemental heat during pressure treatment, the allergen potency was further enhanced. The possible reason is that the conformational changes in β-lactoglobulin might have actually exposed new epitopes for IgE binding.

Kato et al. (2000) found a decreased reactivity of rice allergens and damaged endosperm cells under 300 MPa pressure treatment for up to 120 min. They also observed that apart from pressure-induced structural damage of rice grains, the buffer solution was able to permeate into the rice grain to facilitate allergen solubilization and subsequent extraction into the buffer, but the amount of allergen extraction depended on the solvent used and the solubility of the rice allergens. They also found the use of proteases might further reduce allergen reactivity.

High pressure treatment was found to cause the allergen reactivity of pasteurized liquid whole egg to decrease by 3.3 fold as detected by EAST inhibition. Combined thermal (heating to 70°C) and HHP at 600 MPa, 10 min caused a 8.9 fold reduction compared to control (70°C heating alone) that resulted in only 1.5 fold reduction in allergen reactivity (Hildebrandt et al., 2010). As demonstrated in the study, the combination of heat and HPP allowed for a greater reduction in overall food allergen reactivity.

In the case of soybeans, Penas et al. (2006) determined the effect of HHP of 100, 200 and 300 MPa on the protein content, the degree of enzymatic hydrolysis and the allergen Gly m 1 in the treated soybean whey and the hydrolysates. They found that besides enhanced proteolysis, HHP reduced the immunochemical response of soybean whey to anti-Gly m 1 monoclonal antibodies, which was further decreased after the combined treatment of high pressure and enzymatic hydrolysis. The results showed soybean whey proteins hydrolyzed at high pressure could be used as sources of peptides with low antigenicity when incorporated as food ingredients. Furthermore, sprouts which grew from HHP-treated soybean seeds had significantly reduced antigenicity compared with the ones grown from

untreated seeds. It was suggested that HHP could be used to produce hypoallergenic soybean sprouts without notable nutrient losses.
It was found soy proteins, including the two major storage protein components 7S and 11S globulins, are dissociated under pressure (Kajiyama et al., 1995), leading to new free thiol residues. Swanson et al. (2002) reported that some of the newly created fragments recombine to give –S–S– exchange reactions or new –S–S– binding by oxidation.
Denaturation of proteins under pressure is a complex process due to the disruption of both hydrophobic bonds and salt bridges. This process depends on a multitude of factors like the magnitude of pressure, pH, temperature, solvent used, ionic strength of the solution, and the protein structure (Kajiyama et al., 1995). Jung et al. (2008) found that the profile of storage proteins (7S, 11S) of the soymilks was similar to the control regardless of the pressure levels (100-700 MPa) applied to the beans.
In a pressure enhanced enzymatic hydrolysis study of soybean whey proteins, Penas et al. (2004) found that HHP increases hydrolysis, as Chymotrypsin and trypsin presented five visible peptides lower than 14 kDa after hydrolysis and pepsin about 11 peptides, and a pressure of 100 MPa presented the best condition for the three enzymes used.
Puppo et al. (2004) studied the HHP-induced physicochemical changes of the 7S and 11S soybean protein isolates at pH 3 and pH 8 in terms of protein solubility, surface hydrophobicity (Ho), and free sulfhydryl content (SHF). They found 200-600 MPa pressures caused the protein to aggregate and denature, with changes in secondary structure detected, leading to a more disordered structure. The protein Ho and aggregation were increased, SHF was reduced, and a partial unfolding of 7S and 11S fractions were observed at pH 8. A major molecular unfolding, a decrease of thermal stability, and an increase of protein solubility and Ho were also detected.

6.2.3 Ionized radiation

As a nonthermal method, ionized radiation (irradiation), such as gamma rays, x-rays and e-beams, has been reportedly used to control food antigenicity. Although the exact mechanism is still unknown, it is generally believed that irradiation changes the structure of the IgE-binding epitopes in the allergenic proteins of the foods like egg (Seo et al., 2007), milk (Lee et al., 2001) and shrimp (Byun et al., 2000). One possible way to induce such changes is through the free radicals created during irradiation, which may lead to protein fragmentation and aggregation.
Seo et al. (2007) treated the egg with gamma irradiation (cobalt-60) and found its allergenic ovalbumin was reduced at a dose of 100 kGy, which is rather high for food applications, but unaffected at a dose of 10 kGy, which is the maximal dose that has been shown to be safe for human foods (Byun et al., 2000). They noticed the occurrence of protein crosslinking during irradiation, as evident from the change in molecular weight, which caused the reduction in ovalbumin content. Irradiation has been reported to induce protein crosslinking, including the formation of disulfide bonds, and cause protein aggregation due to hydrophobic interactions (Davies and Delsignore, 1987).
Gamma irradiation (1-15 kGy) supplemented with heat (100°C) was found by Li et al. (2007) to be effective in reducing shrimp allergen reactivity by 5 to 30 fold. However, gamma irradiation or heat treatment alone did not cause a notable decrease in the IgE binding. In a similar study, Byun et al. (2000) looked at the effects of cobalt-60 irradiation on the major shrimp allergen, tropomyosin, and found that the protein band corresponding to

tropomyosin (36 kDa) was undetectable in SDS-PAGE at the irradiation dose of 7 kGy or so. The IgE binding to tropomyosin was minimal at the irradiation dose of 10 kGy. They explained that the proteins ultimately coagulated under irradiation, causing their disappearance on SDS-PAGE and the decreased allergenic potency.

Lee et al. (2002) treated the isolated egg ovomucoid with gamma irradiation at a dose of 10 kGy with or without supplement of heat. It was found irradiation supplemented with heat reduced the ovomucoid concentration to nearly an undetectable level, while irradiation alone or heat alone was not able to reduce the ovomucoid concentration to an undetectable level, with the former more effective than the latter, due possibly to the heat resistance of ovomucoid.

Lee et al. (2001) applied gamma irradiation to milk proteins (Bovine α-casein, ACA and β-lactoglobulin, BLG) and detected changes in their allergenic reactivity using milk-hypersensitive patients' IgE and rabbit IgGs individually produced to ACA and BLG by competitive indirect ELISA. Results showed a change in allergenicity and antigenicity of the irradiated milk proteins and a decrease in the solubility of irradiated proteins, which might be attributed to agglomeration of the proteins during irradiation. These results indicated that epitopes on milk allergens were structurally altered by gamma irradiation.

Shriver and Yang (2011) stated that the tree nut allergens are noticeably stable to gamma irradiation, even when combined with heat treatment. Su et al. (2004) subjected almond, cashew and walnut to gamma irradiation at the doses of 1, 5 10 and 25 kGy with or without heat supplement through one of the heating methods: autoclaving, blanching, frying, microwave, or roasting. They found none of the foregoing conditions, alone or in combination, were able to reduce the allergen reactivity.

Specifically for soybean, Manjaya et al. (2005) treated the soybean variety VLSoy-2 with 250 Gy gamma rays to induce variability to proteins including the two allergenic storage proteins 7S and 11S. A large number of mutants affecting morphological characters were identified and characterized. It was found there were 3 mutants lacking the A3 subunit of glycinin (11S) protein, and among the 3 mutants identified, 2 of them were also characterized by the lack of a 0-subunits of b-conglycinin (7S).

6.2.4 Power ultrasound

Ultrasound can be divided into high frequency, low-intensity diagnostic ultrasound in the MHz range and low frequency, high-intensity or power ultrasound in the kHz range (Feng et al., 2009). During sonication, the intermittent compression and rarefaction of the sonic waves promote formation of sonication bubbles in the sonication media, which eventually implore at critical bubble sizes, creating a localized high pressure (up to 1000 atm), high temperature (e.g., 5000 K), micro jets (e.g., hundreds of kilometers per hour in velocity), and free radicals to induce physical and chemical effects on the surrounding, including the change of allergen conformation and its reactivity. Also, the free radicals generated from water during sonication can contribute to protein modification (Soria and Villamiel, 2010).

The use of power ultrasound in the food processing has been a subject of research and development for many years, and recent applications include increasing extractability of protein for allergen detection (Albillos et al., 2011), tomato peeling (Rock et al., 2010), surface lipid removal and shelf life extension of potato chips (Wambura and Yang, 2009), expedited rice parboiling (Wambura et al., 2008), and expedited xylan extraction from corn cob (Yang et al., 2009).

So far, only a few applications of power ultrasound in food allergen reduction have been found in literature, but none has been reported on soybean allergens.
Li et al. (2006a) applied power ultrasound to change the structure of shrimp proteins and then assessed its effect on the IgE-binding capacity of major shrimp allergen Pen a 1(tropomyosin). The sonication conditions were 30 kHz, 800 W for 1.5 h at 0 and 50 °C, respectively. For comparison, samples were also boiled for 15 min. Allergen reactivity was analyzed by the enzyme allergosorbent test (EAST) and competitive inhibition ELISA (ci-ELISA) using pooled sera of 15 shrimp-allergic patients. Protein extracts treated with power ultrasound at 50°C (treated 2) was 2.2-fold lower than that of untreated shrimp, while protein extracts boiled was 1.2-fold lower than that of untreated shrimp. The results suggest that power ultrasound at 30 kHz, 800 W and 50°C treatment temperature may reduce the allergen potency of shrimp. In a separate but similar study, Li et al. (2006b) found the IgE binding to the power ultrasound treated isolated shrimp allergen decreased by approximately 81.3-88.5%. It was noted that during the treatment of the allergen isolate, formation of a new protein fraction with a low molecular weight increased in quantity as treatment time elapsed. Thus, fragmentation of the shrimp allergen may occur during high intensity ultrasound under the conditions stated (Li et al., 2006b).
Yu et al. (2010) evaluated the effects of combined ultrasound and boiling (CUB) along with boiling alone and high pressure steaming (HPS) on the degradation of crab tropomyosin and reduction of its IgE-binding reactivity. SDS–PAGE analysis indicated that boiling had little impact on the digestive stability of tropomyosin. Both the CUB and HPS accelerated the digestion of tropomyosin. Western blotting and inhibition ELISA also indicated a partial decrease in the reactivity of IgG/IgE-binding of tropomyosin after CUB or HPS treatment. It was concluded that the ultrasound combined method was inferior to the HPS that was found to be the most effective method to accelerate the digestion of tropomyosin in gastrointestinal digestion and reduce the reactivity of IgG/IgE-binding of tropomyosin.

6.2.5 Pulsed electric field

Pulsed electric field (PEF) is a nonthermal method for food processing and preservation that uses short pulses of high frequency electricity for microbial inactivation, with minimal changes on food quality attributes, of liquid or semi-liquid food products. The PEF treatment involves placing foods between electrodes subject to high voltage pulses in the order of 20–80 kV, which results in an electric field that causes microbial inactivation and other changes to food. Although PEF technology has found the most significant application in the inactivation of microorganisms, it has also been studied for other uses such as enzyme inactivation (Yang et al., 2004), drying or enhancing mass transfer rate (Lebovka et al., 2007; Gachovska et al., 2008), wine maturation (Zeng et al., 2008), and allergen reduction (Toshiko et al., 2004). So far, studies on the effects of PEF on allergens or protein structure and functionality in general have been scarce, which becomes an area of immediate research needs as the PEF technology is intensified in food processing applications and moves towards commercialization.
Toshiko et al. (2004) studied the effect of PEF on various allergen-antibody interactions as detected by ELISA. They found the ELISA value was drastically decreased when PEF at 10kV and 50Hz was applied to the ovalbumin solution. However, no significant changes in ELISA values were observed in other allergenic proteins. The ovalbumin concentration

before and after PEF treatments was also examined using the Bradford method. The results show that though the ovalbumin concentration was not changed significantly, ELISA value was decreased. This suggested that conformational changes of ovalbumin might have taken place during the PEF treatments, which resulted in lower incidences of allergen-antibody interactions.

Sun et al. (2011) studied the effect of PEF on the structure and properties of whey protein, including solubility, as it forms the whey protein isolate–dextran conjugation in aqueous solution during the PEF treatment. They found that higher pulsed electric field intensity enhanced the extent of glycosylation, and the secondary structure of whey protein isolate had a considerable loss due to the covalent attachment of dextran. Compared with initial whey protein isolate, the solubility was significantly improved. At the same time, glycosylation also inhibited heat-induced aggregation after treated at 80°C for 20 min.

Johnson et al. (2010) applied PEF, high pressure and thermal treatments to four purified allergens from peanut (Ara h 2, Ara h 6) and apple (Mal d 3, Mal d 1b) and examined their structural changes using circular dichroism spectroscopy and gel-filtration chromatography. For the PEF treatments, electric field strengths were from 0 to 35 kV/cm and specific energy inputs from 0 to 130 KJ/kg to minimize temperature rise during PEF treatment, a frequency of 2 Hz was chosen and samples were removed from treatment chamber immediately after PEF treatment. It was found that PEF did not induce any significant changes in the secondary structure of these four plant-based allergens. However, Johnson et al. (2010) commented, due to the fact there is a lack of studies of PEF effects on protein structure in general, it is difficult to predict whether other allergens may be affected under similar PEF conditions.

6.2.6 Nonthermal plasma

Application of nonthermal plasma (NTP) technology to allergen mitigation is still new. Although no literature has been found on NTP application to soybean yet, recent development on using NTP to reduce allergens in shrimp and wheat protein extracts (Nooji, 2011; Shriver, 2011) may indicate a potential application of NTP to soybean extracts.

Nonthermal plasma is electrically energized and highly energetic gaseous matter, which can be generated by electrical discharge across an electrical field. The NTP species can include electrically neutral gas molecules, free radicals, photons, negative or positive ions, and electrons. A similar principle of bacterial killing by NTP, i.e., oxidation of the reactive oxygen species (Montie et al., 2002), UV radiation, and free radicals that come along with plasma generation (Desmet et al., 2009), may also be applicable to explain allergen reduction.

Shriver (2011) and Nooji (2011) exposed shrimp extract and wheat extract, respectively, to the plasma generated at 30 kV voltage and 60 Hz frequency for 1, 3, and 5 min at ambient temperature. Results showed that the IgE binding to tropomyosin in the shrimp samples was decreased to undetectable levels after 3 and 5 min NTP exposure. Dot blot and ELISA confirmed a decrease in tropomyosin reactivity following the NTP treatments. It was also found 5-min NTP treatment of wheat protein extracts gave a pronounced decrease in allergen reactivity as probed by both ELISA and dot blot.

6.3 Chemical and biological methods

Combination of chemical reaction such as carbohydrate conjugation during Maillard reaction showed some evidences of reducing soy allergenicity (Babiker et al., 1998). Conjugated galactomanan by Maillard reaction was believed to mask the structure of soy

protein, P34, and decreased allergenicity, although the same method was not investigated with other soy allergens. Immunoreactivity of soy protein can be altered through control of pH and ionic strength showing that pH 6 displayed less IgE binding than at pH 2.2 or pH 7.2 (L'Hocine et al., 2007). These researchers investigated the effect of ionic strength and pH on immunoreactivity of pure soy glycinin and found that pH has significant effect on immunoreactivity, possibly due to protein's tertiary structural changes. High immunoreactivity was shown at low pH (2.2) and at neutral pH (7.2). It was suggested that conformational structure change of protein is the main reason at pH 2.2 by revealing epitopes for IgE binding. At pH 7.2 due to possibly highly ordered structure of protein, epitopes may be positioned ideally for binding with antibodies.

Protein hydrolysis and fermentation also show effectiveness on reducing soy allergenicity (Wilson et al., 2005; Penas et al., 2006; Frias et al., 2008). Although the allergen reactivity was reduced substantially, functional properties of resulting soy proteins were also significantly altered. Several studies have confirmed the degradation of soybean allergens during fermentation by microbial proteolytic enzymes in soy sauce, miso products, soybean ingredients and feed grade soybean meals (Yamanishi et al., 1996; Lee et al., 2004; Kobayashi, 2005). Fermentation has the capacity to improve nutritional and functional properties compared to original products. Fermentation may entail the ability to hydrolyze soy protein into smaller peptides. Soy sauce possesses microbial proteolytic enzymes able to degrade soybean proteins, including major soybean allergen (Gly m Bd 30K), into peptides and amino acids (Kobayashi, 2005).

As a biological method, genetic modification has gained much attention despite its controversial nature. It prevents the translation of selected allergens using post-transcriptional gene silencing or co-suppression (Shewry et al., 2001). Herman et al. (2003) used transgene-induced gene silencing to prevent the accumulation of Gly m Bd 30 K (or P34) protein in soybean seeds. It was found the Gly m Bd 30 K-silenced plants and their seeds lacked any compositional, developmental, structural, or ultrastructural phenotypic differences when compared with control plants. The silencing of Gly m Bd 30 almost completely inhibited IgE binding (Herman et al., 2003). Experimental evidences by electron microscopic-immunocytochemical assays, two-dimensional protein analysis, and tandem mass spectrometric identification all indicated no significant differences between the modified soybean and the control in respect to structural morphology and protein composition. Furthermore, comparative testing showed that no new allergens were formed during silencing. Nethertheless, genetic modification method is still under scrutiny about the stability of hypoallergenic foods produced by it, therefore food safety risks exist with foods genetically modified for allergen silencing. Also, the allergen removal process may still accompany the alteration of the foods' functional and physical properties (Shewry et al. 2001), because many allergenic proteins are parts of plant development and metabolism. Genetic modification of IgE epitopes instead of the protein fraction may be a better option for genetic modification to reduce allergen.

Combination of physical (novel technologies), chemical and biological techniques may be an effective approach to maximize the alleviation of allergen and minimize the change in functionality.

7. Future trends

Effective fighting with food allergy, including soy allergy, necessitates blocking any of the critical steps leading towards allergy development. One of the critical steps is preventing

allergen from entering human body. In the current practice, this is achieved through total avoidance of the allergenic foods with increasing difficulty to effectuate, but novel processing technologies, which may significantly reduce allergen reactivity while maintaining the product functionality, offer a hopeful prospect in minimizing allergen level in soy products, thus preventing allergens from entering human body. More research is needed in this area, especially on pulsed UV light that has been shown promising in mitigating allergens in soybean, peanut, shrimp, almond, milk, egg and wheat (Shriver and Yang, 2011).

Application of "hurdle technology" concept in allergen mitigation, where one method is combined with other methods or conditions, may prove quite effective in producing truly hypoallergenic soy products where allergens are down to a significantly low level safe to most allergenic patients. As discussed earlier, combination of physical, chemical and biological techniques may be an effective processing strategy to reduce maximally the soy allergens and yet affect minimally the product functionality.

The accurate detection and identification of soy allergens are still rather difficult, especially when they are hidden in different products including bakeries, infant foods, dairy foods and processed meats. A robust detection method is desired that can meet the foregoing demand and yet identify a multitude of allergens in the food product. Mass spectroscopy is a potential method for this purpose, which deserves more intensified research. In addition, in order for the results to be comparable, the specificity, selectivity and reliability of different methods also require a continued study for addressing.

8. Concluding remarks

Soybean is one of the most healthy and yet allergenic foods. Soybean allergens consist of proteins with molecular weights ranging from 7 to 71 kDa. Among the 34 allergic soy proteins identified, Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K are categorized as major soy allergens, while soy hydrophobic protein (Gly m 1a), soy hull protein (Gly m 2), soy profiling (Gly m 3), glycinin (320-360 kDa), β-conglycinin (140-180 kDa) and tripsin inhibitor (20 kDa) are listed as major soy allergenic proteins. The strongest soy allergen among others is Gly m Bd 30k, also known as P34. Like any allergens in a food system, soy allergen reactivity is dominated by epitopes or IgE binding sites, categorized as linear or conformational. The epitopes are not only fully characterized by their primary protein structure, but also by their tertiary structural conformations, which offer opportunities for different thermal and nonthermal food processing technologies to alter the nature of epitopes and thus allergen reactivity with IgE antibody.

As one feasible approach towards blocking the steps of allergic reaction sequence, removing or reducing allergens in soybean and its derivatives, thermal and nonthermal methods have been gaining momentum worldwide. Postharvest processing techniques, especially nonthermal methods, are especially attractive. Several nonthermal processing techniques, such as PUV, HHP, power ultrasound, ionized radiation, pulsed electric filed and nonthermal plasma, have been tested on a number of food products including soybean to alter allergen reactivity without changing the inherent properties of the products. Nonthermal techniques cause changes to allergen structures and modifications to IgE binding epitopes, including promoting the aggregation or crosslinking of proteins. Pulsed ultraviolet light, among other novel methods, has been outstanding in terms of allergen mitigation, as it has been reported to significantly reduce allergens in soybean, as well as other products like peanut, shrimp, almond, milk, egg and wheat.

Nevertheless, before an effective allergen mitigation approach is securely in place for soybeans, total avoidance is still likely to be the most effective policy for soy allergy control. Because of this, reliable allergen detection methods are critical for ensuring the public health and compliance with food labelling regulations. To date, the majority of detection methods developed for soybean allergens are immunochemical based. The ELISA technique has become a preferred approach for soy allergen detection due to its high precision, simple handling and good potential for standardization. As a newly developed allergen detection technique, the PCR method offers unique features including less cost, faster turnaround and stability of targeted DNA over processing conditions, as compared to ELISA, but its quantitative correlation to ELISA results has so far been low due probably to the fact that nucleic acids are affected differently from allergenic proteins. In contrast, mass spectroscopy has shown a great potential over ELISA and PCR for detection and quantification of soy allergens due to its wide detection concentration range and its capability of detecting a multitude of allergens within one food species or over a number of different species in a composite food system.

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Nutritional and Bioactive Compounds of Soybean: Benefits on Human Health

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1. Introduction

The soybean (*Glycine Max*. (L) Merrill) is a leguminous which originated from China and has been cultivated in this country for around five thousand years. It was considered for centuries the staple food of the countries of East Asia. In the West, its introduction was made experimentally in the Europe in 15th century. In the USA the first reports of cultivation of soybean date from 1765. Unlike East Asia, the USA soybean was initially cultivated as forage plant being used as a source of edible oil starting at 1915. A major technological progress for the use of soybean meal as protein source in nutrition was made in 1917, when Osborne and Mendel demonstrated that unheated soybean meal presents nutritional quality lower than that of heated soybean meal. Thus, the finding of the nutritional value of soybean meal as a feed for animal nutrition, coupled with growing demand for vegetable oils allowed the deployment of many industries for the processing of soybeans in the USA (Müller, 1981).

In Brazil, the introduction of soybeans occurred in Bahia state, in 1882 (Bonetti, 1981). But the first statistics showing the use of soybean for grain production are from 1941. In this same year, the first soybean processing industry in the country was installed in Rio Grande do Sul (Teixeira, 2003). However, only from the 1960, soybean crop became important in the country, initially in the South where it showed better adaptation because of the similarity with regions of cultivation in southern United States. From the 70's, the soybean crop has evolved significantly in the cultivation states, not only in the south, but also in the middle western of Brazil. With the development of new cultivars adapted to different agro-climatic regions of the country, Brazil has become the second largest soybean producer in the world.

Advances in new technologies for increased acceptability of soy proteins as human food occurred as result of protein deficiency worldwide, after the World War II. Since the 1950's, USA and Canadian public research institutions worked on the development of soybean cultivars for human food use for export, mainly to Japan. In 1999, there were 16 breeding programs in public institutions working on soybean for human consumption (food-type). By

1998, dozens of cultivars had been launched specifically for the production of Tofu, Edamame and Natto. In the domestic market, only from mid-1980 genetic breeding became important in reducing the beany flavor, which is the factor that contributes more to low acceptability of soybeans as food.

In the 70s Brazil implemented a series of government programs for the introduction of a soybean beverage in school lunches, but this product was not well accepted because of its unpleasant taste resulting from the use of an inappropriate processing technology and inappropriate soybean cultivars. This led to the failure of this initiative and the deactivation of almost all programs, which reduced for a long time the possibility of using soybeans for human consumption.

The lack of a cultivar more suited to Western market has an explanation in the evolution of the low acceptability of soybean grown in Brazil, as well as in the USA. Soybean was initially grown as a forage plant, then as grain for oil extraction and only in 1960 as a source of protein meal. The development of flavored cultivars, more suited to the Western palate, was never a priority. Hence, the lack of cultivars adapted for this purpose. The cultivars used until this time point had undesirable taste and odor called beany flavor. The beany flavor derives from the formation of hydroperoxides as a result of the degradation of polyunsaturated fatty acids resulted from the action of lipoxigenases enzymes. The heat inactivation of lipoxygenases during the processing of soybean has been used in the food industry to reduce the beany flavor. Several treatments are recommended; however, they promote denaturation of proteins, altering their functional properties. This fact, coupled with the inefficiency of the thermal treatment to completely eliminate the beany flavor encouraged several groups of researchers in the country and abroad to find instead a genetic solution to the problem.

Mindful of the need to adjust the taste of soybean to the Western consumer, testing began in Brazil in 1982, in partnership with foreign researchers, in order to identify soybean genotypes that have a lower beany flavor. So, productive cultivars without lipoxygenase and very low levels of other related compounds to beanny flavor have been developed. Research conducted at the *Universidade Federal de Viçosa*, Brazil, has led to the development of five cultivars devoided of lipoxygenases in the seeds, and also considered as productive cultivars (Cultivars UFVTN 101, UFVTN 102, UFVTN 103, UFVNT 104 and UFVTN 105). These cultivars are currently being recommended for planting in the state of Minas Gerais.

Other aspects relating to quality of soybean, of great relevance for the food industry, subject of genetic improvement, are: higher content and quality of protein, lower oligosaccharides content (stachyose, raffinose and verbascose), higher contents of isoflavones, oleic acid and lower levels of linolenic acid, and absence of inhibitors of protease and lectins (Moreira, 1999). Several cultivars that meet these characteristics are now under development, among them, some derived of UFVTN 105, that besides their absence of lipoxygenases, may contain either high protein (UFVTN 105 AP), low-linolenic acid (UFVTNC 106) or high protein content, and lack of KTI and lectin (UFVTNK 107).

Another improvement program for creation of cultivars destined to human feeding has been developed by *Empresa Brasileira de Pesquisa Agropecuária - Soja* (*EMBRAPA Soja*) since 1987. With the aimed of searching for cultivars with more suitable characteristics for food processing, such as good appearance (clear integument and hilum) and mild flavor, among others, *EMBRAPA Soja* released the following cultivars: BRS 155, which features low content of trypsin inhibitor; BRS 213, which does not contain lipoxygenase enzymes; BRS 216, which has grains of small size, suitable for the production of sprouts and natto; BRS 257, which does not contain lipoxygenase; BRS 267, which has a higher sugar content, and BRS 258, destined to the organic system.

In the private sector the company Naturalle Agro Mercantil Ltda works in the commercialization of special soybean for human consumption and aimed for export. Several cultivars (NT2; NT4, NT10, NT12, among others) with differentiated characteristics of grain size, with clear colors of integument and hilum, and high levels of protein have been already developed.

Traditionally, soybean is not consumed raw. Studies have shown the importance of using appropriate techniques of preparation, preservation and improvement of their nutritional and sensory characteristics. Traditional methods of preparation of soybean for human consumption include germination, cooking, roasting, and fermenting, in order to inactivate thermolabile antinutrients e.g. trypsin and chymotrypsin inhibitors and lectins (Reinwald et al., 2010).

Regarding heat treatment, there is not a recommendation of the best time and temperature to be applied to the grains in order to inactivate and, or reduce their phytochemicals which have antinutricional effects, in detriment of those with functional or beneficial effects. Adequate levels of urease activity have been demonstrated in soybeans subjected to a temperature of 98 ºC for 30 minutes under humid heat, 60 °C for 42 hours, 150 ºC for 30 minutes and 121 ºC for 15 minutes under dry heat indicating denaturation of trypsin inhibitor (Machado et al., 2008). Moreover, the temperature of 150 °C for 30 minutes promotes the dephosphorylation of phytic acid, reducing the concentrations of inositol pentaphosphate and hexa, which have anti-nutritional action, without affecting the concentration of inositol triphosphate and tetra, which are phytochemicals with antioxidant properties (Andrade et al., 2010).

As soybean consumption increases in the Western diet, it is more common to find foods containing soy components rather than whole soy. Soy flour is the soy products less refined, however it is the industrialized most important soy product, since it is used to enrich foods, and for manufacturing textured, concentrated and isolated proteins. The incorporation of soy flour to replace wheat flour in formulation of bakery products increases the nutritional value of these products, especially the levels of protein, lipids, fiber and minerals when compared to conventional product (Dantas et al., 2009). The acceptance of formulations using mixed flours of wheat and soybeans suggests that the use of soy flour, obtained from cultivars without lipoxygenase, minimizes the unpleasant sensory problems which are characteristics of soybean (Dantas et al., 2010).

The use of soybean for human consumption in the form of integral flour is a nutritional and technological advantage, since it presents greater contents of total dietary fiber and iron than soybean hull-less flour (Carvalho, 2009).

2. Chemical composition and nutritional value of soybean

In general, soybean cultivars have approximately 8% bark, 90% cotyledon, which contains the highest percentage of proteins and lipids, and 2% hypocotyledon. The soybean chemical composition and its structural parts vary according to cultivar, growing season, geographic location and environmental stress (Ciabotti et al., 2006).

Protein is the major constituent of the soybean (30 to 50 g/100g), followed by carbohydrates (20 to 35 g/100g) and lipids (15 to 25 g/100g) (Carvalho et al., 2011; Esteves et al., 2010; Felix & Canniatti-Brazaca, 2008; Machado et al., 2008; Martino et al., 2007; Oliveira et al., 2007; U.S. Department of Agriculture, 2009). However, commercially, carbohydrates are less important than the proteins and lipids (Liu, 1997). There is an inverse relationship between the protein content and, contents of lipid and carbohydrate, thus the cultivars with greater protein content present a lower contents of lipids and carbohydrates (Table 17.1).

Compounds	BRS 133[a]	BRS 258[b]	OCEPAR-19[c]	UFV-116[d]	UFVTN 105AP[e]	USDA[f]
Protein	37.4	42.3	41.1	39.4	46.2	39.9
Lipid	21.4	22.7	24.6	20.8	19.9	21.4
Ash	4.9	4.8	4.7	6.6	5.3	5.2
Insoluble dietary fiber	23.2	23.0	0.7	0.8	9.1	nd
Soluble dietary fiber	2.8	2.8	1.7	1.3	1.5	nd
Total dietary fiber	26.0	25.8	2.3	2.1	10.5	9.9
Total carbohydrates	36.3	30.1	28.0	23	13.8	32.3
Linoleic (18:2)	5.6	5.6	5.5	5.5	5.8	9.9
α-Linolenic (18:3)	0.9	0.7	0.8	0.8	0.7	1.3

nd: not determined; a Soybean with low protein content and with hull; b Soybean with high protein content and with hull (Paucar-Menacho et al., 2010); c Conventional soybean with hull thermally treated 80 oC for 20 minutes; d Soybean without LOX 2 and 3, hull-less, thermally treated 80 °C for 20 minutes (Esteves et al., 2010); e Soybean with high protein content without LOXs, with hull, thermally treated 150 °C for 30 minutes (Andrade et al., 2010); f Conventional soybean (U.S. Department of Agriculture, 2009).

Table 17.1. Chemical composition of conventional and genetically improved soybeans (g/100g).

The main soluble carbohydrate of immature soybean is glucose. With the maturation of the grain, this sugar disappears, predominating non-reducing sugars such as sucrose, raffinose and stachyose (Paula, 2007). Most of the complex polysaccharides and oligosaccharides of the grain are composed of fibers, which are located mainly in cell walls and interstitial material of the shell (86%). The cell wall of soybean contains about 30% pectin, 50% hemicellulose and 20% cellulose. The grain also contains approximately 1% starch (Liu, 1997).

Soybean presents a high lipid content and is good source of essential fatty acids. In some soybean cultivars it has been observed they contain approximately 63% polyunsaturated fatty acid, 55% linoleic acid and 8% linolenic acid; 21.5% monounsaturated fatty acid (oleic acid), and 15% fatty acids saturated, 11% palmitic acid and 4% stearic acid (Esteves et al., 2010). The linoleic acids (ω6) and linolenic (ω3) found in soybean are considered essential and are easily oxidized due to the presence of unsaturations. These fatty acids compete with the enzymes involved in the reactions of desaturation and chain elongation. Although these enzymes have a greater affinity for ω3 acids, the conversion of alpha-linolenic acid to polyunsaturated fatty acid of long chain is strongly influenced by levels of linoleic acid in the diet. Thus, the ratio between daily intake of foods rich in ω6 and ω3, such as soybeans, is very important in human nutrition (Martin et al., 2006).

The lipid fraction of grain contains vitamin E isomers, α-tocopherol (42.0 to 291.0 mg/100g), γ-tocopherol (79.0 to 252.0 mg/100g) and δ-tocopherol (27.0 to 89.0 mg/100g) (Andrade, 2010; Baumgartner et al., 2010); and small amounts of vitamin K (47 mcg/100g) and vitamin A (1.0 mcg/100g). There is also the presence of water soluble vitamins such as vitamin C (6.0 mg/100g), folates (375.0 mcg/100g), thiamin (0.9 mg/100 g), riboflavin (0.3 mg/100 g), pyridoxine (0.5 mg/100g), niacin (2.1 mg/100 g) (Lebiedzinska & Szefer, 2006; U.S. Department of Agriculture, 2009).

2.1 Soy proteins

Soybean is the legume that presents the highest protein content and thus contributes to the supply of essential amino acids and nitrogen to human and animals (Liu, 1997). The major components of soy proteins are storage proteins known as β-conglycinin (7S) and glycinin

(11S), which represents 65% to 80% of total seed proteins. In addition, soybean contains are several inhibitors de proteases, lectins and enzymes (such as lipoxygenase and urease). Storage proteins can be classified according to their rate of sedimentation during centrifugation, as 2S, 7S, 11S, and 15S (Table 17.2). S means Svedberg unit, which is a unit of sedimentation rate computed as the rate of sedimentation per unit field of centrifugation strength (Wang & De Mejia, 2005).

Ultrafiltration protein fraction[b]	**Percent in extractable protein**	**Proteins in the fraction**
2S	20%	Kunitz typsin inhibitors; Bowman-Birk typsin inhibitors; Cytochrome C; AL1 and AL3α-Conglycinin
7S	33%	β-Conglycinin; γ-Conglycinin; α-Amylase; Lipoxygenase; Hemagglutinins (or lectins); Soybean vacuolar protein P34
11S	33%	Pure protein: glycinin
15S	10%	Pure protein: polymer of glycinin

[a] Adapted by: Wang et al.,(2005); [b] S = Svedberg unit. A unit of sedimentation rate computed as the rate of sedimentation per unit field of centrifugation strength.

Table 17.2. Soybean protein classification [a]

Soy proteins have high nutritional value and high concentration of lysine. This feature is important because of the its possible use in diets based on cereals, which are poor in lysine. In conventional cultivars sulfur amino acids are limiting, being methionine the most limiting one, followed by cystine and threonine (Liu, 1997). However, some genetically improved cultivars, such as the Brazilian cultivars BRS 133, BRS 258, OCEPAR-19, UFVTN 105 AP and UFV116, do not present this limitation (Carvalho, 2009; Esteves et al., 2010; Paucar-Menacho et al., 2010). In these cultivars, the breeding provided a qualitatively and quantitatively better balance of essential amino acids.

Genetic breeding is able of improve the profile of amino acids of soybean. However, the genetically improved cultivars may still present deficiencies of essential amino acids. By comparing the content of essential amino acids of soybean cultivars, with hull and hull-less (Table 17.3) with the standard of nutritional recommendations of FAO/WHO (2007), it was observed chemical scores above 1 for most of the essential amino acids. However, the cultivar UFVNT 105 AP was deficient in valine. Therefore, the development of new cultivars that meet the recommendations of essential amino acids is necessary.

The antigenic proteins, glycinin and β-conglycinin, may cause transient hypersensitivity reactions in the intestinal mucosa, especially in recently weaned animals, causing changes in the morphology of the intestinal mucosa with shortening of the villous and increased of the crypt depth. As a result, there may be a reduction in the number of mature enterocytes in villous, reduced villous height and increased of the crypt depth and thickness of inner and outer muscle, with consequent reduction of the digestive and absorptive capacity in the gut (Andrade et al., 2010; Caruso & Demonte, 2005). However, fermentation can decrease soy immunoreactivity and can be optimized to develop nutritious hypoallergenic soy products. However, the clinical relevance of these findings needs to be determined by human challenge studies (Song et al., 2008).

Amino acid	BRS 133 [a]	BRS 258 [b]	OCEPAR-19 [c]	UFV-116 [d]	UFVTN 105AP [e]
			Indispensable		
Cystine	0.72	0.71	0.62	0.72	0.66
Histidine	0.98	1.16	1.34	1.34	1.08
Isoleucine	1.66	1.90	2.32	2.66	1.97
Leucine	2.83	3.17	4.03	4.26	3.31
Lysine	2.30	2.58	2.94	3.25	2.77
Methionine	0.46	0.43	0.93	0.99	0.55
Phenylalanine	1.80	2.06	2.66	3.1	2.22
Threonine	1.47	1.56	1.99	2.2	1.77
Triptophan	nd	nd	0.86	0.73	0.59
Tyrosine	1.33	1.43	1.76	1.99	1.54
Valine	1.68	1.82	2.32	2.55	2.03
Total of indispensable	15.24	16.82	21.77	23.79	18.49
			Dispensable		
Alanine	1.61	1.81	2.21	2.36	1.92
Arginine	3.25	3.98	3.78	4.35	3.25
Aspartic acid	4.34	4.90	5.62	6.24	5.11
Glutamic acid	6.93	8.02	9.28	10.51	7.87
Glycine	1.91	2.09	2.03	2.24	1.88
Proline	1.86	2.11	2.51	2.64	2.38
Serine	2.27	2.55	2.44	2.51	2.35
Total of dispensable	22.17	25.48	27.87	30.85	24.66
Total of amino acids	37.41	42.30	49.64	54.64	43.15

nd: not determined; a Soybean with low protein content and with hull; b Soybean with high protein content and with hull (Paucar-Menacho et al., 2010); c Conventional soybean with hull thermally treated 80 °C for 20 minutes; d Soybean without LOX 2 and 3, hull-less, thermally treated 80 °C for 20 minutes (Esteves et al., 2010); e Soybean with high protein content without LOXs, with hull, thermally treated 150 °C for 30 minutes (Andrade et al., 2010)

Table 17.3. Amino acids composition of conventional and genetically improved soybeans (g/100g)

β-Conglycinin is a trimer with a molecular weight (MW) of about 180 kDa. It is composed of 3 subunits, α (63 kDa), α′ (67kDa) and β (48kDa) (Apweiler et al., 2004; Liu, 1997; Nielsen, 1996). These subunits share a large degree of amino acid homologies. Furthermore, β-conglycinins with different subunit composition have also been identified. It is likely that the trimers are composed of randomly assembled mixture of subunits (Nielsen, 1996). Glycinin, on the other hand, is a hexamer with MW of about 320 to 375 kDa and with 5 major subunits, G1, G2, G3, G4, and G5. Each subunit consists of 2 polypeptide chains, an acidic chain (about 40 kDa) and a basic chain (about 20 kDa), joined by a single intra-chain disulphide bond. G1, G2, and G3 can be grouped as they share about 90% sequence

homologies. Similarity, G4 and G5 share about 90% sequence homologies. However, sequence homologies between these 2 groups (G1, G2, G3 and G4, G5) are only about 50% (Nielsen, 1996).

Whole soybean contains about 7-9% of protease inhibitors that are in the grain cotyledon (Brandon & Friedman, 2002; Esteves et al., 2010; Penha et al., 2007). There are two types of inhibitors: the Kunitz trypsin (KTI), and trypsin inhibitor and chymotrypsin Browman-Birk (BBI) (Miura et al., 2005; Monteiro et al., 2004; Monteiro et al., 2003). Although cultivars without protease inhibitors already exist (Barros et al., 2008), heat treatment alone is able to inactivate them, improving its nutritional value (Miura et al., 2005). These compounds can be removed with heat treatment of the grain and genetic breeding, without prejudice the quality of protein (Andrade et al., 2010; Machado et al., 2008).

Lectins are widely distributed in plants and animals and are present in greater quantities in grain legumes and grasses. They are proteins that do not interact with the immune system, but are able to recognize specific sites on molecules and bind reversibly to carbohydrates without altering covalent structure of the glycosidic links of sites (Silva & Silva, 2000). The lectins can interfere in the metabolism of animals and man. Some lectins, in contrast to other proteins, are resistant to enzymatic hydrolysis in the digestive tract and are recovered intact and still biologically active in animal feces. Since they are not inactivated by the action of enzymes of the digestive tract, these lectins can react with the gut epithelial cells causing complications to those who consume them. The lectins have the ability to bind to specific receptors in the intestinal epithelium, and this interaction interferes in the absorption and utilization of nutrients, causing poor performance in developing animals (Ritt, 2005). Soybean lectins are tetrameric protein with a molecular weight of 110–120 kDa constituted by four subunits of 30 kDa each. The natural content of lectin in soybean ranged from 2.10 to 5.68 mg/g (Gu et al., 2010; Paucar-Menacho et al., 2010), however this content can be reduced during the heat treatment. It has been observed that soybean thermally treated for 25 minutes showed no hemagglutinating activity and its analysis through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) confirmed the absence of lectins. However, activities of trypsin inhibition and urease were detected (Machado et al., 2008). Thus, lectins demonstrated larger sensibility to heating than trypsin inhibitors and urease.

Some proteins, such as lipoxygenase and urease, have enzyme activity. Lipoxygenases (LOX 1, 2 and 3) are enzymes belonging to a group of nonheme iron containing proteins widely distributed in soybean, representing up to 2% of soybean seed protein (Loiseau et al., 2001). They catalyze the oxidation of polyunsaturated fatty acids such as linoleic (18:2) and a-linolenic (18:3) to produce unsaturated fatty acid hydroperoxides (Liavonchanka & Feussner, 2006). Those hydroperoxides are further decomposed into volatile compounds such as hexanal aldehyde and other carbonyl compounds that are responsible for the beany flavors. With the genetic breeding, cultivars have been developed without lipoxygenase in order to decrease the production of beany flavor. The reduction of beany flavor also may occur in function of processing, since lipoxygenase is an enzyme thermolabile.

Urease is an enzyme thermolabile which correlates directly with levels of phytochemicals with antinutritional action presents in foods. Therefore, with the inactivation of the enzyme urease possibly some phytochemicals with anti-nutritional effect such as trypsin inhibitors and lipoxygenases, could be destroyed (Wiriyaumpaiwong et al., 2004). The analysis of urease is used as an indication that the thermal process was sufficient to inactivate phytochemicals with anti-nutritional effect present in soybean. The analysis of activity ureatic is a good indicator of adequate heat processing when it is between the cut points

from 0.01 to 0.30 (Mendes, 2004; Runho, 2009). It has been demonstrated that two soybeans cultivars presented urease activity of 2.39 and 2.86, however, after subjected to 15 minutes in an autoclave, the urease activity in this cultivars was reduced to 0.10 and 0.12 (Machado et al., 2008).

2.2 Other soybean components

Soybean contains other compounds, among them isoflavones, oxalate, phytic acid and bioactive peptides, which act in different ways in the human body. Isoflavones are a subclass of a large group called flavonoids. Because of its estrogenic activity, are also known as phytoestrogens (Cederroth & Nef, 2009). In soybean there are basically three types of isoflavones that are normally present in four different isoforms: glucosides (daidzin, genistin and glycitin); acetylglucosides (acetyldaidzin, acetylgenistin and acetylglycitin); malonylglucosides (malonyldadzin, malonylgenistin and malonylglycitin) and structure unconjugated aglycone (daidzein, genistein and glycitein) (Kao et al., 2007; Pereira & Oliveira, 2002). Soybean is the main source of isoflavones (genistein, daidzein and glycitein), and its concentration range from 1.45 to 4.59 mg/g (Table 17.4) Traditional fermented products (miso, tempeh) contain high concentrations of aglycone forms, genistein and daidzein, resulting from the enzymatic action of β-glucosidase, while non-fermented products such as dried beans, aqueous extracts of soybeans, soy flour and tofu, contain high concentrations of the glucosides, genistin and daidzin (Wang & Murphy, 1994).

The majority of species in the Leguminosae family contain oxalate in one or more of their tissues. The greater concentrations of oxalate in soybean are found in the mature seeds (Ilarslan et al., 2001; Nakata, 2003). The oxalate content of some plants is known to vary by genotype of soybean and possibly within cultivars under different growth conditions. Some Brazilian soybean cultivars, such as UFV-116 and OCEPAR-19 present oxalate content of 0.38 and 0.20 mg/100g, respectively (Esteves et al., 2010). Absorbed oxalate cannot be metabolized by humans and is excreted in the urine. In humans, high urinary oxalate increases the risk of calcium oxalate (CaOx) kidney stones because CaOx is poorly soluble in the urine. Saturated oxalate in solution binds to Ca to form crystals that aggregate, often becoming large enough to block the urinary stream (Massey et al., 1993).

The phytic acid, also known as phytate, inositol hexaphosphate (IP^6), myo-inositol, myo-inositol and inositolphosfate, is the main form of phosphorus stocks in soybean who is essential for germination of the grain (Domínguez et al., 2002) (Table 17.5).

During processing, storage, fermentation, germination and digestion of grains and seeds, IP^6 may be partly dephosphorylated to produce compounds as pentaphosphate (IP^5), tetraphosfato (IP^4), triphosphate (IP^3) and possibly inositol diphosphate (IP^2) and monophosphate (IP^1), by action of endogenous phytases (Chiplonkar & Agte, 2005; Domínguez et al., 2002) (Table 17.5). Only IP^6 and IP^5 exert a negative effect on the bioavailability of minerals. The other compounds formed such as IP^4 and IP^3 has low capacity to bind to the minerals or complexes formed are more soluble. Furthermore, these compounds may have antioxidant properties (Andrade et al., 2010; Domínguez et al., 2002; Martino et al., 2007).

During gastrointestinal digestion or food processing of proteins, small bioactive peptides can be released. Food-derived peptides commonly contain 2 to 9 amino acids (Kitts & Weiler, 2003). However, this range may be extended to 20 or more amino acid units (Korhonen & Pihlanto, 2003). Numerous biologically active peptides have been identified in foods, and may act as regulatory compounds with hormone-like activities. Soybean is a

potential source of bioactive peptides which play an important role in physiological activities, particularly those related to the prevention of chronic diseases. Peptides with other biological activities, such as opioid agonistic and antagonistic, antioxidative, anticancer, and immunomodulatory actions have also been identified in soybean (Wang & De Mejia, 2005).

Types	Isoforms	BRS 133 [a]	BRS 258 [b]	OCEPAR-19 [c]	UFV-116 [d]	UFVTN 105AP [e]
Glucosides	Daidzin	424.6	147.1	237.5	74.5	308.6
	Genistin	361.2	230.9	382.0	325.5	498.1
	Glycitin	10.4	60.2	59.0	70.0	106.4
Malonyl	Daidzin	131.6	575.6	211.0	59.5	395.1
	Genistin	1007.5	729.6	263.0	213.5	526.2
	Glycitin	419.6	226.4	27.5	33.0	38.5
Acetyl	Daidzin	nf	nf	32.0	nf	227.8
	Genistin	nf	nf	40.5	28.5	233.9
	Glycitin	nf	nf	nf	nf	99.9
Aglycones	Daidzein	109.8	76.9	112.0	26.5	143.3
	Genistein	144	154.1	124	86.5	34.5
	Glycitein	13.1	23.0	10.5	nf	9.1
Total Isoflavones		2621.8	2223.8	149.09	917.5	2621.4

nf: not found; a Soybean with low protein content and with hull; b Soybean with high protein content and with hull (Paucar-Menacho et al., 2010); c Conventional soybean with hull thermally treated 80 °C for 20 minutes; d Soybean without LOX 2 and 3, hull-less, thermally treated 80 °C for 20 minutes (Esteves et al., 2010); e Soybean with high protein content without LOXs, with hull, thermally treated 150 °C for 30 minutes (Andrade et al., 2010)

Table 17.4. Isoflavone composition of conventional and genetically improved soybeans (μg/g).

Compounds	Embrapa 48 [a]	BRS-213 [b]	BRS-155 [c]	UFVTN 105AP [d]
Phytate	0.78	1.75	1.40	1.11
IP^6	0.62	1.01	0.82	0.67
IP^5	0.12	0.43	0.43	0.27
IP^4	0.04	0.23	0.12	0.12
IP^3	nf	0.08	0.03	0.05

nf: not found; a Conventional soybean with hull, with hull, thermally treated 150 °C for 30 minutes; b Soybean without LOXs, with hull, thermally treated 150 °C for 30 minutes; c Soybean with reduced trypsin inhibitor content, with hull, thermally treated 150 °C for 30 minutes; d Soybean with high protein content without LOXs, with hull, thermally treated 150 °C for 30 minutes (Andrade et al., 2010)

Table 17.5. Phytate content of conventional and genetically improved soybeans (mg/100g).

2.3 Protein quality

Protein content among studied soybean cultivars (commercial, triple zero, not without LOX and KTI, without LOX and KTI, with and without LOX KTI with LOX and KTI) varies from 31.32 to 44.07 g /100g (Ciabotti et al., 2006; Felix & Canniatti-Brazaca, 2008; Martino et al., 2007; Mendes et al., 2007; Monteiro et al., 2004; Oliveira et al., 2007; Silva et al., 2006; Toledo et al., 2007). The biological utilization of soy protein is lower than that animal protein due to limitations in some essential amino acids and the presence of phytochemicals with anti-nutritional effects such as protease inhibitors (KTI and BBI) (Monteiro et al., 2004; Wang et al., 2000). These compounds inhibit the proteolytic enzymes and, consequently, reduce protein digestion leading to a decrease in weight gain and growth of animals (Miura et al., 2005; Monteiro et al., 2004). However, these compounds are thermolabile and can be inactivated by heat treatment.

With genetic breeding, new cultivars of soybean have been developed without KTI, lectins and lipoxygenase to improve their protein quality and flavor, making them more suitable for human consumption. Heat treatment is also a determinant of protein quality since it can change the structure of compounds with effect anti-nutritional in conventional soybean cultivars and thus inhibit their negative nutritional effect by increasing protein digestibility.

Some animal studies demonstrated that conventional soybean cultivars or genetically improved heat treated soybean showed values of Relative Protein Efficient Ration (R-PER) ranging from 30 to 77 and Relative Digestibility True (R-DV) from 82 to 90 (Carvalho, 2009; Monteiro et al., 2004). These variations in protein quality are related to genetic breeding of cultivars and processing, including heat output, binomial time: temperature, and peeling. *In vivo* studies showed that heat treatment of grains reduced the activity of trypsin inhibitor, urease and did not affect protein solubility (Andrade et al., 2010; Carvalho, 2009). In these studies, soybean with hulls presented higher nutritional quality, indicating that soy can be used in integral form as a source of quality protein, and in addition contributing to improving the intake of dietary fiber and minerals.

The soybean breeding programs have concentrated on developing more productive cultivars, and more recently, there is also a concern with quality characteristics such as content and composition of storage protein. These new cultivars need to be characterized from the nutritional point of view (Gonçalves et al., 2007), because the use of vegetable proteins influences the processing of the product, its amino acid profile and the presence of phytochemicals with potential anti-nutritional effect.

2.4 Mineral bioavailability

Besides the evaluation of protein quality, it is also of great importance the study of mineral content and its bioavailability in different soybean cultivars (Table 17.6). In the five cultivars presented in the following table, it stands out they contain relevant levels of calcium, iron and zinc, whose intake is marginal for some population groups in social vulnerability. It appears that the contents of some minerals, especially the so-called antioxidants, have not yet been quantified.

A comparative study of two genetically improved soybean cultivars (without LOX2 and LOX3) and a conventional cultivar, using an animal model and radioactive markers, revealed a lower iron and zinc bioavailability in the improved cultivar likely due to higher phytate content (Martino et al., 2007). However, in a study of a new cultivar, without lipoxygenase, a lower concentration of phytate and phytate:iron molar ratio and high protein content, using an animal model of depletion and repletion of hemoglobin,

demonstrated that the Relative Biological Values of hull and hull-less soybean flour were approximately 68% (Carvalho, 2009). These studies demonstrate the importance of combining the grain processing with the genetic breeding to promote the bioavailability of minerals. Stands out that soybean hull flours resulted in a good source of dietary fiber allowing elimination the initial hulling step in the industrial processing of soybean flour.

Minerals	BRS 133 [a]	BRS 258 [b]	OCEPAR-19 [c]	UFV-116 [d]	UFVTN 105AP [e]
Ca	290.4	335.4	208.0	171.0	177.9
Fe	22.3	26.3	40.0	40.0	nd
Zn	7.4	8.3	nd	nd	nd
K	nd	nd	1880.0	1740.1	1925.6
Na	nd	nd	2.4	2.5	3.2
P	524.4	1.2	nd	nd	nd
Mg	nd	nd	317.0	261.0	282.4
Cu	2.88	3.14	nd	nd	nd

nd: not determined; a Soybean with low protein content and with hull; b Soybean with high protein content and with hull (Paucar-Menacho et al., 2010); c Conventional soybean with hull thermally treated 80 °C for 20 minutes; d Soybean without LOX 2 and 3, hull-less, thermally treated 80 °C for 20 minutes (Esteves et al., 2010); e Soybean with high protein content without LOXs, with hull, thermally treated 150 °C for 30 minutes (Andrade et al., 2010)

Table 17.6. Mineral content of conventional and genetically improved soybeans (mg/100g in dry basis)

The heat treatment of soybean by removing the peel after soaking improved the availability of iron *in vitro* (Felix & Canniatti-Brazaca, 2008). A study using the femur ^{45}Ca uptake method, showed similar calcium bioavailability between genetically breed (without LOX 2 e LOX 3) and conventional cultivars thermally treated (80 °C for 20 minutes). In these study, the higher oxalate:calcium molar ratio and the higher content of oxalate and phytate in the improved cultivar did not affect calcium absorption (Martino et al., 2008). These observations are very important for individuals and populations that use soy diets since they demonstrate that the adequate heat treatment could be a good strategy for improving the minerals bioavailability of soybean. Also, the use of whole soybean, including the hull, is a good strategy to increase dietary fiber consumption in the human diet without impairing iron bioavailability.

3. Studies on functional properties of soybean

A food is defined as functional if, "in addition to providing nutrients, it contains bioactive compounds that act promoting health and/or reducing the risk of chronic diseases" (Diplock et al., 1999). In this context, soybean is considered a functional food with high potential. Experimental and epidemiological studies have provided evidence for a variety of health benefits derived from the consumption of soybean and soy food products.

3.1 Improvement in serum lipid profile

The consumption of soybean or its bioactive compounds has been reported to contribute significantly to reducing cholesterol and triglyceride levels in laboratory animals and humans (Esteves et al., 2011; Reynolds et al., 2006; Sirtori et al., 2007; Sirtori et al., 2009). The

beneficial effect on lipemia has been explained by the action of various constituents present in soybean which act via different mechanisms:

Amino acid protein profile. The high content of arginine and low methionine content (Wilson et al., 2007) can promote higher levels of nitric oxide (Gornik & Creager, 2004) and lower levels of homocysteine (Torres et al., 2006), favoring vessel relaxation and reduction of the risk of cardiovascular disease. There are suggestions that soy protein exerts hypolipidemic and antiatherogenic effects because the relationship between amino acids lysine/arginine alters the relationship insulin/glucagon which, when elevated, increases the risk of cardiovascular disease (Demonty et al., 2002; Torres et al., 2006).

Action of non-digestible peptides. The presence of peptides from soybean not digested in the gastrointestinal tract, has also explained their hypocholesterolemic effect. Peptides increase the fecal excretion of steroids, elevating both the hepatic synthesis of bile acids and receptors of LDL-c, as well as the uptake and oxidation of cholesterol in the liver (Belleville, 2002). Hypocholesterolemic effect has been found in peptide Leu-Pro-Tyr-Pro-Arg, a protein fragment derived from soybean glycinin, which reduced serum cholesterol in mice (–25.4% in total cholesterol and –30.6% in LDL-c) (Yoshikawa et al., 2000). This peptide has structural homology to enterostatin (Val-Pro-Asp-Pro-Arg). Although both have hypocholesterolemic activities, enterostatin did not increased excretion of bile acids in feces, suggesting that they may act by different mechanisms (Takenaka et al., 2004).

Bioactivity of isoflavones. Isoflavones, acting on the β-estrogen receptors present in the liver, lead to increased number of hepatic receptors of LDL-c, and favors the catabolism of cholesterol and β-oxidation of fatty acids (Dewell et al., 2002; Douglas et al., 2006; Torrezan et al., 2008). The antioxidant effect of isoflavones can act protecting the oxidation of copper-dependent LDL-c and favor a serum lipid profile associated with protection against atherosclerosis (Teixeira Damasceno et al., 2007).

Hypolipidemic action of storage proteins. β-conglycinin and glycinin showed hypolipidemic effect, through increased fecal excretion of fatty acid, induction of β-oxidation in the liver and decreased hepatic synthesis of fatty acids by down regulation of fatty acid synthesis and up regulation of liver VLDL receptors (Duranti et al., 2004; Fukui et al., 2004; Moriyama et al., 2004).

Hypolipidemic action of phytate. The phytate intake improves the serum lipid profile and reduces the hepatic lipid deposition in animal model of atherosclerosis (Lee et al., 2007). It is known that phytate exerts effect on hepatic glucose-regulating enzyme activities and reduce the risk of high fat diet-induced hyperglycemia (S.M. Kim et al., 2010).

The evidences commented above show that the beneficial effects of soybean on lipid profile improvement is mediated by various constituents present in the grain and thus the intake of whole soybean has potential to exert greater effect in comparison with supplementation of their components. This idea is supported by some evidence.

The hypocholesterolemic effect of soy consumption may be attributed not only to the presence of bioactive compounds (intrinsic effect) but also to replacing animal foods rich in saturated fat and cholesterol (extrinsic effect). A recent study estimated the intrinsic and extrinsic effects of soybean to reduce cholesterol and verified that the combined effects are important to reduce cholesterol in approximately 4% (Jenkins et al., 2010). In fact, the synergistic action of amino acids and isoflavones in improving lipid metabolism and activation of the LDL-c has been demonstrated (Bertipaglia de Santana et al., 2008).

A study performed with a group of postmenopausal women found no significant hypocholesterolemic effect of consuming soy milk for 4 weeks, despite good adherence of the participants (Beavers et al., 2010). The authors discussed that the baseline cholesterol status, supplement type, dosage, and duration, as well as dietary control, are all potential confounding factors that have been identified as determinants of the conflicting results.

3.2 Protection against oxidative stress

Isoflavones and its metabolites formed by bacterial action in the intestinal lumen, have structural determinants to exert strong antioxidant activity (Arora et al., 1998). Studies in humans have shown that the antioxidant effect of soy products. The intake for 2 weeks, of soy bars containing 7 and 12 milligrams of daidzein and genistein, respectively, increased the resistance of oxidation of isolated LDL (Tikkanen et al., 1998), although the ingestion of a product isolated soy containing protein and isoflavones did not show antioxidant effects of plasma and LDL-c (Steinberg et al., 2003). The antioxidant effects are mediated by direct elimination of free oxygen radicals by isoflavones (Teixeira Damasceno et al., 2007) and by increasing the concentrations of antioxidant enzymes, improving the body's antioxidant defense (Engelman et al., 2005; Rios et al., 2008). It is believed that a diet rich in isoflavones causes LDL-c to become resistant to peroxidation, that is responsible for the generation of a cascade of events that produces atherosclerotic plaques, making the consumption of soy a protective factor against atherosclerosis (Mateos-Aparicio et al., 2008; Sacks et al., 2006).

Several amino acids that composed bioactive peptides, such as Tyr, Met, His, Lys, and Trp, have antioxidant properties. Saito et al. (2003) evaluated 108 peptides containing either 2 His or Tyr residues and 114 peptides structurally related to Pro-His-His. They observed that the tripeptides containing Trp or Tyr residues at the C-terminus had strong radical scavenging activities, but very weak peroxynitrite scavenging activity. They also found that the antioxidative peptides may exert strong synergistic effects with some other antioxidants, such as phenolic compounds.

Phytates present in soybeans also have a positive effect on health, exerting a protective action against oxidative stress (Lee et al., 2007; Martinez Dominguez et al., 2002). Phytate binds to iron, suppressing the catalysis of this ion in oxidative reactions, which favors the preservation of the seed. Through this mechanism, there is inhibition of lipid peroxidation and consequent acceleration of the autoxidation of ferrous ions to ferric ions, with the formation of ferric chelates (Quirrenbach et al., 2009).

It is known that the protective effect of soybean against oxidative stress involves the action of several bioactives compounds and once again, it is reasonable to state that soy intake may offer greater antioxidant protection mediated by the synergistic effect of several components: isoflavones and their metabolites generated in the intestinal lumen, peptides and phytic acid. In addition, the action of other antioxidants such as vitamin E and minerals like copper, iron, zinc and manganese, although not present in high concentrations in soybean, help in the antioxidant protection of the body. All these factors suggest that eating whole soybean would be more effective than the intake of their isolated components.

3.3 Effect on glycemic control

Soybean has shown a potential to exert physiological effects through mechanisms that stimulate insulin production and decrease the glycemic index of diet, suggesting the possibility of their health claim to act in the prevention and control of diabetes and obesity and its metabolic complications.

The effect of isoflavones in reducing blood glucose may be explained by the stimulus by genistein to pancreatic β cells which increases insulin production and consequently also the glucose uptake by cells (Esteves et al., 2011). These effect has been demonstrated in studies with animals that received genistein (Jonas et al., 1995) and also in a *in vitro* study, which used adipocytes and insulinoma cells, showing that bioactive compounds formed from isoflavonoids and soy peptides during the fermentation process to produce meju, activated signaling cascades that stimulated insulin release (Kwon et al., 2011).
The dietary fiber contained in soybeans is effective in controlling blood glucose (Penha et al., 2007). It can reduce the rate of emptying of the digestive tract, increase the rate of peristalsis of the bowel and slow down the rate of glucose uptake and therefore its importance in the control of blood glucose (Takahashi et al., 2003). In a clinical study, the intake of soybean fiber resulted in decreased the blood glucose levels after ingestion of a glucose solution. Thus, soy fibers may adsorb glucose in the intestine and slow their release for absorption (Messina et al., 1992). A cake made with whole soybean flour showed low glucose and insulin indexes in study with a group of 20 individuals (Oku et al., 2009).
Phytate also shows potential in controlling diabetes, as demonstrated by its hypoglycemic effect in diabetic mice (S.M. Kim et al., 2010; Lee et al., 2007). Lee (2006) observed that consumption of diets containing phytate reduced the high blood glucose levels and glycated hemoglobin in diabetic mice.
In summary, various bioactive soy compounds may act on mechanisms that improve the metabolism of carbohydrates, working in reducing the risk of diabetes and metabolic complications of obesity. As an adjunct to control diabetes, it stands out the importance of dietary fiber to improve the glycemic index of diet as well as the antioxidant components to minimize the oxidative stress that is present in this disease. However, in diabetic individuals, studies are needed to prove whether pancreatic β cells of these individuals are responsive to stimulation of soy isoflavones to increase insulin release.

3.4 Antihypertensive effects and protection of the endothelium

Antihypertensive soybean peptides are the most extensively studied. Peptides as Tyr-Val-Val-Phe-Lys, Pro-Asn-Asn-Lys-Pro-Phe-Gln, Asn-Trp-Gly-Pro-Leu-Val, Ile-Pro-Pro-Gly-Val-Pro-Tyr-Trp-Thr, Thr-Pro-Arg-Val-Phe, Ile-Ala, Tyr-Leu-Ala-Gly-Asn-Gln, Phe-Phe-Leu, and Ile-Tyr-Leu-Leu show their activity by inhibiting angiotensin-converting enzyme-ACE, which is a nonspecific dipeptidyl carboxypeptidase associated with the regulation of blood pressure by modulating the rennin-angiotensin system (Chen et al., 2004).
Some peptides such Val-Pro-Pro and Ile-Pro-Pro, released during the fermentation of soybean meal, added of casein to produce of miso, showed hypertensive action by inhibiting ACE (Inoue et al., 2009).
Black soybean extract showed potent inhibitory activity of the platelet aggregation induced by a collagen *in vitro* model, using platelets isolated from humans (K. Kim et al., 2010).
Animal studies suggest that soybean may exert a protective effect against cardiovascular disease when the fetus is exposed to isoflavones via maternal diet (Bonacasa et al., 2011).

3.5 Chemopreventive effects

Epidemiologic studies have demonstrated the direct association between the protection of breast cancer and soy intake (Dong & Qin, 2011). This effect has been attributed to the bioactivity of isoflavones and their estrogenic effect. However, intervention studies do not always demonstrate benefits of supplementation of isolated soy isoflavones in reducing the

risk of breast cancer. In a study with 406 premenopausal women, who received 80 or 120 mg/day of isoflavones or placebo, during two years, did not showed modification in mammographic density, which is a marker of risk of breast cancer. However, there was a significant reduction of the marker over time (Maskarinec et al., 2009). There is evidence that early exposure to the bioactive compounds of soybean, via maternal diet, may alter the paracrine signaling in adipocytes of breast tissue and increase the differentiation of epithelial tissue, with implications in prevention of breast cancer associated with obesity (Su et al., 2009).

In prostate cancer, the chemopreventive effect of the soybean mediated by mechanisms of modulations of gene expression was evidenced in a study in which isoflavones derived from soybean cake exhibited antiproliferative effects on prostate cancer cells, decreasing the expression of cyclin B1 (Wang et al., 2009). A recent study investigated the effect of administration of soy isoflavones as an adjunct in hormone therapy and verified that for postmenopausal women there was lower risk of cancer recurrence, for a monitoring period of 5.1 years (Kang et al., 2010).

Although components of soybean have demonstrated potential to act as antiproliferative agents, their effect in reducing cancer risk is still controversial. It is possible that the metabolic imprint is the main mechanism to explain the inverse association between cancer and soy intake. In fact, it was found that for breast cancer, the association is present for the Asian population and not for the western population (Dong & Qin, 2011). For this reason, the habit of soy intake by the population through successive generations could be a useful strategy to obtain the chemopreventive benefits of the bioactive compounds in soybeans.

3.6 Effects on obesity and metabolic syndrome

Several anorectic peptides have been already identified to exert antiobesity activity through decreasing food intake, fat and lean body mass, and body weight (Challis et al., 2004). For example, Leu-Pro-Tyr-Pro-Arg, a peptide from soybean glycinin A5A4B3 subunit (Takenaka et al., 2004) and Pro-Gly-Pro have been found to have anorectic activities.

The consumption of soy protein may also lead to low hepatic deposits of triglycerides (Ascencio et al., 2004), reducing the risk of hepatic lipotoxicity and therefore of steatosis (Tovar et al., 2005). In animals, soybean protein modulated the expression of genes that regulated lipid metabolism and thyroid hormone, promoting weight loss (Simmen et al., 2010). These mechanisms of action may attenuate metabolic changes that occur in obesity, which are related to insulin resistance (Oh et al., 2011).

Soy protein isolate was found to lower plasma triglycerides, increase adiponectin (Nagasawa et al., 2003), accelerate lipid metabolism, and decrease body fat in obese rats and mice (Aoyama et al., 2000).

Thus, there is enough scientific evidence to motivate soybean research for exploring their potential for its use as a coadjuvant in the treatment of obesity and its complications.

3.7 Other diverse effects

Soybean peptides with immunomodulatory activities have also been identified from soybean protein hydrolysates. For example, immunostimulating peptide preventing the alopecia induced by cancer chemotherapy has been isolated from an enzymatic digest of soybean protein (Tsuruki et al., 2005; Tsuruki & Yoshikawa, 2004). Recently, it was shown that a lectin of 48-kDa purified soybean black Koreana inhibited the activity of reverse transcriptase of HIV-I with an IC (50) of 1.38 micromolar (Fei Fang et al., 2010).

Although there are suggestions of the benefits of soy on bone health, clinical studies do not always prove such effects (Alekel et al., 2010; Kenny et al., 2009). A prospective cohort study of the Chinese population showed that the intake of soybean and its products was associated with protection against hip fracture in women but no association in men (Koh et al., 2009). There are doubts if the intake of soybean can provide superior beneficial effect on bone health (Reinwald & Weaver, 2010).

Prebiotic effect with improvement in the balance of minerals especially calcium and magnesium was demonstrated in rats, which consumed the 120mg/day/animal of galactooligossacarides contained in soybean serum obtained from by-product of the manufacturing of tofu (Tenorio et al., 2010).

Soy isoflavones derived from soybean cake exhibited effect in inhibiting apoptosis and inflammation induced by UVB in *in vitro* and *in vivo* mice models, suggesting bioactivity of soy components against aging (Chiu et al., 2009).

In animal models of neurotoxicity induced by beta-amyloid, neuroprotective effect of soy isoflavones were demonstrated, indicating the possible benefit of bioactive compounds as co adjuvant in disease (Ma et al., 2009).

The benefits of soy isoflavones as an alternative to estrogen therapy for the treatment of menopausal symptoms are still uncertain (Steinberg et al., 2010). A recent review on the subject states that the number of studies is limited to assess the effectiveness of soy foods in alleviating vasomotor and urogenital symptoms (Levis & Griebeler, 2010). Study on animals showed that the soybean extract rich in isoflavones increased the epithelium and vaginal collagen (Carbonel et al., 2011).

4. Conclusion

Soybean is a legume with great potential for use as human food, offering especially protein, lipids, fiber, minerals and bioactive compounds. Studies have shown their nutritional value and health properties, with potential of reducing the risk of non-communicable diseases. Many studies focus on the use of isolated soy constituents, especially isoflavones, bioactive peptides and protein. Therefore, it is necessary to intensify research that allows the use of whole soybeans for the supply of nutrients and bioactive compounds and to evaluate the synergistic effect of these. Studies with different processing techniques should continue, since they can optimize the nutritional and functional quality of conventional soybeans or genetically improved varieties.

Health policy in Western countries should be implemented to encourage increased consumption of whole soybean, such as a partial substitute for refined ingredients.

5. References

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22

Polyamines - The Principal Candidate Substance of Soybean-Induced Health

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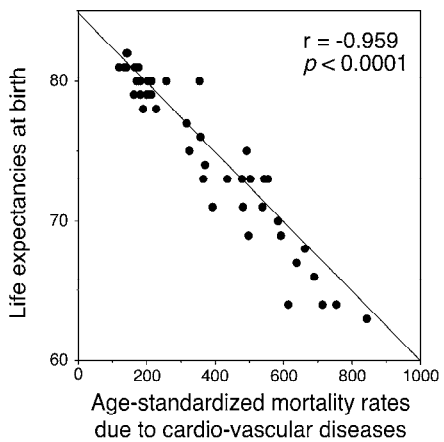
1. Introduction

In countries with sufficient medical and public health resources, cardiovascular diseases (CVDs), cancers, and other chronic metabolic diseases are major killers. The incidence of these diseases increases with aging and, therefore, they are called age-associated diseases. Importantly, the rate of mortality from these diseases ranges widely, even in countries with similar social and economic conditions. And, life expectancy at birth is greatly affected by the incidence of age-associated diseases, especially CVDs (Figure 1). The close inverse correlation between these two variables indicates that prevention of chronic age-associated diseases is the most effective way to improve health and prolong lifespan.



Close inverse correlation was found between life expectancy at birth in 2006 and age-standardized mortality rates due to CVDs per 100,000 population in 2002 (available date close to 2006) in 49 European and Western countries.
Data are collected from World Health Organization (WHO).

Fig. 1. The relationship between CVD mortality and longevity.

Variation in the rates of mortality from age-associated diseases is considered to be largely due to lifestyle differences, especially differences in eating pattern. There is clear epidemiological evidence indicating that soybeans have beneficial effects on health and delay the onset of age-associated diseases, especially CVDs . However, in spite of numerous and extensive studies, the substance(s) in soybeans and mechanism(s) by which soybeans improve health are still obscured.
In our studies, we have shown that polyamines contained abundantly in soybeans inhibit the progression of age-associated pathological changes and prolong mouse lifespan.

Importantly, we found that the favorable effects of food polyamines on murine health and longevity were not due to differences in food intake or changes in body weight, but rather considered due to the polyamine-mediated suppression of inflammatory mediators (Soda et al., 2005, 2009a, 2009b).
In this chapter, I discuss the possible contributions to human health of polyamines present in dietary soybeans.

2. Foods that prolong life

Epidemiologic studies have suggested the relationship between intake of several foods and prolonged longevity, and numerous interventional studies have been done to test the effect of food on human health. The beneficial effects of soybean consumption on human health are well recognized. For instance, epidemiological studies have shown that consumption of soybean products is closely correlated with lower incidence of CVDs, type 2 diabetes, and decreased risk of certain types of cancers such as breast and prostate cancers as well as with better bone health and relief of menopausal symptoms. Human and animal intervention trials have also shown that consumption of soybean products has beneficial impacts on the risk factors for cardiovascular diseases (Anthony et al., 1998; Lin et al., 2004).
At one time, isoflavones were considered one of the most promising health-promoting nutritional factors in soybeans. Isoflavones are polyphenols found in many plants. They are antioxidants (i.e., chemicals that inhibit the transfer of electrons from a substance to an oxidizing agent). Because oxidative stress is involved in many age-associated diseases, isoflavones were previously considered to help decrease the incidence of age-associated diseases and help increase longevity. However, recent studies in animals as well as humans have shown that isoflavones are not responsible for the favorable effects of soy products, such as improvement of lipid metabolism, blood pressure, etc(Balmir et al., 1996; Sacks et al., 2006; Song et al., 2003). The US Food and Drug Administration (FDA) and the American Heart Association (AHA) Nutrition Committee have concluded from the findings of numerous intervention studies that isoflavones have no efficacy for preventing or treating age-associated diseases, despite the demonstration that consumption of soybeans and soy products does have efficacy (US Food and Drug Administration (FDA), 1999; Sacks et al., 2006).
Similarly, the effects of fruit polyphenols are not established yet. Red wine consumption is purported to underlie the "French paradox", which refers to the relatively lower mortality from CVDs in France and among individuals who consume much more wine, especially red wine, despite their high intake of animal products rich in saturated fat. A large number of *in vitro* studies have shown the favorable effects of fruit polyphenols on human health. Moreover, animal experiments have shown many favorable effects of increased intake of polyphenols, such as decrease in carcinogenesis and attenuation of cerebro- and cardiovascular deterioration. However, in most of these experiments, the concentrations vastly exceed those physiologically achievable by diet, and therefore it is considered to be impossible for humans to benefit from fruit intake. The evidence from human intervention studies is limited, mostly inconsistent, and inconclusive. Most studies fail to show any effects leading to the prevention of CVDs. In addition, the strongest anti-oxidants, vitamin E and beta-carotene, even increased mortality, including mortality from CVDs (Miller et al., 2005; Vivekananthan et al., 2003).
These findings suggest that substance(s) other than isoflavones or fruit polyphenols help prevent age-associated diseases. Consequently, our focus since 2005 has been on the

polyamines in soybeans. In 2005, we first noticed that they have biological activities capable of delaying the onset of age-associated diseases (Soda et al., 2005).

3. What are polyamines

Polyamines (spermine, spermidine, and putrescine) are organic compounds having two or more primary amino groups $-NH_2$. Polyamines are synthesized from arginine and contained in almost all cells (Figure 2). They are indispensable for cell growth and differentiation, and are involved in diverse functions such as DNA synthesis and stability, regulation of transcription, ion channel regulation, and protein phosphorylation. With aging, the enzymatic activities required for polyamine synthesis decrease gradually.
Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase ((3)) but not spermine/spermidine synthases (which catalyze the synthesis of spermine from spermidine and spermidine from putrescine) ((1) and (2) in Figure 2) are rate-limiting enzymes and under regulatory control. Intracellular *de novo* synthesis and concentrations of polyamines in cells and tissues, especially those of spermine and spermidine, decrease with aging (Das & Kanungo, 1982).

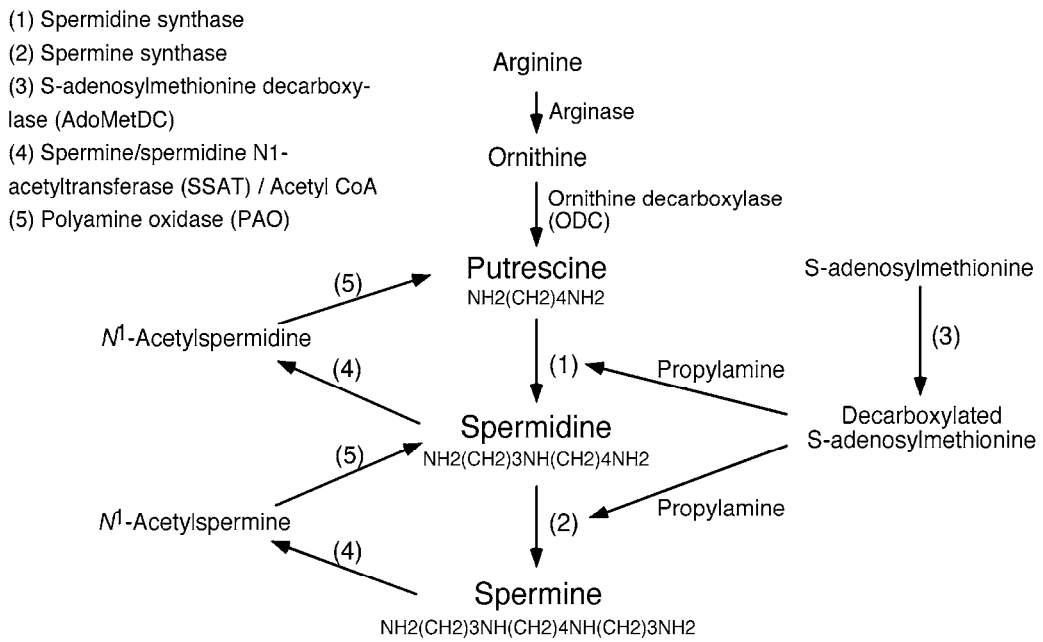


Fig. 2. Polyamine biosynthesis and catabolism.

In addition to polyamines from *de novo* synthesis, polyamines are obtained from the environment (Bardocz, 1990a; Colombatto et al., 1990). White blood cells and red blood cells, because they circulate in all tissues and organs, should reflect, at least somewhat, the amount of polyamines in the body. However, the interindividual variation in polyamine concentration in human blood cells ranges widely and does not appear to decrease in an age-related manner (Elworthy & Hitchcock, 1989; Soda et al., 2005). Although the factors underlying this difference are not fully understood, one is considered to be the amount of

polyamine intake from food and/or the diversity of intestinal microflora (which serve as a source of endogenous polyamines). In fact, increasing polyamine intake increases blood polyamine concentrations in animals and humans (Soda et al., 2009a, 2009b).

4. Food is an important source of polyamines

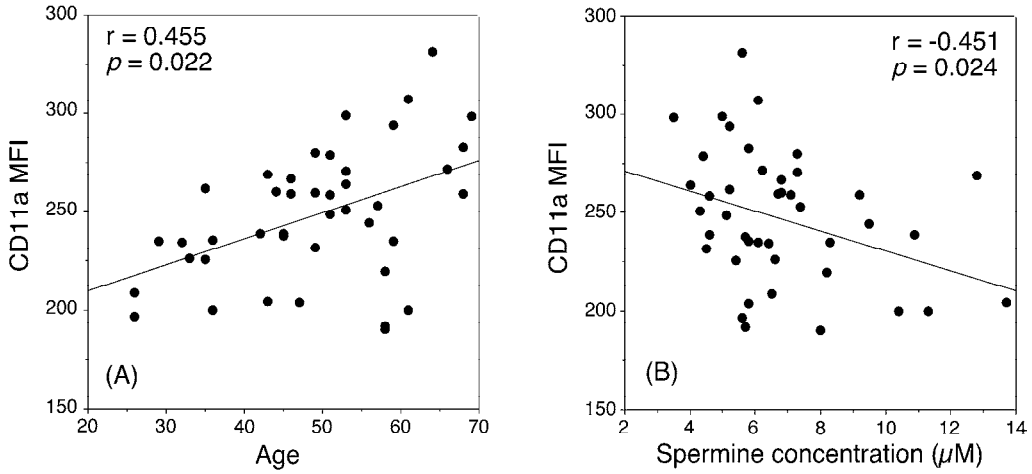
Because most foods originate from microorganisms, plants, or animals, almost all foods contain polyamines, but in widely varying concentrations. Of all foods, beans, especially soybeans, have the highest amount of the polyamines, especially spermine and spermidine. Spermine and spermidine are not enzymatically degraded in the alimentary tract, and they are absorbed quickly from the intestinal lumen and distributed to all organs and tissues (Bardocz, 1990b; Uda et al., 2003). In animals, polyamine restriction in conjunction with elimination of intestinal bacteria by antibiotics successfully decreases blood polyamine levels, suggesting the importance of polyamines from foods and microbes in the intestinal lumen.

However, short-term ingestion of polyamine rich foods fails to increase blood polyamine levels (Brodal et al., 1999; Soda et al., 2009a, 2009b). Cells contain polyamines, and erythrocytes and leukocytes contain almost all the polyamines in blood. Intracellular polyamine concentration is regulated very tightly by mechanisms such as synthesis, degradation, and transmembrane transport; therefore polyamine concentration can be stable. However, long-term intake of polyamine-rich chow gradually increases blood polyamine levels in mice, and continuous intake of the polyamine-rich food called "Natto" (a traditional Japanese dish made from fermented soybeans) gradually increases blood polyamine concentration in humans (Soda et al., 2009a). The slow increase in blood polyamine concentrations after long term increased polyamine intake suggests that the process is an adaptative response to polyamine influx from extracellular sources.

5. Polyamines as anti-inflammatory substances

We have found that polyamnines, especiaqlly spermine and spermidine, suppress the expression of lymphocyte function-associated antigen-1 (LFA-1) on the surface of peripheral blood mononuclear cells (PBMCs), i.e., monocytes and lymphocytes in humans (Figure 3), and inhibit LFA-1 associated cellular functions (Kano et al., 2007; Soda et al., 2005). LFA-1 consists of two membrane molecules, CD11a and CD18. Among the many membrane molecules (i.e., membrane proteins) whose expression we studied on human PBMCs, only CD11a and CD18 were suppressed by spermine and spermidine. More than 300 membrane proteins with independent cellular functions were identified. LFA-1 is crucial for immune cell activation. Without this protein, immune cells are not activated and pathogens cannot be eliminated. In addition, spermine and spermidine have been shown to suppress the production of pro-inflammatory cytokines, both *in vitro* and *in vivo*. (M. Zhang et al., 1997; Zhu et al., 2009)

The suppressions of LFA-1 expression and of cytokine production was not due to decreases in cell activity and viability. Polyamines even enhance the mitogenic activities of immune cells responding to lectins, such as concanavalin A and phytohaemagglutinin (Soda et al., 2005). Both LFA-1 (a leukocyte cell surface protein) and pro-inflammatory cytokines promote systemic inflammation. Thus, polyamines, especially spermine and spermidine, are natural anti-inflammatory substances.



Blood samples were collected from 42 healthy male volunteers. Concentration of polyamines in whole blood was measured by HPLC (High Performance Liquid Chromatography), and the expression of CD11a on peripheral mononuclear cells (lymphocytes and monocytes) was determined by flow cytometry.
(A) The positive correlation between age and CD11a MFI (mean fluorescence intensity).
(B) The inverse correlation between CD11a MFI and blood spermine concentration.

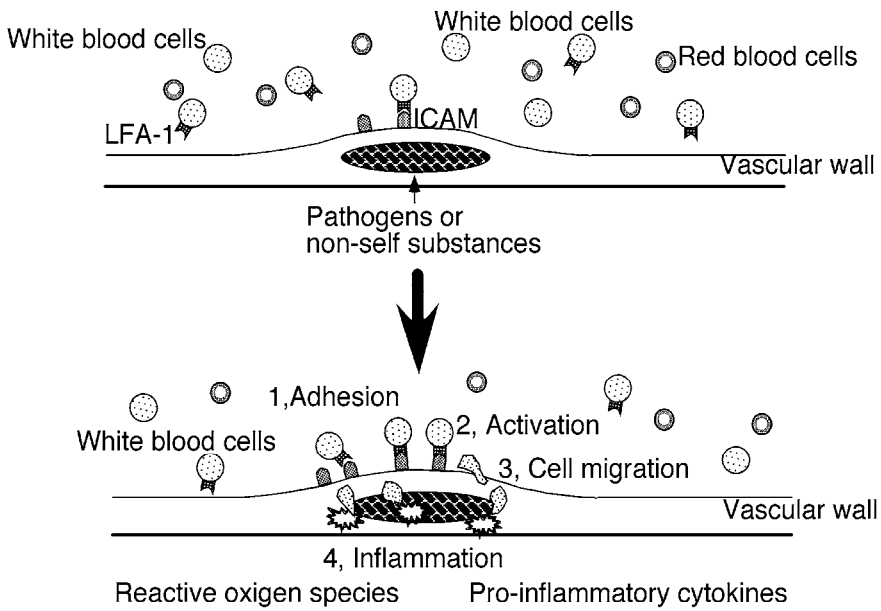
Fig. 3. Polyamine inhibits CD11a (i.e. LFA-1) expression.

6. The role of inflammation in the pathogenesis and progression of age-associated diseases

Inflammation is a part of the biological response to harmful stimuli, such as pathogens, damaged cells, or irritants. Redness, swelling, heat, and pain are symptoms and signs of acute inflammation. Inflammation is the result of transmigration of immune cells from the blood into the inflamed site. Initially, leukocytes circulating in the bloodstream adhere to endothelial cells lining the blood vessels. Leukocyte adhesion is mediated by the binding of molecules, such as LFA-1 on the surface of immune cells and intercellular adhesion molecules (ICAMs) on the surface of endothelial cells. The adhesion stimulates immune cell activation, and the activated cells migrate to the sites of harmful stimuli and produce chemical substances, such as pro-inflammatory cytokines, to localize and eradicate such stimuli (Figure 4). Therefore, the primary objective of inflammation is to protect organisms from harmful stimuli.

Recent investigations have shown that chronic inflammation is involved in the pathogenesis of many age-associated diseases. Unlike acute inflammation, chronic inflammation manifests few symptoms and signs. Increased pro-inflammatory activities, such as age-dependent increase in the expression of LFA-1 on human PBMCs (Figure 3-A), are considered to be a factor accelerating the progression of age-associated pathologies, and "inflamm-aging" is a term coined to express the close association between inflammation and aging. For example, atherosclerosis, the underlying cause of CVDs, was formerly considered

to be merely a lipid storage disease. However, a fundamental role for inflammation in all stages of atherosclerosis and, ultimately, in thrombotic complications leading to CVD events has recently been established (Granger et al., 2004). Therefore, increased chronic inflammation in conjunction with impaired lipid metabolism is the principal factor contributing to initiation and progression of atherosclerosis (Figure 4).
In addition, an association of chronic inflammation with several other age-associated metabolic disorders has been shown. For example, obesity is not merely a problem related to increased fat storage but also involves increased production of inflammatory mediators in fat tissues. Therefore, strategies for preventing or treating age-associated diseases must include suppression of inflammation.



1. LFA-1 is an adhesion molecule on the leukocyte surface that exclusively recognizes the intercellular adhesion molecule (ICAM).
2. Binding between LFA-1 and ICAM initiate activation of immune cells.
3. The activated immune cells transmigrate into the site where pathogens or non-self substances exist.
4. Interaction between migrated immune cells and pathogens provokes the production of chemical substances, resulting in inflammation.

In the pathogenesis of atherosclerosis, monocytes in the intima are transformed to macrophages, which take up oxidized-low density lipoprotein (LDL) to generate lipid-laden macrophages, also known as foam cells. Chronic inflammation disrupts endothelial and muscle cells and destroys the normal structure of the vessel wall.

Fig. 4. Role of inflammation in the pathogenesis of atherosclerosis.

7. Food, food nutrients, and chronic inflammation

Several foods, including soybeans, are confirmed to have favorable effects on human health. Foods and food components that inhibit the progression of age-associated diseases are shown to have anti-inflammatory properties. Examples are soybeans, mono-unsaturated fatty acids in olive oil, n-3 unsaturated fatty acids in fish oil, and components of the "Mediterranean diet" (MD) (Table 1).

• High olive oil consumption
• High consumption of legumes
• High consumption of fruits
• High consumption of vegetables
• Moderate consumption of dairy products (mostly as cheese and yogurt)
• Moderate to high consumption of fish plus low consumption of meat and meat products
• Moderate wine consumption
• High consumption of unrefined cereals
• Less sweets

Table 1. The principal aspects of a Mediterranean diet

The benefit of dietary soybeans has been attributed primarily to reducing serum cholesterol levels, especially low density lipoprotein (LDL) cholesterol level, which if high promotes atherosclerosis in humans and animals (Jenkins et al., 2002; McVeigh et al., 2006; Reynolds et al., 2006). However, studies have shown that atherosclerotic lesions are reduced in atherosclerosis-susceptible apolipoprotein E knockout (apoE-/-) mice fed a soy-containing diet despite unchanged serum lipid levels (Adams et al., 2002; Nagarajan et al., 2008). Moreover, intervention studies in humans have shown that the favorable effects of soy protein on vascular function are independent of lipid and antioxidant effects (Steinberg et al., 2003). Soy protein has anti-inflammatory properties, and the mechanisms of soy-mediated inhibition of atherosclerosis are considered to be anti-inflammatory (Nagarajan et al., 2008). The importance of isoflavones has been disputed because the favorable effects of soy can be elicited by soy protein devoid of isoflavones (Balmir et al., 1996; Nagarajan et al., 2008; Song et al., 2003).

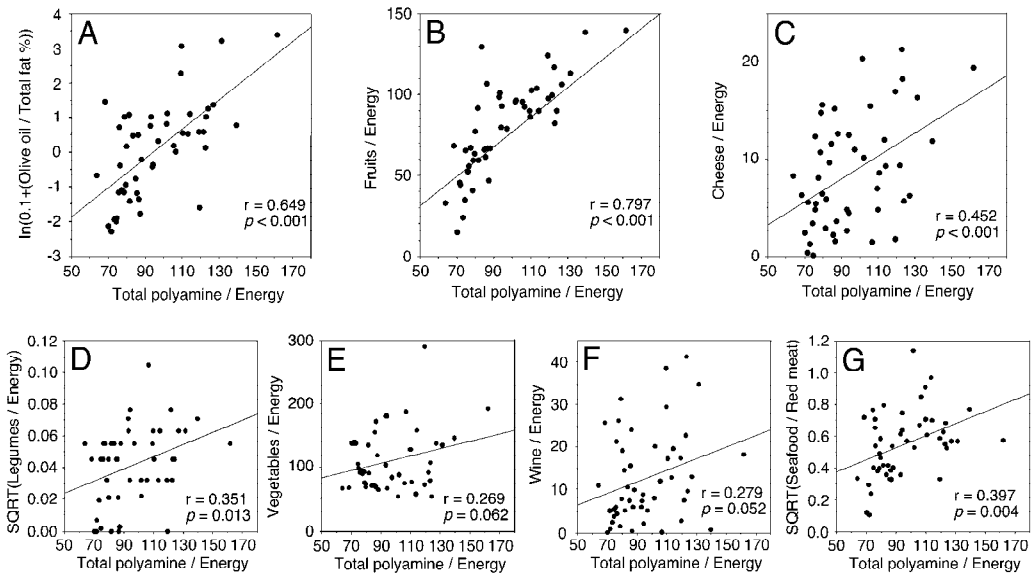
The effects of various fatty acids on inflammation are well established. Recent investigations have linked the health benefit of olive oil and fish oil to anti-inflammatory effects. Mono-unsaturated fatty acids in olive oil and n-3 polyunsaturated fatty acids in fish oil inhibit prostaglandin-mediated inflammation (Lee et al., 1985; Mehta et al., 1988).

The dietary patterns of several countries in the Mediterranean Basin are similar (Table 1) (Trichopoulou & Lagiou, 1997). In spite of the relatively high amount of fat consumed by individuals in Mediterranean countries, rates of CVDs are far lower in these individuals than in those living in the other European countries and the United States, where similar levels of fat are consumed. In addition to many epidemiological studies, several intervention studies have shown that the Mediterranian diet (MD) is closely associated with prolonged lifespan and decreased mortality from chronic age-associated diseases (Bamia et al., 2007; de Lorgeril et al., 1999; Tunstall-Pedoe et al., 1999). The mechanism underlying this favorable

effect on health has not been fully elucidated. However, the MD has been shown to attenuate inflammation (Esposito et al., 2004; Schulze et al., 2005).
On the other hand, food components that enhance inflammation seem to have deleterious effect on health. Examples are saturated fatty acids from animal fat and trans-fatty acids. Increased intake of these fatty acids is associated with increased incidence of age-associated diseases, especially CVDs (Forman & Bulwer, 2006; Howard et al., 2006).

8. Epidemiological evidence showing a soybean diet improves human health

The results of basic and animal studies have indicated that polyamines are potential candidate molecules responsible for the beneficial effects of soybeans on human health. Epidemiological evidence other than that indicating an association between soybean intake and human health provides further support for the role of polyamines in human health.
The MD pattern has attracted considerable interest because of its association with a lower mortality from all causes in prospective studies (Lasheras et al., 2000; Trichopoulou et al., 2003) and also with lower incidence of coronary events in recent case–control studies (Martinez-Gonzalez et al., 2002; Panagiotakos et al., 2002). Although there is no one MD, the principal components of the MD pattern are shown in Table 1. Health benefits of the MD can be partially explained by the relatively high intake of mono- and n-3 poly-unsaturated fatty acids from olive oil and fish oil. The MD does not include many animal meats rich in saturated fatty acids and emphasizes foods rich in n-3 polyunsaturated fatty acids and monounsaturated fatty acids, including legumes, fresh fruits and vegetables, seafood, and olive oil. However, they do not sufficiently explain the beneficial effects of the MD. The "French paradox" shows that mortality from CVDs can be lower despite the high intake of animal products rich in saturated fatty acid.
To evaluate the association between the MD and increased polyamine intake, the amounts of food in 49 European and other Western countries were collected from the United Nations' database, and the amount of food polyamine was estimated using polyamine concentrations in foods from published sources. For all 49 countries and for foods such as olive oil (Figure 5-A), fruits (B), and cheese (C), the ratios of the amounts of these foods to total calories were all positively associated with the amount of polyamines per total food energy supplied (Figure 5). The amount of legumes (beans and nuts) per energy unit (1000 kcal/capita/day) (Figurre 5-D), vegetables per energy unit (E), wine per energy unit (F), and the amount of seafood relative to red meat (G) tended to have a positive association with the amount of polyamines per energy unit, while several foods in the non-MD group tended to have no or negative association. Examples are the amounts of whole milk per energy unit ($r = -0.291$, $p = 0.043$), other alcohols per energy unit ($r = -0.181$, $p = 0.215$), and potato per energy unit ($r = -0.174$, $p = 0.234$) (Binh et al., 2011).
Because polyamines content of wine is low, the positive correlation between the amount of wine and amount of polyamines can be interpreted as indicating that consumption of wine tends to accompany consumption of foods rich in polyamines. Many epidemiological studies have shown that milk consumption is positively correlated with CDV incidence, however several studies have indicated that such a positive correlation is attenuated or lost when the relationship is to dairy foods (including cheese) (Tholstrup, 2006). Epidemiological studies indicate that individuals who prefer whole milk prefer foods containing less polyamines and those who prefer cheese prefer foods rich in polymines.



Energy = 1000 kcal/capita/day; Olive oil, and Wine = mL/capita/day; Fruit, Cheese, Legumes, Vegetables, Seafood, and Red meat = g/capita/day.

Total polyamines (Putrescine + Spermidine + Spermine) = μmol/capita/day.

Some data were transformed to approximate a normal distribution. ln = natural logarithm, SQRT = Square root.

All data are obtained from the statistical database of the Food and Agriculture Organization (FAOSTAT) of the United Nations.

Fig. 5. Mediterranean diets and the amount of polyamine

In addition, two other elements of the MD in Table 1 (high consumption of unrefined cereals and less sweets) have a relationship with the amount of polyamines. Polyamines are abundant in cereal germ and bran; however, refined cereals and Western sweets (a combination of refined flour, sugar, milk, egg, and butter) contain only a small amount of polyamines (Cipolla et al., 2007; Nishimura et al., 2006).

9. Other background information concerning the health effects of polyamine

In addition to anti-inflammation, dietary polyamines play a role in the prevention of age-associated diseases, especially CVDs. Endothelium covers the inner surface of the vessel wall, and the status of endothelial function can be predictive of future cardiovascular events. Endothelium uses nitric oxide (NO) to signal the surrounding vascular smooth muscle to relax, thus resulting in vasodilation and increasing blood flow. Decreased bioavailability of NO is involved in the pathogenesis of various disorders (John & Schmieder, 2003). Conversely, increased NO synthesis by arginine administration restores vascular function (Cooke et al., 1992; Drexler et al., 1991).

Nitric oxide synthase (NOS) enzymes use only L-arginine to synthesize NO. Simultaneously and competitively, arginase catalyzes the first step of polyamine synthesis, which is the conversion of L-arginine to ornithine (Figure 2). Therefore, polyamines and their metabolic

and catabolic enzymes affect the synthesis of NO by increasing or decreasing the amount of L-arginine available. An increase in intracellular polyamines from extracellular sources suppresses polyamine synthesis and increases arginine availability for NO synthesis (Mendez et al., 2006; Stojanovic et al., 2010). Conversely, decrease in arginine availability as a consequence of increased arginase activity inhibits endothelial NO synthesis (Thengchaisri et al., 2006; C. Zhang et al., 2001). Therefore increased polyamine intake enhances the bioavailability NO, essential for normal vascular physiology.

10. Conclusion

Polyamines have many biological activities for the possible inhibition of age-associated diseases. And, recent studies have shown the contribution of polyamines on longevity of various living organisms including mammals. In addition, epidemiological studies show the close positive correlation between increased polyamine intake and increased components of healthy dietary pattern, Mediterranean diet. Soybean is one of the foods containing the largest amount of polyamines in nature. Therefore, I believe that soybean-induced inhibition of age-associated diseases is mainly produced by polyamines. Polyamines, in association with the appropriate amount of and ratios of various fatty acids, e.g. n-3 polyunsaturated fatty acids, saturated fatty acids, may be two major food components that help inhibit the progression of age-associated diseases and prolong human lifespan.

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Edited by Hany El-Shemy

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ISBN 978-953-51-5171-5

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