ANTIOXIDATIVE ISOFLAVONES FROM FERMENTED THAI SOYBEAN

SASIKHAN TOSO

MASTER OF SCIENCE

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A THESIS SUBMITTED TO MAE FAH LUANG UNIVERSITY
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ABSTRACT

The study was conducted to compare the transformation of isoflavones and their glycoside in Thai soybean, which fermented with *Lactobacillus casei*, *Rhizopus oryzae* and *Aspergillus* spp. Results revealed that the fermentation of soybean caused a marked increase in the content of aglycones (daidzein and genistein) and a significant reduction in the content of glycoside isoflavones (daidzin and genistin) compared with those in the non-fermented soybean. The extent of increased aglycones and reduced glycoside isoflavones contents were varied with the used strain of microorganisms. The percentage of aglycones to total isoflavones increase 21.31 %, 33.56 % and 25.94 % after the fermentation with *L. casei*, *R. oryzae* and *Aspergillus spp,* respectively. The cause of change is the hydrolyzation of glycoside isoflavones (daidzein and genistein) into aglycones (daidzein and genistein) by β-glucosidase produced form *L. casei*, *R. oryzae* and *Aspergillus* spp. From the study of antioxidative activities by ABTS method the results shown that soybean fermented with *L. casei*, *R. oryzae* and *Aspergillus* spp. possessed higher antioxidant activity than non-fermented soybean. Thus, Soybean products fermented *with L. casei R. oryzae* and *Aspergillus* spp. can therefore be used in health promotion product, food

product or the product for external used such as anti-aging cosmetic product because they possessed higher antioxidant activity than non-fermented product**s.**

Keyword : Soybean/ Fermentation/ Isoflavone/ Antioxidant

ชื่อเรื่องวิทยานิพนธ์	สารต้านออกซิเดชั่นกลุ่มไอโซฟลาโวนที่ได้จากถั่วเหลืองหมักสายพันธุ์ในประเทศไทย	
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	อาจารย์ ดร. พรรณรวี พรหมนารท	กรรมการ

บทคัดย่อ

งานวิจัยนี้เป็นการศึกษาการเปลี่ยนแปลงของสารกลุ่มไอโซฟลาโวนทั้งอะไกลคอน (แดทซิอีน และจีนิสติอีน) และไกลโคไซด์ไอโซฟลาโวน (แดทซิน และจีนิสติน) ที่ได้จากการหมักถั่วเหลืองสายพันธุ์ในประเทศไทยด้วยเชื้อที่ผ่านการคัดเลือกแล้วได้แก่ *Lactobacillus casei, Rhizopus oryzae* และ *Aspergillus* spp. ซึ่งพบว่ากระบวนการหมักทำให้สารกลุ่มอะไกลคอนในถั่วเหลืองหมักมีปริมาณเพิ่มขึ้นและสารกลุ่มไกลโคไซไอโซด์ฟลาโวนมีปริมาณลดลงเมื่อเปรียบเทียบกับถั่วเหลืองที่ยังไม่ผ่านการหมัก ซึ่งปริมาณการเพิ่มของอะไกลคอนและการลดของไกลโคไซไอโซด์ฟลาโวนแตกต่างกันตามชนิดของเชื้อที่ใช้หมัก ภายหลังจากการหมักด้วยเชื้อ *L. casei*, *R. oryzae* และ *Aspergillus* spp. พบว่าปริมาณของอะไกลคอนเมื่อเทียบกับปริมาณไอโซฟลาโวนทั้งหมด เพิ่มขึ้น 21.31, 33.56 และ 33.56 เปอร์เซ็นต์ ตามลำดับ การเปลี่ยนแปลงดังกล่าวเกิดจากกระบวนการไฮโดรไลด์เซชั่นของสารกลุ่มไกลโคไซด์ไอโซฟลาโวนไปเป็นอะไกลคอนโดยเอนไซม์เบต้าไกลโคซิเดสที่เกิดจากเชื้อ *L. casei*, *R. oryzae* และ *Aspergillus* spp. และจากการศึกษาฤทธิ์ต้านออกซิเดชั่นด้วยวิธี ABTS พบว่าผลิตภัณฑ์ถั่วเหลืองที่หมักด้วยเชื้อ *L. casei R. oryzae* และ *Aspergillus* spp. มีฤทธิ์ต้านออกซิเดชั่นสูงกว่าถั่วเหลืองที่ยังไม่ผ่านกระบวนการหมัก ดังนั้นผลิตภัณฑ์ถั่วเหลืองที่หมักด้วยเชื้อทั้ง 3 ชนิดสามารถนำไปใช้เป็นผลิตภัณฑ์ส่งเสริมสุขภาพทั้งในรูปของผลิตภัณฑ์อาหารหรือผลิตภัณฑ์ที่ใช้ภายนอกเช่น ผลิตภัณฑ์เครื่องสำอางกลุ่มต้านความชรา

เนื่องจากผลิตภัณฑ์จากถั่วเหลืองทั้ง 3 ชนิดนี้มีฤทธิ์ต้านออกซิเดชั่นสูงกว่าผลิตภัณฑ์ถั่วเหลืองที่ยังไม่ผ่านการหมัก

คำสำคัญ : ถั่วเหลือง/ หมัก/ ไอโซฟลาโวน/ ต้านอนุมูลอิสระ

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CHAPTER I

INTRODUCTION

1.1 Introduction

Soy or soybean (Glycine max (L) Merrill) belongs to plants of Leguminosae family, Papilionoideae sub-family (Nwokolo & Smartt, 1996[1]). Soybeans were first grown as a crop in China 5000 years ago. Now they are commonly cultivated throughout the world. Over 75% of global requirements are produced in USA, Brazil and Argentina (Roebroeck, 2002[2]).

There are many soybean breeds in Thailand, including Chiang Mai 60, Chiang Mai 1, S.J. 5, S.J. 9 (4), S.J. 2 (Tadang), Rajamongkon, Khonkaen and Nakhonsawan. They are used for the foodstuff productions, for feeding animals and as an industrial raw material. Soybean is an important source of nutrition; 36.5% of the bean is vegetable protein. About 20% consist of fix oil, which is rich in polyunsaturated fatty acids and contains no cholesterol. The rest of the beans are moisture, dietary fiber (soluble fiber and insoluble fiber), and isoflavones (Nwokolo & Smartt, 1996[1]).

There are 3 types of major form of isoflavones in soybean, including genistein, daidzein, and glycitein and their glycosides. Daidzin, genistin and glycitin are the glycosylate forms of daidzein, genistein and glycitein, respectively. Most isoflavones in soybean were reported to present an antioxidant activity, preventing an oxidative damage in human body (Disilvestro , et al., 2004[3], Lee, et al., 2004[4], Genovese and Hassimotto & Lajolo, 2005[5]).

The causes of oxidative damage are the oxygen free radicals, known as reactive oxygen species (ROS), including superoxide, peroxyl, alkoxyl, hydroxyl, and nitric oxide. They are generally unstable and very reactive. The ROS are produced from the internal factors, *i.e.* our metabolism and the existence of some physiopathological situation and the external factors, *e.g.* cigarette smoke, air pollution, UV radiation, high polyunsaturated fatty acid diet, inflammation, ischemia/reperfusion, etc. (Disilvestro , et al., 2004[3], Lee, et al., 2004[4], Genovese and Hassimotto & Lajolo, 2005[5]).

ROS can be very damaging, since they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates, and DNA, to induce oxidations, which cause membrane damage, protein modification (including enzymes), and DNA damage (Pietta, 2000[6] and Fang, Yang & Wu, 2002[7]). Humans have evolved with antioxidant systems to protect against the free radicals that are to produce antioxidants in the body (endogenous). Sometimes ROS are produced in excess at the wrong time and place, causing an imbalance between the production of ROS and a biological antioxidant system, which is called “oxidative stress”. This is considered to play a causative role in aging and several degenerative diseases associated with it, such as heart disease, cataracts, cognitive dysfunction, and cancer (AFAR, 2003[8]). Thus the dietary antoxidations (exogenous) are needed for diminishing the cumulative effect for oxidative damage over the life span. Soybean is very cheap product that contains high quantities of antioxidant compound and is an important source of nutrition. For these reasons, soybean product is the popular foodstuffs in the world.

There are many traditional soybean fermented products available, especially in Asia countries, such as Miso, Tempeh, Nutto, etc. Most studies reported that the fermented soybean products were found to be stronger antioxidant activity than non-fermented soybean because of the enzyme that produced by microorganism changes isoflavones structures into the highly antioxidative compounds (Esaki, et al., 1999[9], Choi, Kim & Rhee, 2002[10], Miyake, et al., 2003[11], Lee & Chou, 2006[12], Lin, Wei & Chou, 2006[13] and Kuo, 2006[14]). It is thus of interest to study the change of the amount of isoflavones after the fermentation in Thai soybean and to

compare their antioxidative activities. Interested isoflavones in this study were daidzin, genistin, daidzein and genistein. They were analysed by using HPLC technique according to the method described by AACC method 20-20. The study of antiovidative activity of fermented Thai soybean product determined by ABTS radical-scavenging activity

1.2 Research Aims

The aims of these studies are as follows:

1.2.1 To study the quantities of isoflavones in Thai legumes and soybean products.

1.2.2 To study the change of isoflavones quantities and to compare the antioxidative activities of soybean fermented with *Rhizopus oryzae*, *Lactobacillus casei* and *Aspergillus* spp.

CHAPTER II

LITERATURE REVIEWS

2.1 Soybean

Soybean is probably the world's most valuable crop, it is such a source of protein and fat, and such a good source of energy, vitamins and minerals. Soybean seed is used for the extraction of edible oil, human food, livestock feed and in the industrial manufacturing of thousand of products. The highest human consumption of soybean is in Asia, where it has traditionally been processed into fermented and non-fermented products. After the introduction to the United States in the early 1800s, it gradually became very popular in the Southern and Midwestern states. The United States has remained by far the world's largest producer of soybean for more than two decades (Nwokolo & Smartt, 1996[1] and Shahidi & Naczk , 2004[15]).

2.1.1 Soybean Description

Soy or soybean (Glycine max (L) Merrill), belongs to Leguminosae family, Papilionoideae sub-family. The plant originates from Asia (Nwokolo & Smartt, 1996[1] and Shahidi & Naczk, 2004[15]). It is a herbaceous annual with determinate as well as indeterminate growth habits. The trunk of soybean is a small, erect, hirsute plant usually 30-70 cm in height, with trifoliolate and alternate leaves and ovate to lanceolate leaflets. The flowers are purple or white. The soybean seeds grow in pods that are oblong, slightly elongated, about 3-8 cm long and 8 – 15 mm broad. They develop in clusters with each pod contains two or three seeds. They are hairy, green when immature but turning to a yellowish brown color on maturity. The physical characteristic of soybean is shown in Figure 2.1.

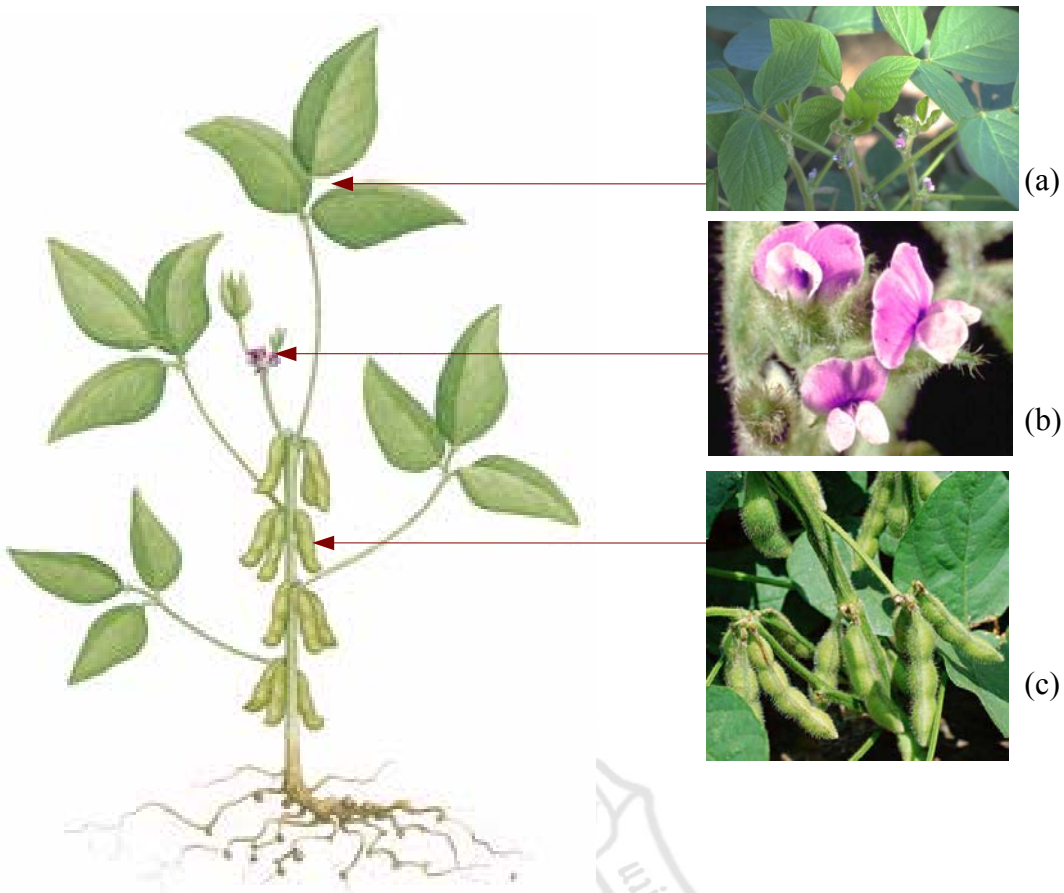


Figure 2.1 The physical characteristic of soybean (IITA Research to Nourish Africa,2007[16]), (a) the leaves (Raloff, 2003[17]), (b) the flowers (Crop Garden – Soybeans, 2008[18]), and (c) the pods (Germes Online, 2008[19]).

The soybean seeds (Figure 2.2) are ovoid to subspherical in shape, a few seeds being irregular. Seeds are smooth. Seed colors varies, common colors being tan, brown, and to a lesser extent black and green. The cotyledons are usually yellow or green.

Figure 2.2 The physical characteristic of soybean seed

2.1.2 Soybean cultivation

Soybeans were first grown as a crop in China 5000 years ago. Now they are commonly cultivated throughout the world. Over 80 % of global requirements are produced in USA, Brazil and Argentina. The production of soybean in 2000 is shown in Table 2.1.

Table 2.1 Production of soybean in 2000 (Roebroeck, 2002[(2)])

Country	Production of soybean	
	Million Tons	**% Total**
United States	75.4	45
Brazil	36.5	21
Argentina	26.0	15
China	15.7	9
Paraguay	3.4	2
Other	9.2	5

Soybean is a crop of mild climates, growing well with an average production cycle of 90-110 days from planting to harvesting and yielding profusely in subtropical in temperate regions. Although it has been introduced not quite successfully to tropical regions of the world, it has, however, been very successful in Brazil where it performs exceptionally well. Soybean performs optimally in the temperature range of 25-32^{o}C. It has a moderate moisture requirement of 400-800 mm rainfall and can therefore be cultivated in a variety of ecological zones (Nwokolo & Smartt, 1996[(1)]). It is a shot-day plant yet thrives in day-natural environments as occurs in the immediate environment of the equator. Although it grows best on fertile, well-drained soils, it tolerates various soil types, preferring mildly acidic soils. Moreover, soybean is the nitrogen-fixing plant. It is fixing nitrogen by specific *nitrogen-fixing rhizobia*, a bacteria live inside legumes root nodules (shown in Figure 2.3), and it fixes nitrogen from the air into a form usable by the plants (Dalton, 2007[(20)]).

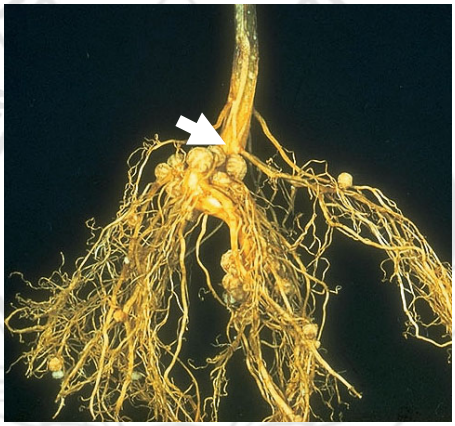


Figure 2.3 The root nodules of soybean with *nitrogen-fixing rhizobia* (Dalton, 2007[(20)])

2.1.3 Nutrition composition in soybean

Soybean is an important source of nutrition. It contains almost twice the protein content of cowpea, the other legume plants that are used for the foodstuffs production (Nwokolo & Smartt, 1996[(1)]). The proximate composition of soybean and soybean products is shown in Table 2.2. Soybean is a good source of protein, carbohydrate and fat. It also has minimal amount

of water, fiber and ash. Soybean is a good source of folic acid, shown in Table 2.3. It is also a fairly good source of thiamine, riboflavin and niacin and pantothenic acid. Mineral contents of soybean and other processed soybean products are shown in Table 2.3. Soybean is a good source of potassium, phosphorus, magnesium and calcium but poor source of iron. It also has minimal amounts of zinc, manganese and copper.

Table 2.2 Proximate composition of soybean and soybean products (g/100g) (Nwokolo & Smartt, 1996[1])

Composition	Contents of composition in soybean and soybean products (g/100g)			
	Raw soybean	Defatted flour	Protein concentration	Protein isolate
Protein	36.49	47.01	58.13	80.69
Fat	19.94	1.22	0.46	3.39
Carbohydrate	30.16	38.37	31.21	0.36
Water	8.54	7.25	5.80	4.98
Crude fiber	4.96	4.27	3.77	0.26
Ash	4.87	6.15	4.70	3.58

Table 2.3 Vitamin and mineral contents in soybean and soybean products (mg/g) (Nwokolo & Smartt, 1996[1])

Composition	Vitamin and mineral contents in soybean and soybean products (mg/g)			
	Raw soybean	Defatted flour	Protein concentration	Protein isolate
Vitamin				
Vitamin B1; Thiamine	0.87	0.7	0.31	0.17
Vitamin B2; Riboflavin	0.87	0.25	0.14	0.1

Table 2.3 Vitamin and mineral contents in soybean and soybean products (mg/g) (Nwokolo & Smartt, 1996[1]) (Cont.)

Composition	Vitamin and mineral contents in soybean and soybean products (mg/g)			
	Raw soybean	**Defatted flour**	**Protein concentration**	**Protein isolate**
Vitamin B3; Niacin	1.62	2.61	0.71	1.43
Vitamin B9; Folic acid	375.1	305.4	340	176.1
Vitamin C; Ascorbic acid	6	0	0	0
Mineral				
Calcium	277	241	363	178
Copper	1.65	4.06	0.97	1.6
Iron	15.7	9.24	10.78	14.58
Magnesium	280	290	315	39
Manganese	2.51	3.01	4.19	1.49
Phosphorus	704	674	839	776
Potassium	1797	2384	2202	81
Zinc	4.89	2.46	4.4	4.03

Soybean oil is semidrying oil, which has found popularity as a salad oil, cooking oil, blacking oil and in the preparation of many culinary delights. Contents of fatty acids in soybean oil are shown in Table 2.4. The oil is composed of approximately 16% saturated fatty acids, 24% monounsaturated fatty acids and 60% polyunsaturated fatty acids. Unsaturated fats help to lower levels of total cholesterol and low density lipoprotein cholesterol in the blood (Wikipedia, 2007[21]). The good fatty acid is linoleic acid (Table 2.4), which presents 63% of total unsaturated fatty acid in raw soybean.

Table 2.4 Fatty acid contents in soybean and soybean products (g/ 100 g) (Nwokolo & Smartt, 1996[1])

Composition	Fatty acid contents in soybean and soybean product (g/ 100 g)			
	Raw soybean	Defatted flour	Protein concentration	Protein isolate
Saturated	2.88	0.13	0.05	0.42
Myristic acid	0.05	0.00	0.0	0.0
Palmitic acid	2.11	0.10	0.03	0.31
Stearic acid	0.71	0.03	0.01	0.10
Monounsaturated	4.40	0.21	0.08	0.64
Palmitoleic acid	0.05	0.0	0.0	0.0
Oleic acid	4.35	0.21	0.08	0.64
Polyunsaturated	11.25	0.53	0.20	1.65
Linoleic acid	9.92	0.47	0.18	1.45
Linolenic acid	1.33	0.06	0.02	0.20

Soybean is a good source of isoflavones, which present in raw soybean from several countries. The different content of isoflavones in several soybeans are shown in Table 2.5. Most studies were reported that isoflavones in soybean present an antioxidant activity and decrease effect of oxidative damage in human body (Disilvestro , et al., 2004[3], Lee, et al., 2004[4], Genovese and Hassimotto & Lajolo, 2005[5]). Of these the soybean is of much interest.

Table 2.5 Isoflavone contents in soybeans from different sources (mg/100 g) (USDA 2007[22])

Source of soybeans	Isoflavone contents in different source of soybeans (mg/100 g)			
	Daidzein	**Genistein**	**Glycitein**	**Total isoflavones**
Japan	34.52	64.78	13.78	118.51
Brazil	20.16	67.47	0.00	87.63
Korea	72.68	72.31	0.00	144.99
Taiwan	28.21	31.54	0.00	59.75

2.1.4 Isoflavones

Isoflavone is one of many classes of flavonoid that is a class in plant polyphenols, which is important antioxidant. They are found most often in legumes, including soybeans, black beans, green beans and chick peas. The basic flavonoid structure is the flavan nucleus (Pietta, 2000[6]) (Figure 2.4), which consists of 15 carbon atoms arranged in three rings (C6-C3-C6), labeled in A, C, and B, respectively. The several of flavonoids differ in the level of oxidation and pattern of substitution of the C ring. The structures of the many classes of flavonoids are shown in Figure 2.5.

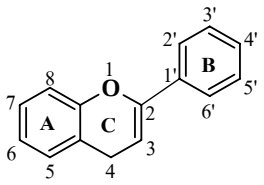


Figure 2.4 The structure of flavan nucleus

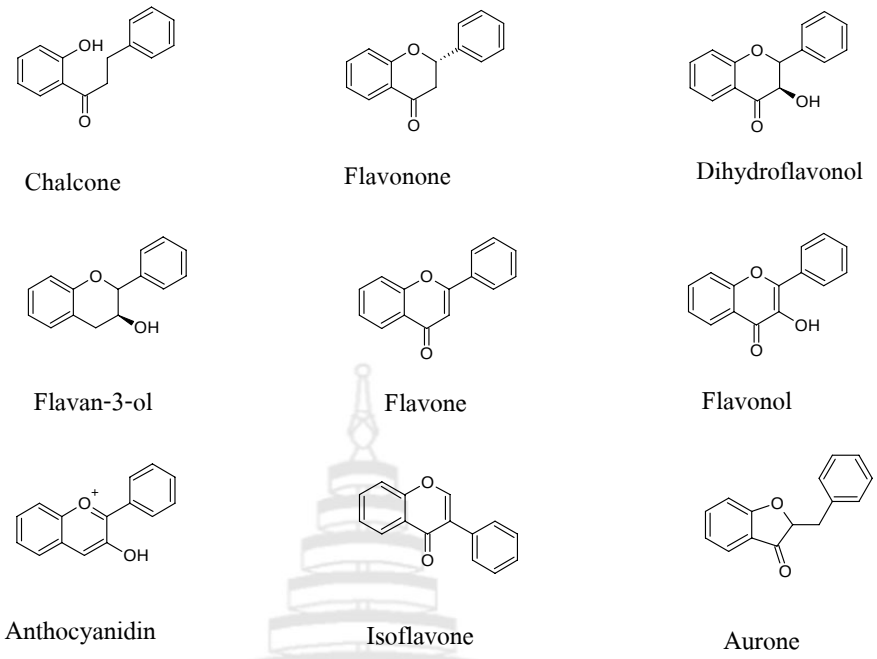


Figure 2.5 The structures of various of flavonoids (Pietta, 2000[6])

The basic structure of isoflavones, consists of 15 carbon atoms arranged in three rings, labeled A, B, and C (Figure 2.6). They are isomeric with flavones (Figure 2.5) and differ in the placement of B ring to the C3 position. The hydroxylation pattern also is similar to flavones. The several of isoflavones differ in the pattern of the substitution of the A and B rings.

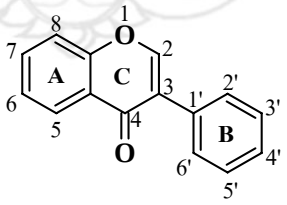


Figure 2.6 The basic structure of isoflavone

Moreover, isoflavones are the compounds in food plants that are structurally similar to mammalian estrogens (Figure 2.7) and that possess estrogen-like biological activity. Isoflavones have both weak estrogenic and anti-estrogenic effects. It was initially suggested that these isoflavones might prevent hormone-dependent breast and prostate cancers by virtue of their potential estrogen-antagonist activity (Dinauer, 2000[23]). The compounds in plant that present estrogen-like biological activity are called as phytoestrogen. These compounds are of great interest, eliciting diverse physiological responses. 5,7-Dihydroxy isoflavone (Figure 2.7 b) is a strong inhibitor of oxidative phosphorylation. Many studies (Atkinson, et al., 2004[24] and Setchell, et al., 1998[25], [26]) reported that the deaths from breast cancer were found significants lower in Asia than in USA. This is because a striking dietary different: Asians consume 20-50 times more soy-based food per capita than Americans do.

Figure 2.7 Similarities in the chemical structures of the mammalian estrogen (a) and the isoflavone, genistein (b) (Dinauer, 2000[23])

2.1.5 Isoflavones in soybean

Isoflavones in soybean called **aglycone,** which are genistein, daidzein and glycitein, are biosynthesized via the phenylpropanoid pathway (Yu, et al., 2003[27]) (Figure 2.8) and stored in the vacuole as glycoside, acetyl-glucose and malonyl-glucose conjugates.

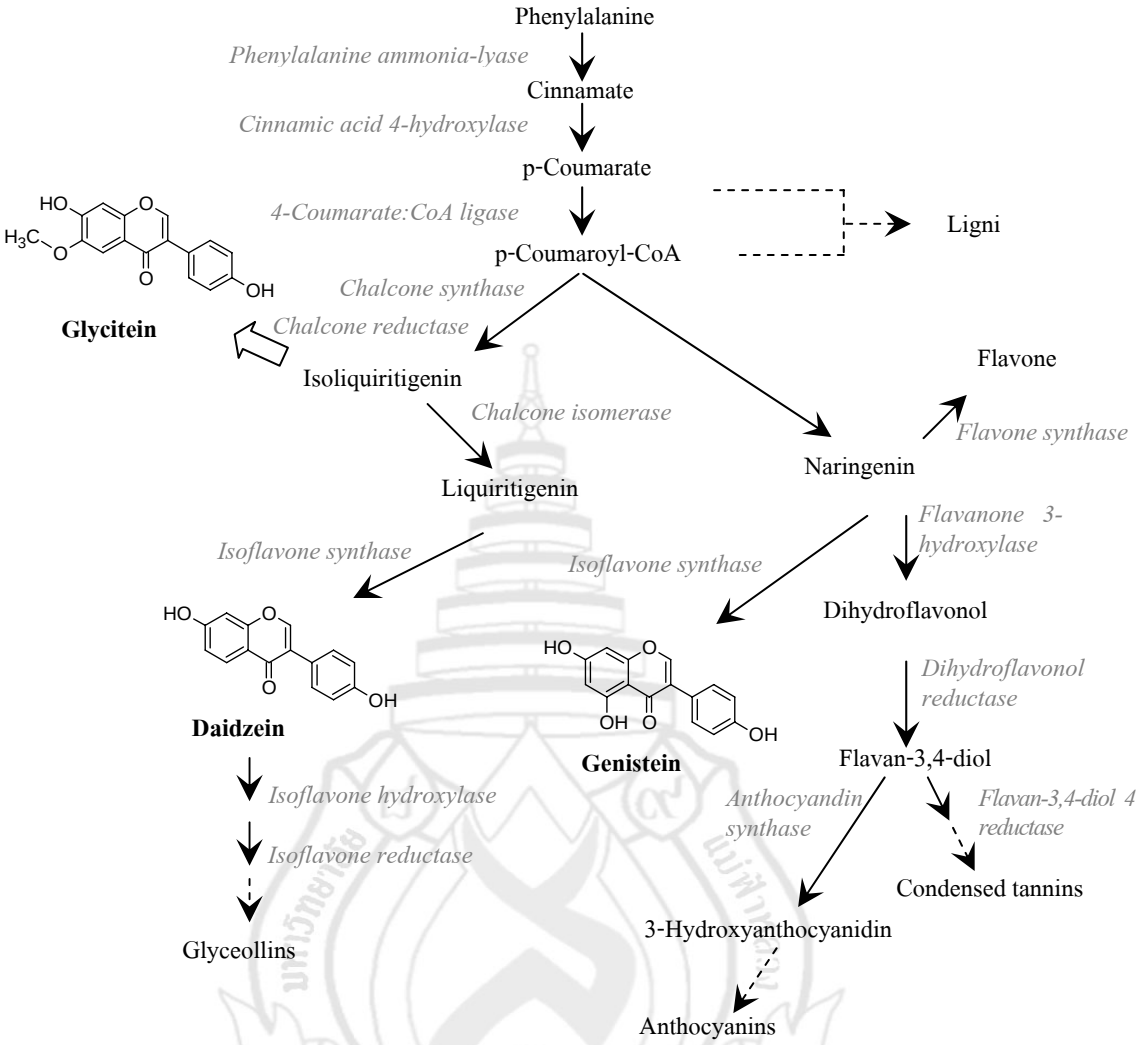


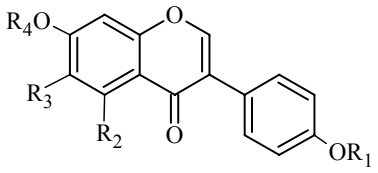
Figure 2.8 The partial diagram of the phenylpropanoid pathway. Intermediates and enzymes involved in isoflavone (aglycones) biosynthesis, as well as some branch pathways, are shown. Dotted arrows represent multiple steps; the block arrow represents speculative steps (Yu, et al., 2003[27])

The phenylpropanoid pathway begins from the amino acid phenylalanine, to form intermediate of the pathway, naringenin, which is sequentially converted in to the isoflavone genistein by legume-specific enzyme, isoflavone synthase. Similarly, another intermediate

isoliquritigenin is converted to the isoflavone daidzein by sequential action of two legume-specific enzymes: chalcone isomerase, and isoflavone synthase. Isoliquritigenin is also converted to the isoflavone glycitein by speculative steps.

The major forms of isoflavonse that present in natural soybean are glycoside isoflavones. Their structures contain aglycones molecule with each one covalently bound to a sugar (glucose) molecule at R4 position, shown in Figure 2.9. Thus the glycoside form of genistein, daidzein and glycitein are genistin, daidzin and glycitin, respectively. Genistin forms about 60 % of the total isoflavone precursors in soybean, daidzin and glycitin forms about 30 % and 8 %, respectively (Majeed & Prakash, 2005[28]). The structures of isoflavones (aglycones) and their glycosides in soybean are shown in Figure 2.9.

Processing of soybean into soy foods causes some loss and change of isoflavones. Soaking soybean in water caused the loss of 10% of isoflavones originally present. Whereas, if soybean was processed into soy protein isolate, isoflavones was reduce 53% of isoflavones originally present in raw materials (Wang & Murphy, 1996[29]). If soybean was fermented, it was found that, glycoside isoflavones in soybean are hydrolyzed into aglycones by enzyme from fermentation microorganisms (Esaki, et al., 1999[9]). Additionally, it was tamed that the glycoside isoflavones are broken down by the bacteria in the gut during the digestive processes and yield the aglycones, when soybean and soy foods are consumed (Majeed & Prakash, 2005[28]). Thus, consumption of and soybean products provides a good effect for human health.



Compounds	R_1	R_2	R_3	R_4
Daidzein	H	H	H	H
Genistein	H	OH	H	H
Glycitein	H	H	OCH_3	H
Daidzin	H	H	H	glucose
Genistin	H	OH	H	glucose
Glycitin	H	H	OCH_3	glucose
6″-*o*-acetyldaidzin	H	H	H	6″-*o*-acetylglucose
6″-*o*-acetylgenistin	H	OH	H	6″-*o*-acetylglucose
6″-*o*-acetylglycitin	H	H	OCH_3	6″-*o*-acetylglucose
6″-*o*-malonyldaidzin	H	H	H	6″-*o*-malonylglucose
6″-*o*-malonylgenistin	H	OH	H	6″-*o*-malonylglucose
6″-*o*-malonylglycitin	H	H	OCH_3	6″-*o*-malonylglucose

Figure 2.9 Isoflavones structures (Shahidi & Naczk, 2004[15]).

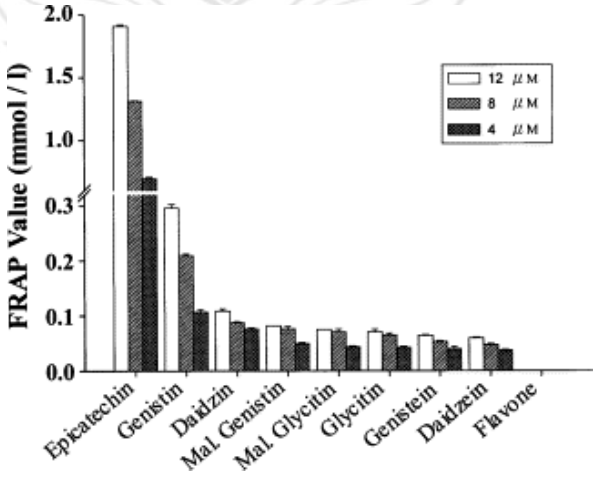


Figure 2.10 Ferric reducing-antioxidant power (FRAP) of isoflavones and their glycoside (Lee, et al., 2005[31])

2.2 Fermented Soybean

Soybean used for the production of foodstuffs, the feeding animals and as an industrial raw material. There are many traditional fermented products produced using soybean, especially in Asia countries. The fermented soybean products were found to be more antioxidant activity than non-fermented steamed soybean (Esaki, et al., 1999[9]). This suggests that the fermentation affects the quantities of isoflavones, which are antioxidants in soybean.

2.2.1 Examples of the popular fermented soybean products

1. Soybean Sauce

Soybean sauce is a fermented product native to Japan, it was fermented with *Aspergillus* species. The fermentation significantly alters distribution of isoflavones in the product. The total isoflavones content in soybean sauce is about 1-14 mg/kg. The major forms are the aglycones (Table 2.6). The glycoside isoflavones were hydrolyzed into aglycones by β-glucosidase produced from *Aspergillus* species (Murphy, 1999, Chiou & Cheng, 2001[32] and Shahidi & Naczk, 2004[33]).

2. Soybean paste

Soybean paste is an important oriental soybean food known as Miso in Japan, Taucho in Indonesia, Jang in Korea, Taotsi in Philippines and Jiang or Chiang in China (Shahidi & Naczk, 2004[15]), it was fermented with *Aspergillus* species (Murphy, 1999[32], Zarkadas, te al., 1997[34] and Chiou & Cheng, 2001[33]). The total isoflavones contents in soybean paste are about 228-235 mg/kg. The distribution of isoflavone forms in soybean paste is summarized in Table 2.6. The isoflavones levels may be different depending on the origin of soybean cultivar used and processing steps employed. More here, the total content of isoflavones in soybean paste in the U.S. ranges forms 22.8 to 23.5 mg/100 g of wet weight but soybean paste purchased in Indonesia have contained 12.8 to 27.6 mg of daidzein per 100 g of wet weight and 10.1 to 23.4 mg of genistein per 100 g of wet weight. (Shahidi & Naczk, 2004[15]).

Table 2.6 Ranges of isoflavones and glycoside isoflavones contents in some soybean food (Murphy, 1999[32])

Isoflavones/ glycoside isoflavone	Isoflavones and glycoside isoflavone contents in soybean foods (mg/kg of wet weight)				
	Soybean milk	Tofu	Soybean sauce	Soybean pasta	Tempeh
Daidzein	1-3	3-22	1-6	25-38	77-85
Genistein	1-4	4-30	0-4	29-43	89-103
Glycitein	0-1	2-4	0-3	12	8-9
Daidzin	20-94	40-105	0	62-66	93-105
Genistin	25-130	64-143	0	89-92	206-226
Glycitin	4-16	11-29	0	10-13	14
6″-*o*-acetyldaidzin	0	0-18	0	8-14	35-49
6″-*o*-acetylgenistin	4-10	8-17	0	20-23	46-50
6″-*o*-acetylglycitin	0	0	0-3	0-9	0
6″-*o*-malonyldaidzin	6-42	64-156	0	14-32	64-66
6″-*o*-malonylgenistin	14-45	40-158	0	17-37	111
6″-*o*-malonylglycitin	0-5	7-19	0	0-8	8
Total	80-165	202-347	1-14	228-235	531-538

3. Tempeh

Tempeh is a fermented product native to Indonesia. Fermentation by genus *Rhizopus* species significantly alters distribution of isoflavones in the final product. Tempeh contains 6.5 times higher levels of aglycones and 57% lower levels of glycoside isoflavones than those in the original raw material (Wang & Murphy, 1996[29]). This increase in aglycones may be due to the hydrolytic action of β-glucosidase from fungi (Murakami, et al., 1984[35]). The total isoflavones content in Tempeh is about 531-538 mg/kg, shown in Table 2.6. Tempeh purchased

in Indonesia and Australia contained 74.5 and 17.6 mg/100 g of daidzein, and 85.4 and 33.3 mg/100 g of genistein respectively, on a dry weight basis. These differences in the isoflavone levels may originate from the soybean cultivar used and processing steps employed (Shahidi & Naczk, 2004[15]).

2.2.2 Antioxidative isoflavones from fermented soybean

Most studies reported that after the fermentation the antioxidant activity of fermented soybean such as Tempeh, Miso and Natto, soybeans fermented with *Rhizopus oligosporum*, *Aspergillus oryzea* and *Bacillus natto,* was significantly higher than that in non-fermented steamed soybean. It was suggested that daidzin and genistin, which each have a β-glucosidic linkage at the 7-position of the A ring, in steamed soybeans were decreased with fermentation period, whereas daidzein and genistein, were increased during fermentation. This is because daidzin and genistin were hydrolyzed into daidzein and genistein, respectively, by β-glucosidase, (Figure 2.11) produced from microorganism, *Rhizopus oligosporum*, *Aspergillus oryzea, Aspergillus awamori, Aspergillus sojae* and *Bacillus natto* (Esaki, et al., 1999[9], Choi, Kim & Rhee, 2002[10], Miyake, et al., 2003[11], Lee & Chou, 2006[12], Lin, Wei & Chou, 2006[13] and Kuo, 2006[14]).

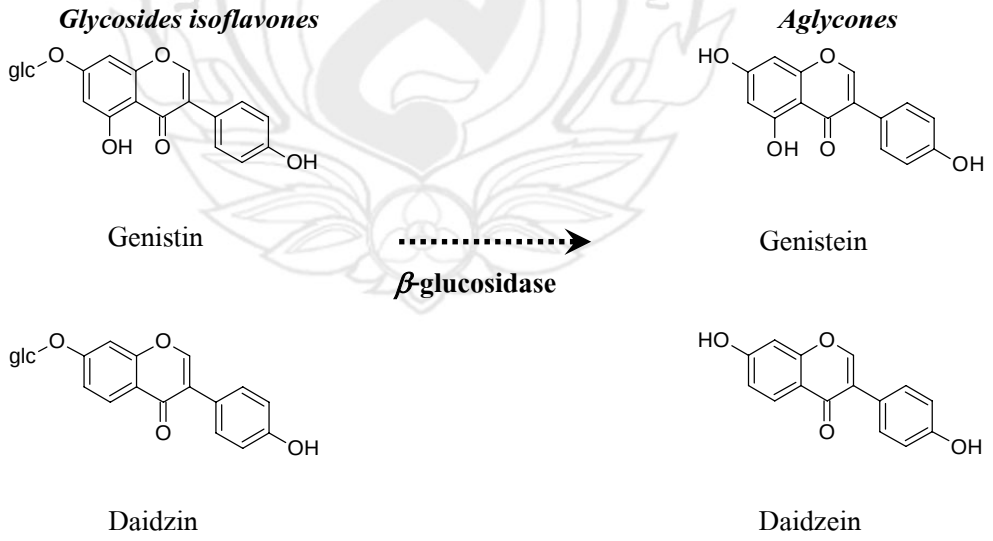
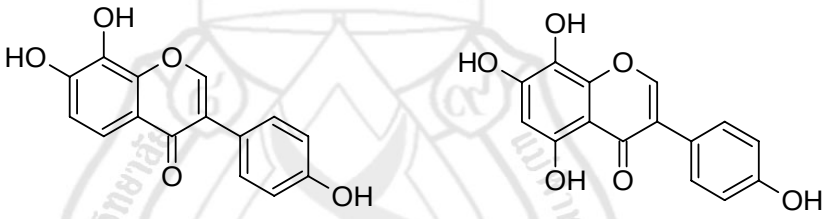


Figure 2.11 The formation mechanism of isoflavones hydrolyzation with β-glucosidase produced from microorganism, including, *Rhizopus oligosporum*, *Aspergillus oryzea, Aspergillus awamori, Aspergillus sojae* and *Bacillus natto.*

Recent studies reported that soybean fermented with *Aspergillus saitoi* produced the new potent antioxidative isoflavones. Daidzein and genistein were hydrolyzed into 8-hydroxydaidzein and 8-hydroxygenistein, respectively, (Figure 2.12) by hydroxylase produced from *A. saitoi*. They have an *o*-dihydroxy structure between the 7- and 8- position at the A ring. 8-Hydroxydaidzein and 8-hydroxygenistein showed stronger antioxidative activities than those of daidzein and genistein, respectively (Esaki, et al., 1999[9]).



8-hydroxydaidzein 8-hydroxygenistein

Figure 2.12 New potent antioxidative isoflavones, 8-hydroxydaidzein and 8-hydroxygenistein structures

The changing mechanism of isoflavones after fermentation with *A. saitoi* is shown in Figure 2.13. Daidzin and genistin were gradually hydrolyzed into daidzein and genistein, respectively, by β-glucosidase. The resulting daidzein and genistein were then hydroxylated to produce the potent antioxidants, 8-hydroxydaidzein (8-OHD) and 8-hydroxygenistein (8-OHG) by the hydroxylase (Esaki, et al., 1999[9]). Both of β-glucosidase and hydroxylase were produced from *A. saitoi*.

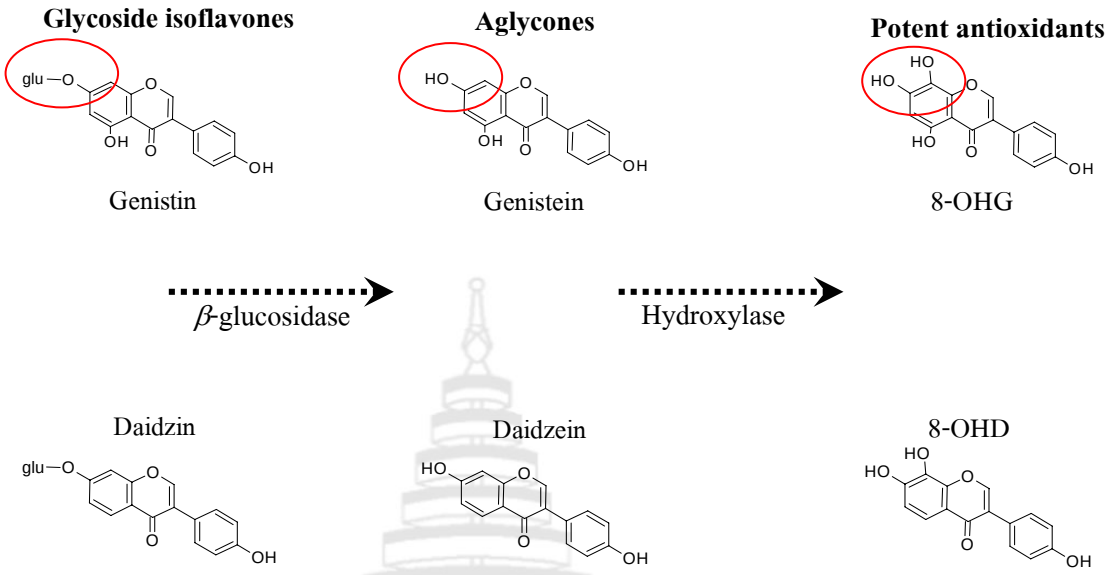


Figure 2.13 The mechanism of isoflavones hydrolyzation with *β*-glucosidase and hydroxylase, produced from *A. saitoi*

2.3 Free radicals and Antioxidants

2.3.1 Free radicals

Oxygen is essential for life and provides the energy, which all cells in the body operate. The downside is that the ordinary oxygen molecule easily turns into oxidizing agents, called **reactive oxygen species** (ROS) that possess great potential danger. In simple terms, oxidizing agents (also called pro-oxidants) are short of electrons and will steal them from any neighboring molecules in the body that do not keep a tight hold on its own electrons. This damages the neighboring molecule severely. Reactive oxygen species play normal roles in the body, but in excess they develop highly poisonous properties.

The most important ROS in the body are the superoxide ion ($O^{\bullet-}$), the hydroxyl radical ($-OH^{\bullet}$), and hydrogen peroxide (H_2O_2) (Pietta, 2000[6]). These ROS are all short of electrons and are thus powerful oxidizing agents. Hydrogen peroxide is different, as it is not itself that is short of electrons, but it is easily converted in the body to the hydroxyl radical, that are short of electrons and that do the damage. Technically, compounds that are short of electrons are called free radicals. The superoxide ion and the hydroxyl radical are free radicals. Thus, ROS include both oxygen-derived free radicals and compounds like hydrogen peroxide that are not free radicals themselves but easily generate them. ROS will attack and damage key molecules in the body, such as fats, proteins, and DNA the molecule that carries genetic information.

To understand why ROS are potentially so harmful for cells, we have to look closely at the cell itself. Each cell in the body is composed of a membrane boundary, a fluid interior, and a central nucleus, along with a set of other small structures called organelles. The membrane of the cell is made largely of fat. When the fats in the membrane are oxidized by a ROS, the membrane becomes brittle and leaky; eventually, it falls apart and the cell dies. Proteins are located either embedded in the fatty membrane or throughout the interior of the cell. Many proteins are small machines that perform important functions in the cell. Some proteins are enzymes that manufacture substances needed by the cell, and some are hormones that act as signals to other cells. Others control the entry and exit of substances across the cell membrane. When a ROS attacks a protein and damages it, some key function of the cell will be jeopardized. DNA, the molecule that carries the genetic information of the cell, is found in the cell's nucleus. Oxidative damage to it can cause mutations that predispose the cell to cancer formation. In these ways, through damage to fats, proteins, and DNA, various parts of the body can be weakened by oxidative attack and will succumb to a wide variety of diseases (Smythies, 1998[36]).

2.3.2 Antioxidant

The biochemical prevents reactions that produce efficient defenses prevent ROS from killing the cells. These defenses consist of a set of specialized molecules known as **antioxidants**. The structure of the cell is required to understand how antioxidants perform their role and why different types are needed. The interior of the cell and the fluid between cells are composed mainly of water. The cell membrane is made largely of fat. As antioxidants can strike

either at the fatty cell membrane or at the watery cell contents, needed to work either in the fatty membrane or in the watery compartments. Besides solubility, another way in which antioxidants differ from each other is size. Some antioxidants are small molecules, whereas others are large proteins. The small molecule types work in part by mopping up or "scavenging" the ROS and carrying them away, and in part by neutralizing them chemically. A sacrificial protein will absorb ROS and thus prevent them from attacking some essential protein.

The human body gets its essential antioxidants from two main sources:

1. Antioxidants are produced in the body itself, (Smythies, 1998[36], Pietta, 2000[6])

a) Enzymetic defenses, such as Se-glutathione peroxidase, catalase, superoxide dismutase, which metabolize, superoxide, hydrogen peroxide, and lipid peroxide, thus preventing most of the formation of the toxic $OH^{\bullet}$.

b) Nonenzymetic defenses, such as glutathione, histidine-piptides, the iron-binding proteins transferring and ferritin, dihydroliponic acid, reduced CoQ10, melatonin, urate, and plasma protein thiols, with the last two accounting for the major contribution to the radical-trapping capacity of plasma.

2. Antioxidants are obtained from the diet. Some of the dietary antioxidants are called vitamins, which mean "vital amine" because they are essential for life. Low levels of them in the diet cause deficiency diseases, such as curvy and pellagra. There are other dietary antioxidants that the body cannot synthesize, but these are not essential for life, and their absence in the diet does not lead to disease. For this reason these antioxidants are not called vitamins. Although they are not essential ingredients in the diet, they are nevertheless helpful in combating oxidative stress.

Antioxidant flavonoids include catechin (green tea), gossypol (rice), apigenin (chamomile tea), quercetin (apples), hesperetin (oranges), and naringenin (grapefruit). Antioxidant phenols include thymol and carvacuol (thyme), ferulic acid (many herbs), gallic acid (nutgall), hydroxytyrosol (olive oil), fisetin, morin, and many others. Antioxidant polyphenols are found in oranges (as cryptoxanthin) and many other fruits, tea, coffee, chocolate, licorice, and

white wine. Other related plant antioxidants include rhein (rhubarb) and aloe-emodin (aloe). Antioxidant peptides (small, proteinlike molecules) include carnosine (which is particularly associated with the glutamate receptor in the brain) and anserine. Rosemary contains compounds that stimulate the production of the antioxidant glutathione in the liver and act as antitumor compounds that block cancer-producing chemicals from binding to DNA.

Soy beans contain a variety of anticancer compounds, such as genistin, which are effective at mopping up ROS but are also likely to act in additional ways to kill cancer cells. Genistin and related compounds are also found in whole grain cereals, seeds, berries, and nuts (Smythies, 1998[36]).

2.3.3 Oxidative stress

Many diseases were caused by the disturbation of the delicate balance between ROS and antioxidants. If ROS are produced at a rate higher than what the antioxidant defense can cope, **the oxidative stress** results, this may lead to the tissue damage and diseases. Medical science is exploring the possibility of using antioxidants in the prevention and cure of disease (Smythies, 1998[36]).

Oxidative stress can result from four main causes:

1. Reactive oxygen species can be ingested in excess from some environmental source (such as tobacco smoke or diesel oil fumes);

2. The diet may contain insufficient antioxidants;

3. There may be some disturbance in the elaborate biochemical systems that control their production and distribution (as in the case of many diseases);

4. There may be a failure in protective anti-oxidant mechanisms (as in the case of many other diseases).

Thus, we are protected against such free radical-mediated oxidative stress by an array of defense systems. As shown in Figure 2.14, the preventive antioxidants, such as peroxidase and metal chelating proteins, suppress the generation of free radicals and act as the first line defense. The radical-scavenging antioxidants, such as vitamin C and vitamin E scavenge radical to inhibit chain initiation and break chain propagation. They are the second line defense.

The repair and de novo enzymes act as the third line defense. Furthermore, the appropriate enzymes are generated and transferred to the right site at the right time and in the right concentration by an adaptation mechanism.

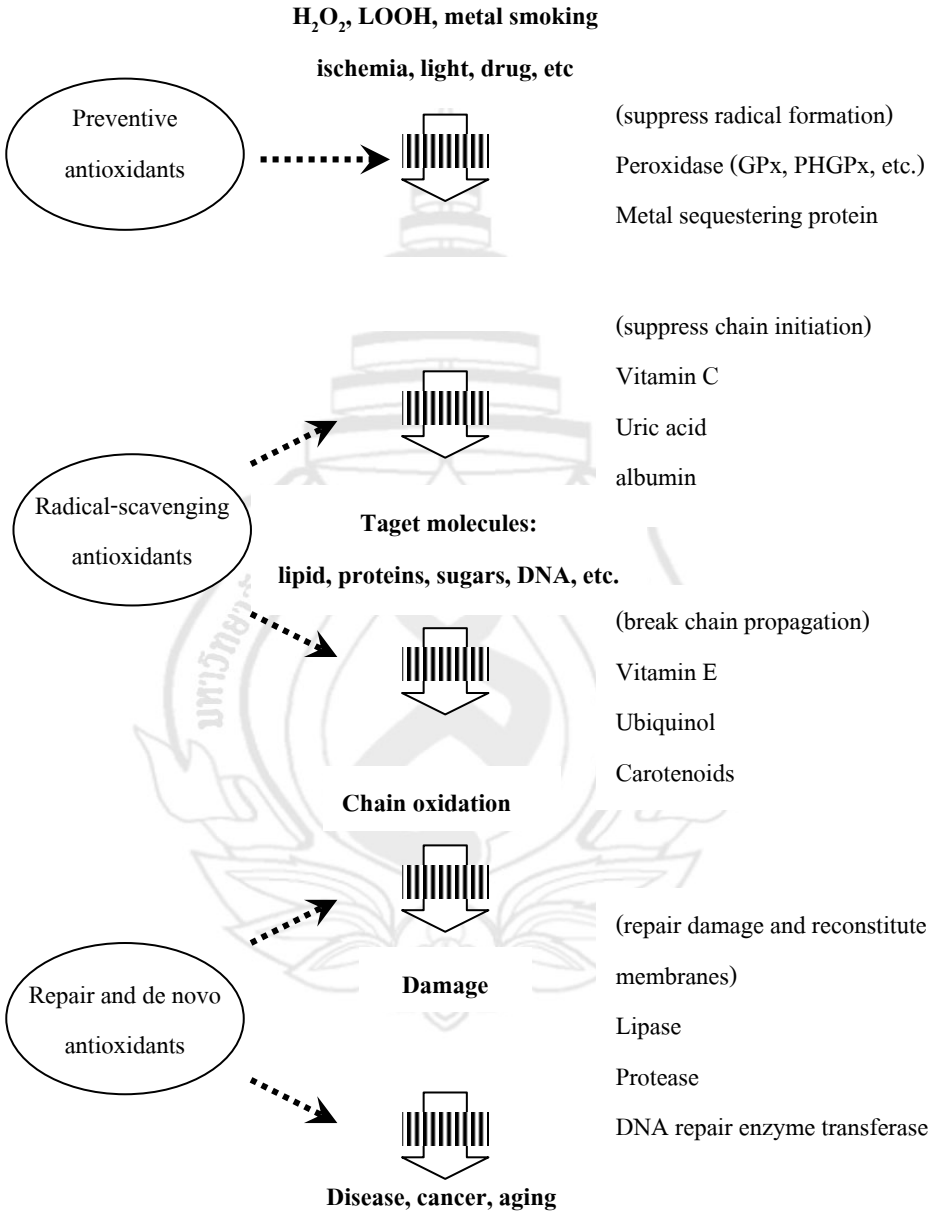


Figure 2.14 Defense systems *in vivo* against oxidative stress (Hiramatsu, Yoshikawa & Inoue, 1997[37])

2.3.4 Antioxidant activity (radical scavenger potential) assay

Natural antioxidants, particularly in fruits and vegetables, have gained increasing interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer (Renaud, et al., 1998[38] and Temple, 2000[39]). The defensive effects of natural antioxidants in fruits and vegetables are related to three major groups: vitamins, phenolics, and carotenoids. Ascorbic acid and phenolics are known as hydrophilic antioxidants, while carotenoids are known as lipophilic antioxidants (Halliwell, 1996[40]).

Several assays have been frequently used to estimate antioxidant capacities in fresh fruits and vegetables and their products and foods for clinical studies, including 2,2- azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Re, et al., 1999[41] and Leong & Shui, 2002[42]), 2,2- diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams, Cuvelier & Beraet,1995[43], Gil, et al., 2002[44] and Prakash, 2007[45]), ferric reducing antioxidant power (FRAP) (Jiménez-Escrig, et al., 2001[46] and Guo, et al., 2003[47]), and the oxygen radical absorption capacity (ORAC) (Ou , Hampsch-Woodill & Prior, 2001[48] and Prior, et al., 2003[49]). The ORAC assay is said to be more relevant because it utilizes a biologically relevant radical source (Prior, et al., 2003[49]). These techniques have shown different results among crop species and across laboratories.

In soybean and soybean product, most studies attempted to identify the major antioxidants present, using the ABTS assay as a guide (Long, Kwee & Halliwell, 2000[50], Moure, Domínguez & Parajó, 2006[51] and Wang, et al., 2007[52]). The ABTS decolourisation assay was carried out interact the antioxidants with ABTS radical cation ($ABTS^{+\bullet}$) show in Figure 2.15. The original ABTS and potassium persulfate react stoichiometrically at 1:0.5 leading to an incomplete oxidation to produce the $ABTS^{+\bullet}$ that is a blue-green complex. The interaction between antioxidants and $ABTS^{+\bullet}$ was measured using a spectrophotometer. Results are expressed in terms of stoichiometric factor and Trolox equivalent antioxidant capacity (TEAC). Stoichiometric factor that used in present study is calculated on the basis of extent of $ABTS^{+\bullet}$ scavenged by the

antioxidant and report as percentage inhibition compare with $ABTS^{+\bullet}$ scavenged by blank solution.

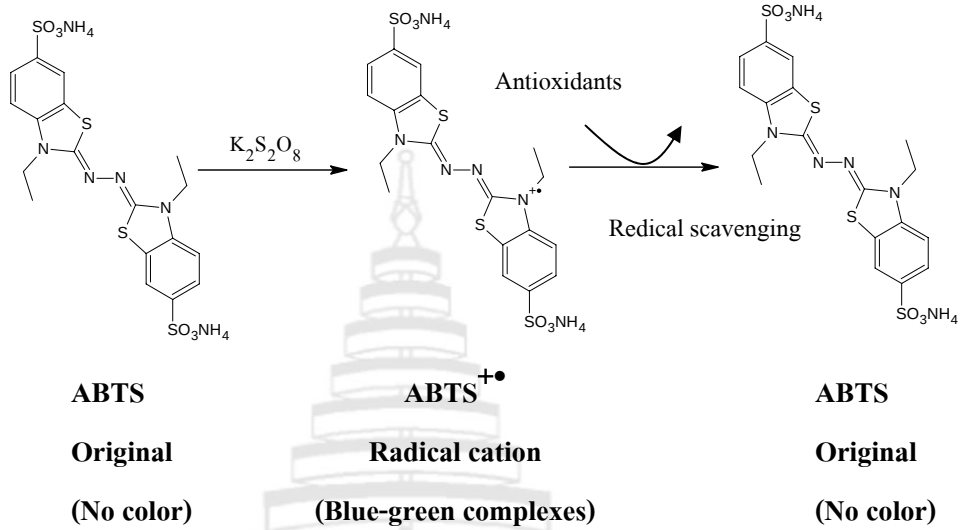


Figure 2.15 The interaction between antioxidants and $ABTS^{+\bullet}$

CHAPTER III

EXPERIMENTAL

3.1 Apparatus

1. Analytical balance, model AB204-S, Mettler Toledo, Switzerland
2. Analytical balance; capable of weighing to 0.0001 g., model AB204-S, Mettler Toledo, Switzerland
3. Autoclave
4. Cellulose filters No. 1, 11 cm, Whatman, UK
5. Centrifuge; model Universal 32 R, Hettich, Germany
6. Glass vials for LC auto-sampler, 2 ml, Agilent, USA
7. Micro-centrifuge tube, 1.5 ml disposable
8. Mobile phase filter, Millipore, France
9. Orbital platform shaker; model KS250 basic, KIKA, Germany
10. Pipette, Witeg, Germany
11. PTFE membrane filters, 0.45 μm, 47 mm, Alltech, USA
12. PTFE syringe filters, 0.45 μm, 13 mm, Whatman, UK
13. Quartz cell; model B0631009, Perkin Elmer USA
14. Vertex mixer; model Genie-2/G-530E, Vortex, USA
15. Volumetric flasks; Schott Duran, Germany
16. Water bath shaker; model SBD 50, Heto, Denmark

3.2 Instrument

HPLC analysis was performed on a Waters Associates chromatograph equipped with model 2695 autosampler, a model 2695 Chromatography pump, and a model 2996 Photodiode Array Detector. Compounds were separated by a reversed-phase C-18, 5 mm (4.6 x 250 mm Waters associates) column.

Antioxidative activities were performed on a Perkin Elmer Associates spectroscopy equipped with model Lambda 35.

3.3 Chemicals

1. Absolute ethanol, C_2H_6O, analytical grade, MERCK, Germany
2. ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)), $C_{18}H_{18}N_4O_6S_4 \cdot (NH_3)_2$ analytical grade, MERCK, Germany
3. Acetic acid, glacial, CH_3COOH, analytical grade, MERCK, Germany
4. Daidzein, $C_{15}H_{10}O_4$, 99.8% HPLC grade, Sigma, Germany
5. Daidzin, $C_{21}H_{20}O_9$, 95% HPLC grade, Fluka, USA
6. Deionization water form Water purifier, model ZMGS5VF01, Milli-Q, France
7. Genistein, $C_{15}H_{10}O_5$, 99.8% HPLC grade, Sigma, Germany
8. Genistin, $C_{21}H_{20}O_{10}$, 99.8% HPLC grade, Sigma, Germany
9. Hydrochloric acid, HCl, analytical grade, J.T. Baker, USA
10. Methanol, CH_4O, HPLC grade, J.T. Baker, USA
11. Potassium persulfate, $K_2S_2O_8$, analytical grade, MERCK, Germany
12. Sodium hydroxide, NaOH, analytical grade, MERCK, Germany
13. Sugar

3.4 Samples

3.4.1 Legume seed

Eight different types of legume seeds, used in this study are black been, red bean, soybean, gram, and peanut, collected from department store, and Pap bean, Pae bean and Noy bean, collected form many villages in Chaing Rai province, Thailand in May, 2004.

3.4.2 Soybean seed

There are 8 different breeds of soybeans, shown in Table 3.1, which are collected from in different division parts of Thailand. They were harvested between March and May in 2004.

Table 3.1 Soybean samples in Thailand, harvested between March and May in 2004

No.	Breed Name	Harvest place
1	Chiang Mai 60	Chiang Mai province, Thailand
2	Chiang Mai 1	Chiang Mai province, Thailand
3	S.J. 5	Chiang Mai province, Thailand
4	S.J. 9(4)	Chiang Mai province, Thailand
5	S.J. 2 (Tadang)	Chiang Mai province, Thailand
6	Rajamongkon	Chiang Mai province, Thailand
7	Khonkaen	Khonkaen province, Thailand
8	Nakhonsawan	Nakhonsawan province, Thailand

3.4.3 Soybean products samples

Four types of soybean products that are popular for cooking in Thailand, namely fermented soy sauce, acid hydrolyzed soy sauce, soy paste and Thua nao were chosen. All samples were purchased from department store in Chaing Rai province, Thailand in May, 2004.

3.4.4 Fermented soybean samples

There are 3 different fermented soybean samples used in this study. They were soybean fermented with *Rizopus oryzae* and *Lactobacillus casei*, produced form Faculty of Pharmacy, Chiang Mai University and the soybean fermented with *Aspergillus* spp., produced from A Driving Force for National Science and Technology Capability (NSTDA) Northern Network, Chiang Mai.

3.5 Procedures

3.5.1 Determination of Isoflavones in Thai legume and Soy products (AACC method 20-20, 2001)

1. Standard solution and calibration curve

Standard isoflavones (daidzin, genistin, daidzein and genistein) were prepared by dissolving 1 mg of each compound in methanol to produce the stock standard solution. The stock standard solution was diluted to 7 levels of working standard solution concentration (see Table 3.2). Calibration curves created from these standards demonstrated a high degree of linearity ($r > 0.995$) upon plotting standard concentration versus peak area obtained from the HPLC analysis with 20 μl injection. Each concentration was analyzed by triplicate injection.

Table 3.2 Concentration of each isoflavone working standard solution

Volume of stock standard (µl)	Volume of water (µl)	Volume of 50% methanol (µl)	Concentration of working standard (ppm)			
			Daidzin	Genistin	Daidzein	Genistein
7.81	7.81	1984.38	0.78	0.78	0.78	0.78
15.62	15.62	1968.75	1.56	1.56	1.56	1.56
31.25	31.25	1937.50	3.12	3.12	3.12	3.12
62.50	62.50	1875.00	6.25	6.25	6.25	6.25
125.00	125.00	1750.00	12.50	12.50	12.50	12.50
250.00	250.00	1500.00	25.00	25.00	25.00	25.00
500.00	500.00	1000.00	50.00	50.00	50.00	50.00

The calibration of standard isofalvones was obtained by using Millenium[32] Version 4.00 (Waters software). The calibrations of standard isofalvones are shown in Figure 3.1.

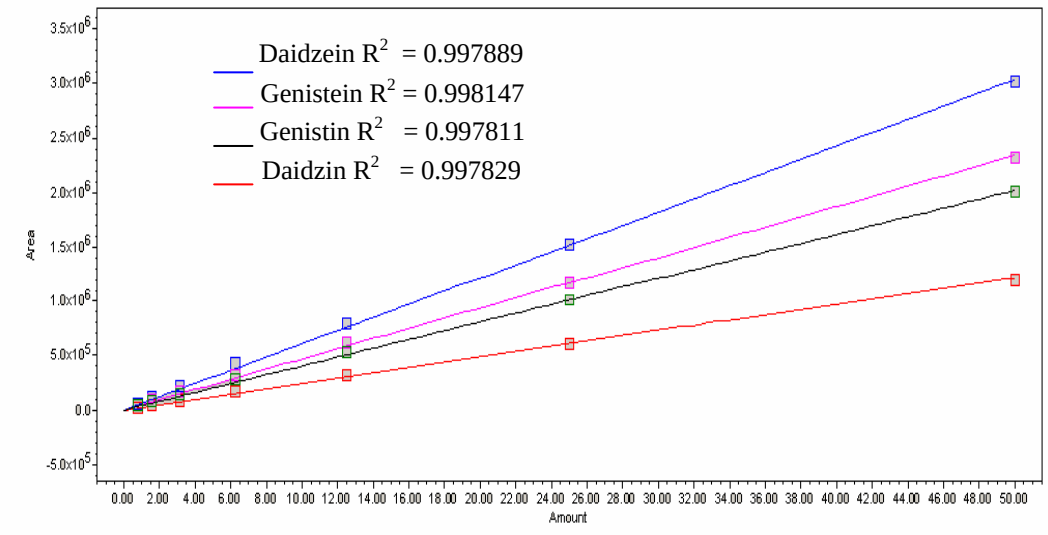


Figure 3.1 Calibration curves of standard isofalvones

2. Samples preparation

The test sample (2 g) was added to 40 ml of 80% methanol and shaken in a 65^{o}C water bath for 2 hours. Following the extraction step, add 3 ml of 2M NaOH and 1 ml glacial acetic acid and then flitter through quantitative grade filter to separate fat from the test sample. 5 ml of filtrate was diluted to 10 ml with 4 ml of water and followed by methanol. Final the sample was centrifuge for 5 min at 7000 x g. The clear supernatant was determent by HPLC analysis.

3. High Performance Liquid Chromatography (HPLC) determination

Compounds were eluted from the column at a flow rate 1.5 ml/min utilizing the following gradient solvent system. The composition of solvent are mobile phase A (H_2O + methanol + acetic acid (88+10+2)) and mobile phase B (methanol + acetic acid (98+2)). The HPLC pump gradient for each run is shown in Table 3.3.

Table 3.3 Liquid Chromatography Pump Gradient for each run.

Step	**Start Time (min)**	**End Time (min)**	**Mobile phase composition at end time**	
			%A	**%B**
Initial	0	0.1	90	10
2	0.1	30	40	60
3	31	31.5	0	100
4	37	37.5	90	10
5	44.5	Stop run	90	10

The samples were monitored by the photo diode array detector at a wavelength of 254 nm. The typical relative retention times (in min) are as follows: daidzin, 12.343; genistin, 19.788; daidzein, 31.223; and genistein, 42.644. The retention times varies with age and condition of column. The chromatogram of standard solutions is shown in Figure 3.2.

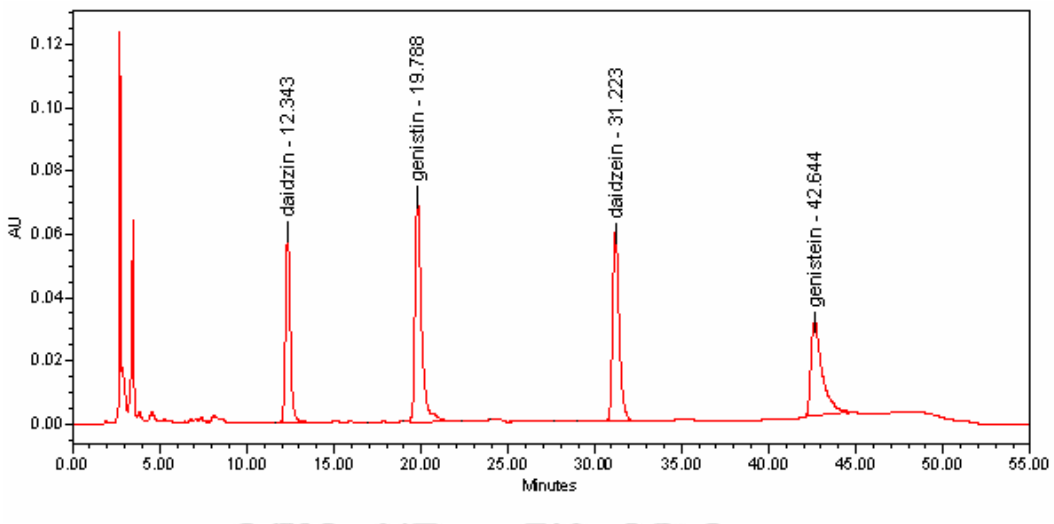


Figure 3.2 The retention time of isoflavones standard solutions

4. Calculation

The response for each isoflavones was determined by calculating the slope (m) and intercept (b), using linear regression analysis of area counts vs. response for five levels of isoflavones standards.

The concentration of each isoflavone in test samples was calculated by using the following equation:

$$Isoflavone(ug/g) = \frac{[(As)(m)+b](50)(10)}{(Ws)(5)}$$

Where

As = area of isoflavones peak in test sample;

m = slope from linear regression for standard response;

b = intercept from linear regression for standard response;

Ws = weight of test sample, g;

50 = dilution volume;

10 = second dilution volume;

5 = aliquot volume.

3.5.2 Fermentation process

1. Dry Fermentation

The S.J. 2 soybean (7 kg) was soaked with water and allowed to stand for 12 h at room temperature. The soaked soybean was incubated for 4 hours at 121 ^{o}C and then fermented with *Rhizopus oryzae* 10^6 spores per gram of soybean at room temperature. A hundred gram of fermented soybean was collected at 0, 6, 12, 18, 24, 48, and 72 hours.

2. Wet Fermentation

S.J. 2 soybean (7 kg) was soaked with water and allowed to stand for 12 h at room temperature. The soaked soybean was incubated for 4 hours at 121 ^{o}C. Incubated soybean (4.86 kg) was mixed with 16.2 kg of water and 1.62 kg of sugar. The mixture was then filtered, resulting in the aqueous part called **liquid soybean. Liquid sugar** was prepared by mixing 1.62

kg of water and 162 g of sugar and the solution was boiled. Liquid soybean and liquid sugar were mixed and heated at 72 ^{o}C for 15 seconds and then fermented with *Lactobocllus casei* 10^{6} spore per gram soybean at room temperature. Two hundreds gram of the fermented soybean was collected at 0, 6, 12, 18, 24, 48, and 72 hours.

3.5.3 Scavenging effect on ABTS (Re, et al., 1999[41] and Patel , et al., 2001[53])

1. Preparation of ABTS radical cation

ABTS stock solution and potassium persulfate solution were prepared by diluting to 7 mM and 2.45 mM, respectively with deionization water. ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting ABTS stock solution and potassium persulfate solution with ratio 2:1. The mixture was allowed to stand at room temperature in the dark for 12-16 h before use. For the study of antioxidant activity, the $ABTS^{+\bullet}$ was diluted with ethanol to an absorbance of 0.70-0.90 at 734 nm.

2. Samples preparation

The test sample (2 g) was added to 40 ml of 80% methanol and shaken in a 65^{o}C water bath for 2 hours. Following the extraction step, add 3 ml of 2M NaOH and 1 ml glacial acetic acid and then and flitter through quantitative grade filter to separate fat from the test sample. The filtrated was determined by UV-visible analysis.

3. Determination of the scavenging effect on ABTS+● at 734 nm

Twenty micro-liters of sample solution was diluted to 100 µl with water; they produce 10% - 30% inhibition of blank absorption. After addition of 2 ml diluted $ABTS^{+\bullet}$ solution (A_{732nm} = 0.7-0.9) the absorbance reading was taken exactly 1 minute after initial and up to 3 minutes. All samples were carried out at least in triplicate. The percent inhibition of sample was calculated by compare with blank (100 µl of water + 2 ml of diluted $ABTS^{+\bullet}$ solution).

CHAPTER IV

RESULTS

4.1. Isoflavones contents of Thai legumes and soybean products

Thai legumes and soybean products were analysed for their isoflavones contents. Interested isoflavones in this study were daidzin, genistin, daidzein and genistein. Their amounts were analysed using HPLC technique according to the method described by AACC method 20-20.

4.1.1 Isoflavones contents of Thai legumes

The legume seeds include soybeans, lentils, lupins, peas, and peanuts are used for human and animal consumption or for the production of oils for industrial uses. There are five kinds of legume seeds found in Thailand. They are black bean, read bean, gram, peanut and soybean, which are usually eaten as processed foods. Besides them, in Northern Thailand there are indigenous legumes used as food ingredients namely, Pap bean, Pea bean and Noy bean. Quantities of isoflavones in the Thai legumes have not been reported. Thus it is of interest to determine their isoflavones contents.

The results show that the highest isoflavones contents were found in soybean. The isoflavones contents of other legume seeds shows in Table 4.1

Table 4.1 Isoflavones contents of Thai legume seeds (* local name)

Type of legume seeds	Isoflavones contents (mg/kg) ± S.D. (n=2)			
	Daidzin	**Genistin**	**Daidzein**	**Genistein**
Black bean	N.D.	11.24 ± 0.26	12.55 ± 0.42	8.33 ± 4.26
Red bean	N.D.	10.06 ± 1.60	16.62 ± 4.70	2.83 ± 4.01
Gram	N.D.	N.D.	N.D.	8.13 ± 4.26
Soybean	626.35 ± 47.62	813.70 ± 1.40	32.00 ± 1.81	24.97 ± 2.71
Peanut	10.42 ± 14.74	5.64 ± 7.90	3.55 ± 0.70	10.38 ± 5.97
Pap bean*	14.25 ± 20.15	7.70 ± 10.89	N.D.	8.99 ± 4.40
Pea bean*	13.20 ± 3.04	N.D.	N.D.	10.16 ± 5.07
Noy bean*	N.D.	N.D.	N.D.	13.65 ± 0.91

N.D. = Not Detected

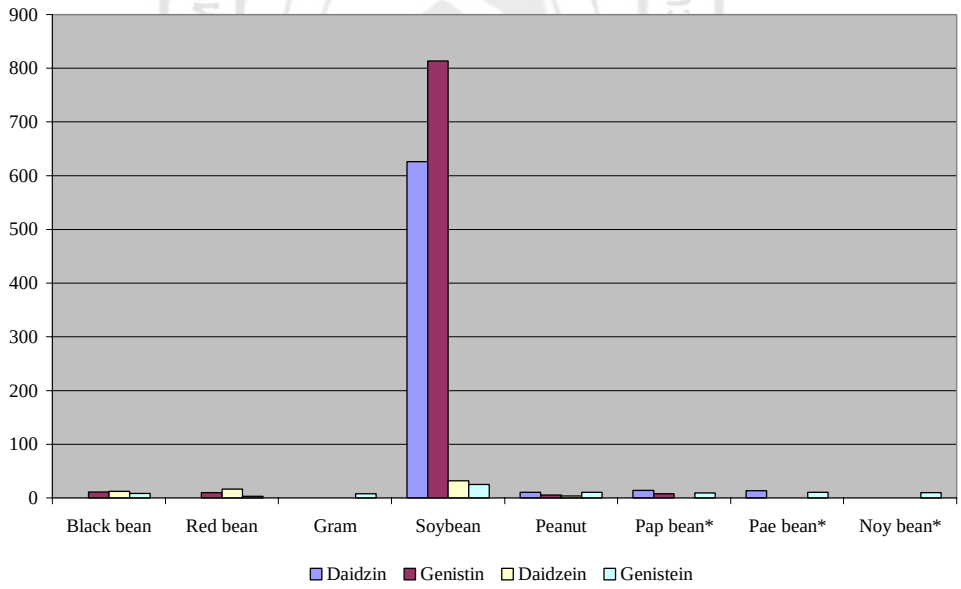


Figure 4.1 Isoflavones levels of 8 different Thai legume seeds

Table 4.1 and Figure 4.1 show that the content of isoflavones in soybean is approximately 100 times higher than those in other legumes. This indicates that soybean is a good source of natural isoflavones. Soybean, therefore, was chosen to use as a raw material for the further study.

4.1.2 Isoflavones contents of Thai soybean

From section 4.1.1, it was found that soybean contains the highest isoflavones contents among legume plants. Thus, soybean was chosen as a raw material in this study. However, in Thailand, there are 8 different breeds of soybeans namely, Chiang Mai 60, Chiang Mai 1, S.J. 5, S.J. 9 (4), S.J. 2 (Tadang), Rajamongkon, Khonkaen and Nakhonsawan. The suitable Thai soybean had to be selected by compared isoflavones contents in all Thai soybeans. The eight breeds of soybean were collected from soybean's cultivated farms of Department of Agriculture (Ministry of Agriculture and Cooperatives) between March and May in 2004. Each sample was subjected to HPLC for quantitative analysis of isoflavones.

The isoflavones contents of Thai soybean are shown in Table 4.2. The results show that a highest glycoside isoflavones contents were found in Rajamongkon, followed by those in S.J. 2, Nakhonsawan, Chiang Mai 60, Khonkaen, S.J. 9 (4), Chiang Mai 1, and S.J. 5 in decreasing order. S.J. 2 gave the highest daidzin content but Rajamonkol gave the highest genistin content. The highest aglycones contents were found in S.J. 2, followed by those in Chiang Mai 60, S.J. 9 (4), Khonkaen, Nakhonsawan, S.J. 5, Rajamongkon, and Chiang Mai 1, in decreasing order. The highest level of total isoflavones contents was found in S.J. 2. Therefore, S.J. 2 was chosen as the raw material in the present study.

Table 4.2 Isoflavones contents of 8 different breeds of Thai soybeans

Soybean breeds	Isoflavones contents (mg/kg) ± S.D. (n=4)			
	Daidzin	**Genistin**	**Daidzein**	**Genistein**
Chiang Mai 60	588.55 ± 68.42	825.62 ± 8.90	32.00 ± 0.70	26.04 ± 2.10
Chiang Mai 1	446.32 ± 104.70	360.23 ± 25.71	23.80 ± 0.63	4.10 ± 2.80
S.J. 5	303.21 ± 18.04	281.10 ± 11.03	20.00 ± 0.88	11.17 ± 3.51
S.J. 9 (4)	408.42 ± 65.26	610.35 ± 95.02	33.78 ± 0.60	18.88 ± 2.20
S.J. 2 (Tadang)	948.64 ± 19.01	834.53 ± 14.02	35.38 ± 1.22	23.84 ± 4.55
Rajamongkon	927.11 ± 63.50	949.18 ± 31.54	23.16 ± 0.92	7.12 ± 1.03
Khonkaen	740.27 ± 91.42	609.87 ± 103.20	28.82 ± 7.44	9.81 ± 6.09
Nakhonsawan	936.31 ± 154.00	667.71 ± 111.02	21.51 ± 2.49	9.79 ± 5.11

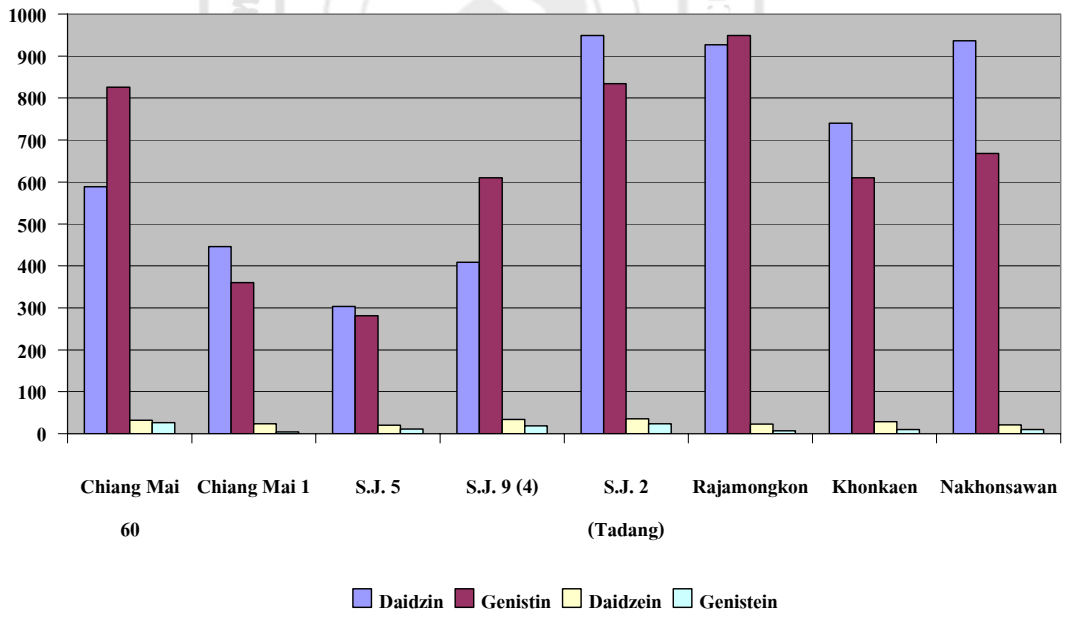


Figure 4.2 Comparison of isoflavones contained in 8 different Thai soybean breeds

4.1.3 Isoflavones contents of soybean products

In Thailand, soybean products are usually used as seasoning and food ingredients. There have been reported (Wang & Murphy, 1996[29] and Esaki, et al., 1999[9]) that the processing of soybean into soy foods causes some loss and change of isoflavones. The study of isoflavones contents in soybean products available in Thai market was of interested.

This section studied the content of isoflavones in Thai soybean products compare with soybean raw material. The soybean products (soy sauce, acid hydrolyzed soy sauce, soy paste and in Northern of Thailand, fermented soybeans that called "*Thua nao*") were collected from department store in Chiang Rai. Each soybean product was analysed for their isoflavones using HPLC. It was found that Tua nao contained the highest amount of glycoside isoflavones, 207.73 ± 6.72 ppm of daidzin and 215.42 ± 7.49 ppm of genistin. The highest contents of aglycones, 91.81 ± 3.17 ppm of daidzein and 108.21 ± 6.92 ppm of genistein, were found in TKC soy paste. The isoflavones contents of soybean products are shown in Table 4.3

Table 4.3 Isoflavones contents of soybean products

Soybean fermented products	Sample code	Percent of Soybean in product	Isoflavones contents (mg/kg dry weight) ± S.D. (n=3)			
			daidzin	genistin	daidzein	genistein
Soybean seed	SBS	100	626.35 ± 47.62	813.70 ± 1.40	32.00 ± 1.81	24.97 ± 2.71
Acid hydrolyzed soy sauce	SPT	64	4.94 ± 2.55	2.10 ± 2.90	2.00 ± 0.52	0.87 ± 0.17
	SDJ	40	16.31 ± 2.10	1.05 ± 0.90	4.67 ± 0.83	5.48 ± 0.26
	SCV	68	8.71 ± 6.77	16.48 ± 2.23	4.69 ± 0.10	6.33± 2.93
Fermented soy sauce	SLK	11	34.57 ± 2.38	55.41 ± 1.12	39.88 ± 1.32	13.28 ± 3.63
	SKK	12	120.92 ± 16.04	25.30 ± 2.82	54.14 ± 6.01	19.72 ± 0.52
Soy paste	TDS	62	37.07 ± 9.32	59.17 ± 10.46	84.76 ± 9.22	66.52 ± 16.48
	TKC	84	14.74 ± 1.24	17.48 ± 4.76	91.81 ± 3.17	108.21 ± 6.92
Thua nao	STN	100	207.73 ± 6.72	215.42 ± 7.49	52.12 ± 1.90	86.73 ± 5.48

Table 4.4 Comparison of glycoside isoflavones and aglycones in soybean and fermented soybean products

sample	Glycoside isoflavones		Aglycones	
	Value (ppm)	**% of contents**	**Value (ppm)**	**% of contents**
Acid hydrolyzed soy sauce	21.27	66.34	10.58	33.66
Fermented soy sauce	118.09	64.65	63.51	35.35
Soy paste	63.84	26.38	173.27	73.62
Thua nao	423.15	75.29	138.85	24.71
Soybean seed	1304.65	96.65	41.15	3.35

Table 4.4 shows the percentage of glycoside isoflavones and aglycones contents in soybean and fermented soybean product. In unprocessed soybean, there is 96.65% of glycoside isoflavones and 3.35% of aglycones. It is very interesting that all the fermented soybean products showed the decreasing of percentage of glycoside isoflavones and increasing of aglycones.

It was suggested that the quantities of aglycones in soybean products were changed with fermentation process. The glycosides were hydrolyzed by β-glucosidase found in fermented material led to the increasing aglycones contents (Esaki, et al., 1999[9], Choi, Kim & Rhee, 2002[10], Miyake, et al., 2003[11], Lee & Chou, 2006[12], Lin, Wei & Chou, 2006[13] and Kuo, 2006[14]). The levels of aglycones in the products are different because of the process and fermented material. The results show that the highest aglycone isoflavones contents of soybean products were found in soy paste, followed by acid hydrolysis soy sauce, fermented soy sauce, and Thua nao, in decreasing order.

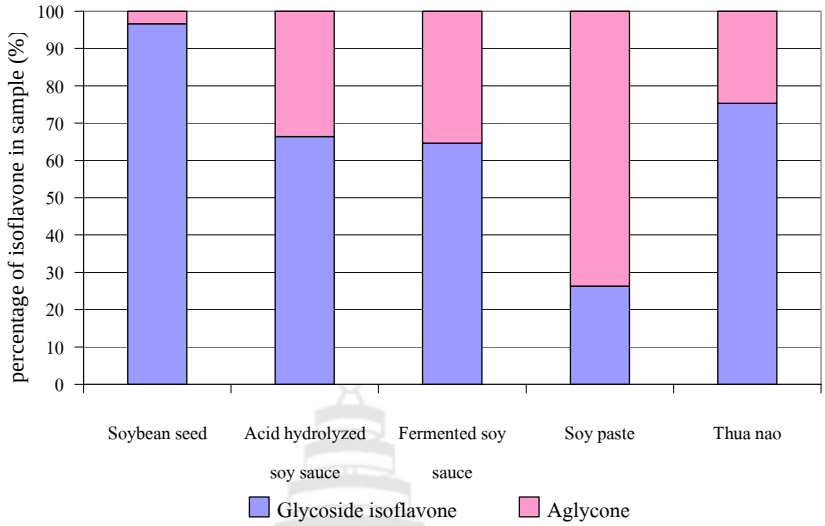


Figure 4.3 Percentage of glycoside isoflavones and aglycones contents in soybean products

4.2. Isoflavones in fermented Thai soybeans

The results of the isoflavones contents in fermented Thai soybean products are presented here. There are two parts of the study of each sample. The first part is the study of isoflavones content in fermented Thai soybean product. Interested isoflavone were daidzin, genistin, daidzein and genistein. They were analysed by using HPLC technique according to the method described by AACC method 20-20. The second part is the study of antiovidative activity of fermented Thai soybean product determined by ABTS radical-scavenging activity.

4.2.1 Soybean fermented with *Lactobacillus casei*

The change of each isoflavones contents and the change of antioxidant activity in soybean fermented with *L. casei*, the lactic acid bacteria found in drinking yogurt, were studied. The soybean milk produced from S.J.2 (Tadang) soybean seed was used as raw material in this

study. Each sample was collected at different fermentation periods and collected from Faculty of Pharmacy, Chiang Mai University.

1. Determination of isoflavones contents

L. casei was used as the test microorganism in this study. Non-fermented soybean (0 hour fermented) was the control sample. The quantitative analysis of isoflavones contents in different fermentation periods were investigated by HPLC according to the method described by AACC method 20-20. The glycoside isoflavones and aglycones contents of fermented soybean with *L. casei* are shown in Table 4.5.

Table 4.5 Isoflavones contents of fermented soybean with *L. casei*

Fermentation Periods	**isoflavones contents (mg/kg dry weight) ± S.D. (n=3)**			
(hour)	**Daidzin**	**Genistin**	**Daidzein**	**Genistein**
control	65.08 ± 5.09	42.56 ± 3.46	17.04 ± 1.81	7.49 ± 1.44
6	57.45 ± 4.91	37.57 ± 2.29	15.70 ± 0.07	7.56 ± 0.28
12	57.54 ± 9.27	31.97 ± 1.95	16.69 ± 4.27	11.91 ± 4.75
18	61.83 ± 8.44	34.72 ± 1.36	18.75 ± 3.49	13.03 ± 4.38
24	60.44 ± 11.07	33.65 ± 0.85	21.17 ± 0.41	19.52 ± 0.64
48	45.68 ± 1.78	15.35 ± 0.48	18.39 ± 0.30	20.81 ± 1.17
72	38.54 ± 0.38	11.85 ± 0.22	16.09 ± 0.27	17.31 ± 0.67

Table 4.5 shows that the highest daidzin and genistin contents was found in control sample (65.08 ± 5.09 ppm and 42.56 ± 3.46 ppm, respectively). After the fermentation, the quantities of glycoside isoflavones decreased during fermentation period. The lowest glycoside isoflavones contents were found at 72 hours (38.54 ± 0.38 ppm of daidzin and 11.85 ±

0.22 ppm of genistin). On the other hand, daidzein and genistein increased during fermentation periods and the highest contents were 21.17 ± 0.41 ppm of daidzein at 24 hours and 20.81 ± 1.17 ppm of genistein at 48 hours of the fermentation.

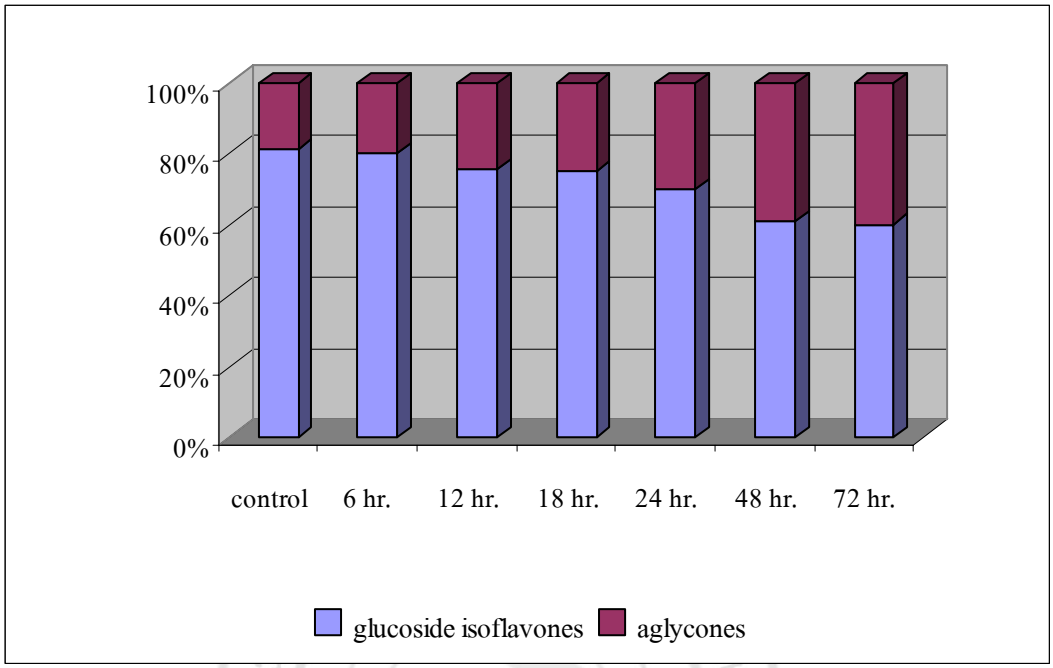


Figure 4.4 Change of isoflavones contents in soybean fermented with *L. casei*

Figure 4.4 shows the percentage of aglycones and glycoside isoflavones of soybean fermented with *L. casei*. The percentage of aglycones contents increased during fermentation periods. The increase percentage of aglycones was 21.31 % from unfermented soybean.

2. Scavenging effect on ABTS radical

The antioxidant activity of fermented soybean product with *L. casei* was determined by ABTS radical-scavenging activity. Non-fermented soybean (0 hour fermented) was the control sample. The results are shown in Table 4.6

Table 4.6 Isoflavones contents of fermented soybean with *L. casei*

Fermentation period (hour)	**% Inhibition ± S.D. (n=3)**
control	16.32 ± 0.25
6	15.62 ± 2.28
12	15.96 ± 2.22
18	24.16 ± 0.02
24	23.12 ± 2.60
48	17.00 ± 1.52
72	14.69 ± 0.11

Figure 4.5 shows that the antioxidative activities gradually increased during 18-24 hours of incubation with *L. casei*

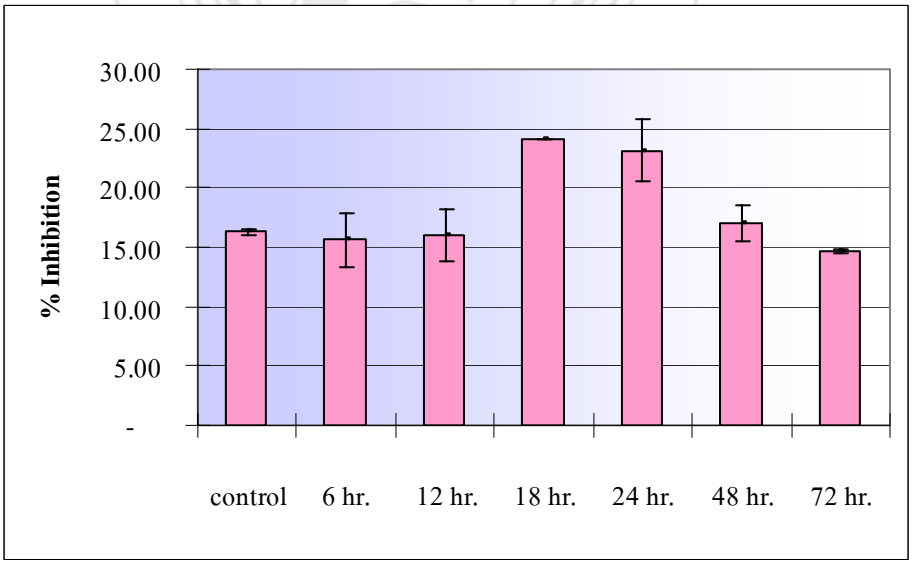


Figure 4.5 Change of the antioxidant activity in soybean after fermented with *L. casei*

4.2.2 Soybean fermented with *Rhizopus oryzae*

The change of each isoflavones contents and the change of antioxidant activity in soybean fermented with *R. oryzae***,** commonly employed as the starter organisms for some fermented food products; soy sauce, Tempe and Saka, were studied. Sojo.2 (Tadang) soybean seed was prepared by solid-state fermentation. Each sample was collected at different fermentation periods and collected from Faculty of Pharmacy, Chiang Mai University

1. Determination of isoflavones contents

R. oryzae was used as the test microorganism in this study. Non-fermented soybean (0 hour fermented) was the control sample. The quantitative analysis of isoflavones contents in different fermentation periods were investigated by HPLC according to the method described by AACC method 20-20. The glycoside isoflavones and aglycones contents of fermented soybean with *R. oryzae* are shown in Table 4.7.

Table 4.7 Isoflavones contents of fermented soybean with *R. oryzae*

Fermentation Periods	**Isoflavones contents (mg/kg dry weight) ± S.D. (n=3)**			
(hour)	**Daidzin**	**Genistin**	**Daidzein**	**Genistein**
Control	117.78 ± 0.53	110.34 ± 0.81	87.89 ± 1.74	81.04 ± 1.09
6	121.24 ± 3.76	122.98 ± 1.05	100.38 ± 1.05	94.71 ± 0.25
12	115.99 ± 1.02	109.46 ± 1.00	85.03 ± 1.47	75.77 ± 1.39
18	109.94 ± 2.00	108.13 ± 2.59	100.16 ± 2.75	86.88 ± 1.99
24	77.63 ± 1.46	85.36 ± 0.64	131.57 ± 0.58	111.11 ± 0.63
48	68.89 ± 0.35	65.31 ± 1.97	145.02 ± 0.69	115.95 ± 1.24
72	10.74 ± 3.69	46.67 ± 3.19	169.53 ± 3.15	136.53 ± 1.88

Table 4.7 shows that the highest daidzin and genistin contents was found in control sample (117.78 ± 0.53 ppm and 110.34 ± 0.81 ppm, respectively). After the fermentation, the quantities of glycoside isoflavones decreased during fermentation period. The lowest glycoside isoflavones contents were found at 72 hours (10.74 ± 3.69 ppm of daidzin and 46.67 ± 3.19 ppm of genistin). On the other hand, daidzein and genistein increased during fermentation periods and the highest contents were 169.53 ± 3.15 ppm of daidzein and 136.53 ± 1.88ppm of genistein at 72 hours of fermentation.

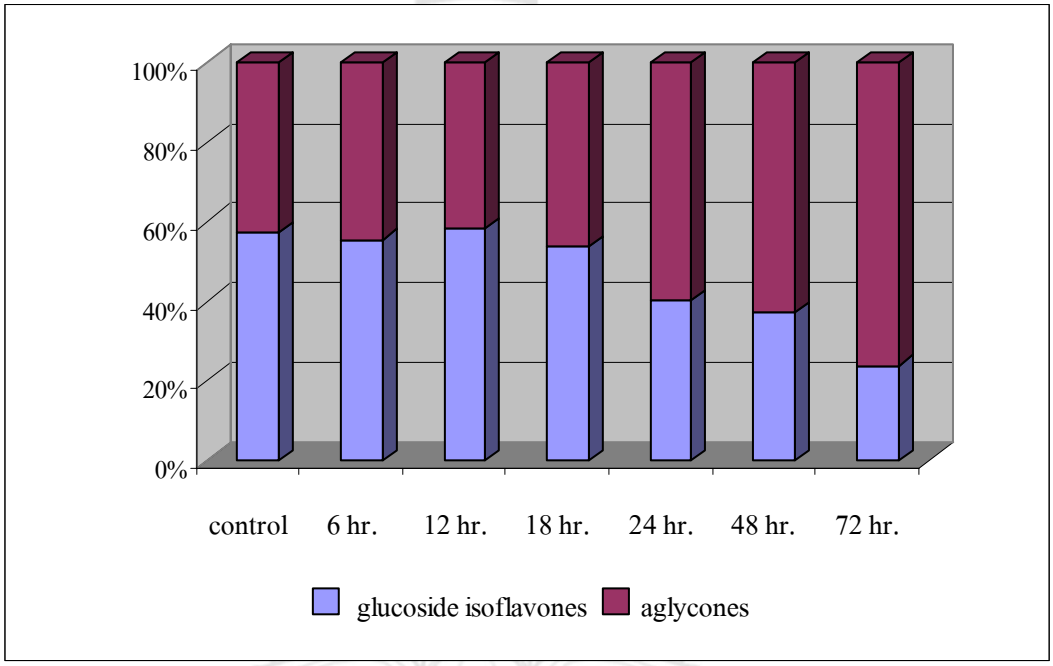


Figure 4.6 Change of isoflavones contents in fermented soybean with *R. oryzae*

Figure 4.6 shows the percentage of aglycones and glycoside isoflavones of soybean fermented with *R. oryzae*. The percentage of aglycones contents increased during fermentation periods. The increase percentage of aglycones was 33.56 % from unfermented soybean

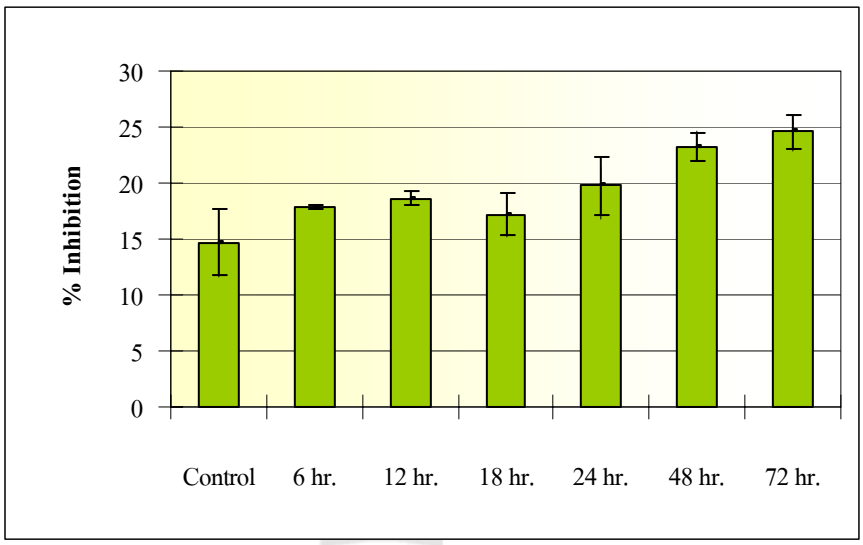
2. Scavenging effect on ABTS radical

The antioxidant activity of fermented soybean product with *R. oryzae* was determined by ABTS radical-scavenging activity. Non-fermented soybean (0 hour fermented) was the control sample. The results are shown in Table 4.8.

Table 4.8 Antioxidant activity in soybean fermented with *R. oryzae*

Fermentation period (hour)	**% Inhibition ± S.D. (n=3)**
control	14.73 ± 2.89
6	17.84 ± 0.11
12	18.61 ± 0.61
18	17.18 ± 1.91
24	19.79 ± 2.57
48	23.16 ± 1.24
72	24.60 ± 1.52

Figure 4.7 shows that the antioxidative activities gradually increased during 18-24 hours of incubation with *R. oryzae.*



Figuer 4.7 Change of the antioxidant activity in soybean after fermented with *R. oryzae*

4.2.3 Soybean fermented with *Aspergillus* spp.

The change of each isoflavones contents and the change of antioxidant activity in soybean fermented with *Aspergillus* spp.**,** commonly employed as the starter organisms for some fermented food products; Japan soy sauce, and Thuo nao, were studied. Sojo.2 (Tadang) soybean seed was prepared by solid-state fermentation. Each sample was collected at different fermentation periods and collected from Faculty of Pharmacy, Chiang Mai University

1. Determination of isoflavones contents.

Aspergillus spp. was used as the test microorganism in this study. Non-fermented soybean (0 hour fermented) was the control sample. The quantitative analysis of isoflavones contents in different fermentation periods were investigated by HPLC according to the method described by AACC method 20-20. The glycoside isoflavones and aglycones contents of fermented soybean with *Aspergillus* spp. are shown in Table 4.9.

Table 4.9 Isoflavones contents of soybean fermented with *Aspergillus* spp.

Fermentation Periods (hour)	Isoflavones contents (mg/kg dry weight) ± S.D. (n=3)			
	Daidzin	**Genistin**	**Daidzein**	**Genistein**
control	69.43 ± 2.21	84.07 ± 2.18	5.82 ± 1.15	6.49 ± 1.13
6	63.58 ± 4.43	69.67 ± 5.47	25.61 ± 1.02	40.90 ± 5.00
12	89.00 ± 10.02	70.38 ± 11.36	29.17 ± 0.90	50.64 ± 5.38
18	93.72 ± 10.86	93.65 ± 11.74	26.37 ± 2.17	42.70 ± 0.34
24	105.02 ± 2.38	107.65 ± 3.28	26.11 ± 0.87	43.46 ± 2.71
48	91.71 ± 0.00	92.22 ± 0.00	29.55 ± 12.04	44.09 ± 7.52
72	89.80 ± 13.31	85.85 ± 17.37	28.39 ± 3.02	39.74 ± 3.17

Table 4.9 shows that the highest daidzin and genistin contents was found in control sample (69.43 ± 2.21 ppm and 84.07 ± 2.18 ppm, respectively). After the fermentation, the quantities of glycoside isoflavones decreased during fermentation period. The lowest glycoside isoflavones contents were found at 12 hours (89.00 ± 10.02 ppm of daidzin and 70.38 ± 11.36 ppm of genistin). On the other hand, daidzein and genistein increased during fermentation periods and the highest contents were 29.55 ± 12.04 ppm of daidzein at 48 hours and 50.64 ± 5.38 ppm of genistein at 12 hours of the fermentation.

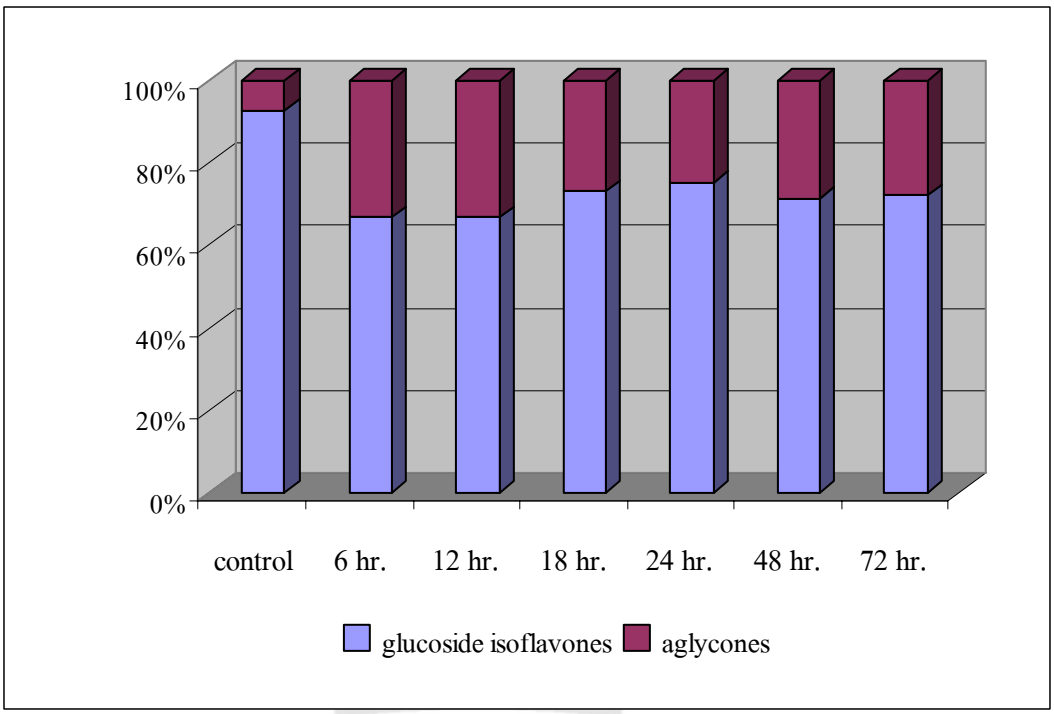


Figure 4.8 Change of isoflavones contents in soybean after fermented with *Aspergillus* spp.

Figure 4.8 shows the percentage of aglycones and glycoside isoflavones of soybean fermented with *Aspergillus* spp. The percentage of aglycones contents increased during fermentation periods. The increase percentage of aglycones was 25.94 % from unfermented soybean

2. Scavenging effect on ABTS radical

The antioxidant activity of fermented soybean product with *Aspergillus* spp. was determined by ABTS radical-scavenging activity. Non-fermented soybean (0 hour fermented) was the control sample. The results are shown in Table 4.10.

Table 4.10 The antioxidant activity in soybean fermented with *Aspergillus* spp.

Fermentation periods (hour)	% Inhibition ± S.D. (n=3)
control	15.39 ± 0.79
6	20.05 ± 1.26
12	20.21 ± 2.33
18	22.14 ± 2.20
24	20.37 ± 2.88
48	25.18 ± 1.17
72	20.45 ± 2.91

Figure 4.9 shows that the antioxidative activities gradually increased during 18-24 hours of incubation with *Aspergillus* spp.

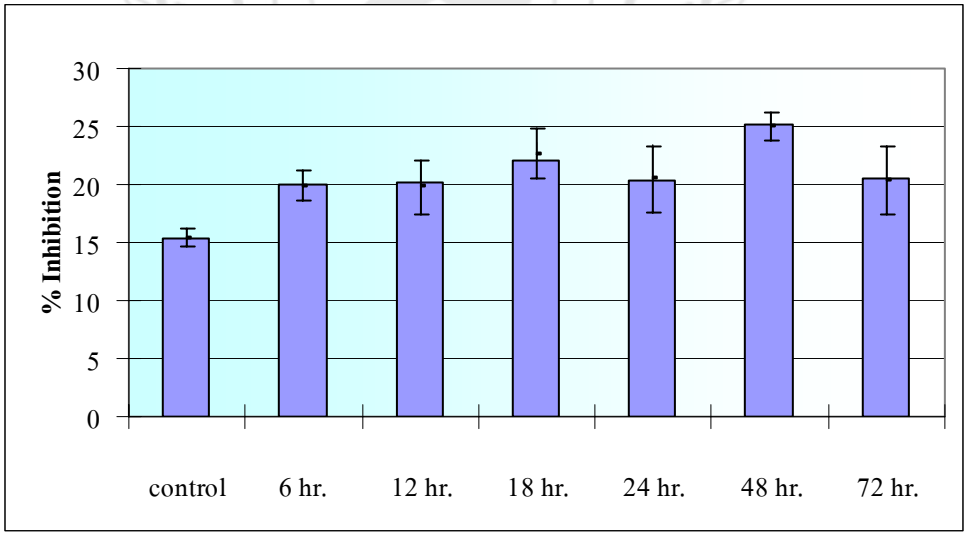


Figure 4.9 Change of the antioxidant activity in soybean after fermented with *Aspergillus* spp.

CHAPTER V

DISCUSSION

Legume seeds especially soybeans are used as food ingredients and seasoning in many countries. In Thailand, a traditional processed food containing soybean and other legume seeds such as thua nao (ถั่วเน่า) is available especially in the northern part. Soybean contains many kinds of polyphenols. The main polyphenols and isoflavone analogues are daidzein, genistein, daidzin and genistin. As mentioned in the reported data (Kuo, 2006[14]) isoflavones in soybean are an important antioxidant and some of them act as phytoestrogens. Although in Thailand many legume seeds are widely used, the quantities of isoflavones contents have not been reported. In the present study, seven kinds of Thai legumes were collected and determined their isoflavones contents compared with those of soybean. The results shown that, soybean has 100 times higher isoflavones contents than the other legumes. Interestingly, from the study of isoflavones contents in legumes plant by using HPLC, the chromatogram of gram and red bean were found the high intensity of unknown peaks with suggested being isoflavones. In this study, the total isoflavones mean the summary of the quantity of daidzein, genistein, daidzin and genistin, If these unknown peaks are real isoflavones, the quantity of total isoflavones containing in gram and red bean will higher than this report.

Generally, the quality of soybean was decided with mass product, protein content, resistance to insect and potential of nitrogen fixing. Besides them, isoflavones contents and antioxidative activity should be additional criteria for quality of soybean which used as healthfood. The eight breeds of soybeans were subjected to HPLC for quantitative analysis of isoflavones. The highest isoflavones (aglycones) was found in S.J. 2. A higher total isoflavones

contents of Thai soybean followed by those in S.J. 2, Chiang Mai 60, S.J. 9 (4), Khonkaen, Nakhonsawan, S.J. 5, Rajamongkon, and Chiang Mai 1, in decreasing order.. Although, Rachamongkol breeding gave the highest total isoflavones, in this study S.J. 2 (Tadang) was used as the raw material because of the highest isoflavone glycosides. The aim of this study was to study the antioxidative activity after fermentation which suggested that glycosides will break glycisidic linkage with β-glucosidase containing in microorganisms.

From the present study, the isoflavones (aglycones) and their glycosides containing in soybean were higher than other legume seeds. As mentioned in the reports (Kerry & Abbey, 1998[30]) and Lee, et al., 2005[31]), aglycones gave higher antioxidative activity than glycoside isoflavones. In human consumption, the glycoside isoflavones was changed to aglycones by the bacteria in the gut during the digestive processed (Majeed & Prakash, 2005[28]). In Asia countries, many food products and seasoning made from soybean, such as soy sauce, soy past, thua nao. The processing of soybeans into soy foods causes some loss and change of isoflavones (Murphy, et al., 1999[32] and Chiou R Y-Y, Cheng, 2001[33]). Quantity of isoflavones contents in soybean products was studied. From 4.2, the percentage of aglycones to total isoflavones containing in soybean products were higher than raw material (before fermentation) 21.36 - 70.27 %. Fermentation and acid hydrolysis process can increase the level of aglycones. Higher alycones gave higher antioxidative activity. Fermentation and acid hydrolysis process can transform soybean into the rich antioxidant product.

Most studies reported that after the fermentation the antioxidant activity of fermented soybean such as Tempeh, Miso and Natto, the soybeans fermented with *Rhizopus oligosporum*, *Aspergillus oryzea* and *Bacillus natto,* was significantly higher than that in non-fermented steamed soybean. It was suggested that daidzin and genistin, which each have a β-glucosidic linkage at the 7-position of the A ring, in steamed soybeans decreased with fermentation period, whereas aglycones, increased during fermentation. This is because glycoside isoflavones were hydrolyzed into aglycones, by β-glucosidase, produced from microorganism, *Rhizopus oligosporum*, *Aspergillus oryzea, Aspergillus awamori, Aspergillus sojae* and *Bacillus*

natto (Esaki, et al., 1999[9], Choi, Kim & Rhee, 2002[10], Miyake, et al., 2003[11], Lee & Chou, 2006[12], Lin, Wei & Chou, 2006[13] and Kuo, 2006[14]).

This research studied the quantity of isoflavones and their glycoside together with antioxidant acitivity of fermented soybean with 3 different test microorganisms; *Lactobacillus casei,*, the lactic acid bacteria which found in drinking yogurt; *Rhizopus oryzae*, one of fungus used for producing the soy sauce, Tempe and Saka and *Aspergillus* spp., the one of fungus that found in Japan soy. The results indicate that the isoflavones and their glycosides contents of all fermented soybeans changed after fermentation. The enzyme that produces from *L. casei* was transformed glycoside isoflavones structure into aglycones 21.31 % from initial, with the continuous transformation during fermentation period from initial until 72 hours. The $ABTS^{+\bullet}$ inhibition is increasing during fermentation period. The 48 to 72 hours of fermented periods gave aglycones in the highest ratio but the total isoflavones contents are decreased. Thus, the antioxidant activities of 48 to 72 hours of fermented soybean with *L. case*i decreased.

The enzyme that produce from *R. oryzae* in soybean product that fermented with *R. oryzae* transformed glycoside isoflavones structure into aglycones 33.56 % from initial, with the continuous transformation during fermentation period from initial until 72 hours. The $ABTS^{+\bullet}$ inhibition were continuous increasing during fermentation period.

The enzyme that produce from *Aspergillus* spp. in soybean product that fermented with *Aspergillus* spp. transformed glycoside isoflavones structure into aglycones 25.94 % from initial, with high transformation, start from 0 hour to 12 hours and then the transformation were constant until 72 hours. The $ABTS^{+\bullet}$ inhibition was increasing from 0 hour to 12 hours and the trend of antioxidant activity were constant until 72 hours.

Soybean product that fermented *with L. casei R. oryzae* and *Aspergillus* spp. can apply for health promotion product, food product or the product for external used such as anti-aging cosmetic product because they contain higher level of aglycones possessed higher antioxidant activity than those of non-fermented product. Besides the breaking of β-glucosidic

linkage, some of microorganism can also add hydroxyl group (OH) in isoflavones structure that isolated potent new antioxidative isoflavones (Esaki, et al., 1999[9] and Miyake, et al., 2003[11]). A part of this study, *Aspergillus orase* was used as the test microorganism in fermentation process. Examination of chemical compounds by HPLC techniques found that there is a new peak (between 18 to 19 minutes in front of genistin) at 12-72 hours after fermented. Results are shown in Figure 5.1. Unfortunately, the unknown was not made clear because the quantity of the sample was limited.

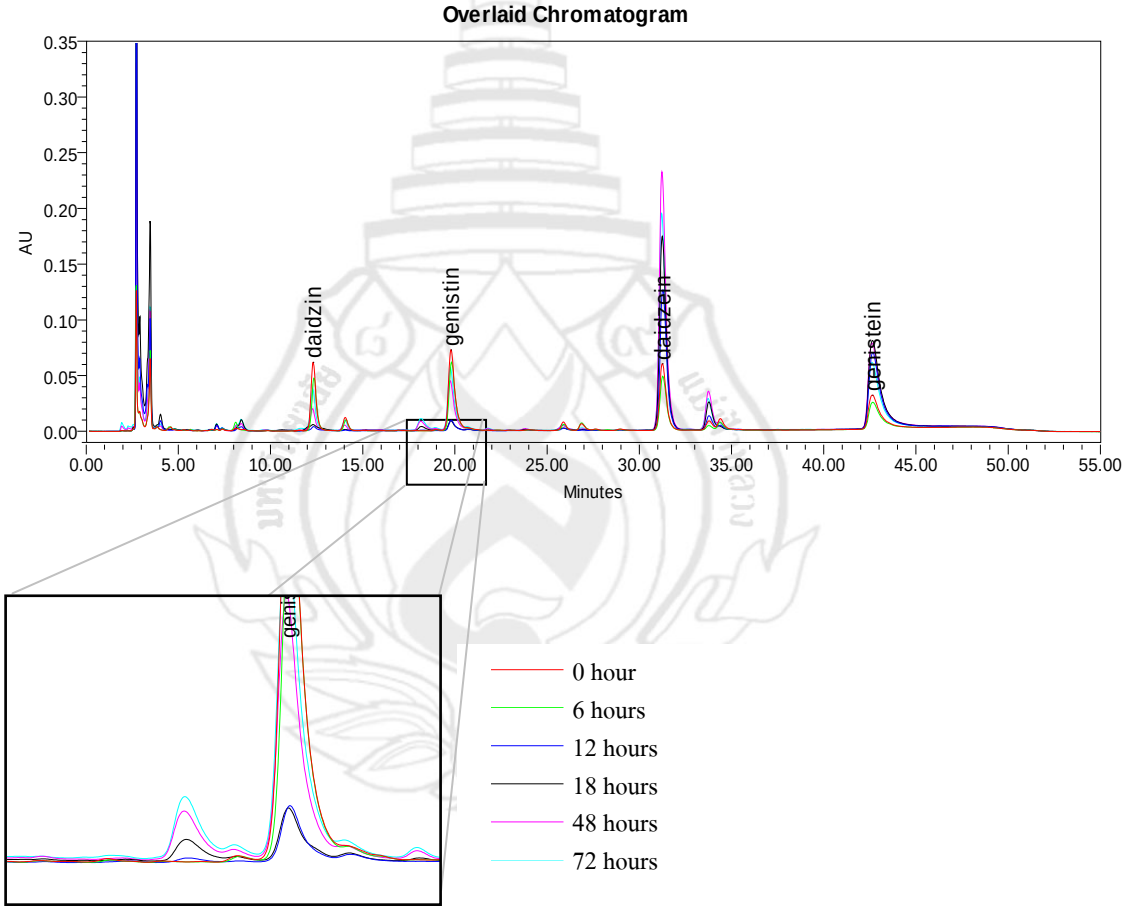


Figure 5.1 The overlaid chromatogram of isoflavones in soybean and soybean fermented with *Aspergillus orase* at 0, 6, 12, 18, 48 and 72 hours.

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APPENDIX

AACC method 20-20

Determination of Isoflavones in Soy and Selected Foods Containing Soy by Extraction, Saponification, and Liquid Chromatography

First approval October 17, 2001

Objective

Test samples are extracted at 65° for 2 hr in 80% methanol solution and then saponified at ambient temperature with sodium hydroxide. The extracts are acidified, filtered, and diluted with water to 50% methanol. They are then centrifuged to clarify and are analyzed by liquid chromatography (LC). Isoflavone glucosides and aglucons are separated using a C-18 reverse-phase column with a methanol-water mobile phase and are measured by UV detection at 260 nm. Results are expressed in aglycon units by summing the concentrations of the aglycon isoflavones (genistein, glycitein, and daidzein) and the concentrations of the aglycon equivalents of the corresponding glucoside forms (genistin, glycitin, and daidzin).

Apparatus

1. HPLC system.
   a. Pump, high-pressure, binary gradient, capable of operating continuously at 0.4–1.5 ml/min, with a flow precision of ±1% or better.
   b. Injector, manual or autosampling, equipped with a 100-µl fixed loop.
   c. Chromatography column, reverse-phase C-18, 5 µm (2.1 × 200 mm, or 4.6 × 200 mm), capable of separating daidzin and glycitin with a resolution of 1.0 or greater.
   d. Detector, capable of monitoring absorbance at 260 nm.
   e. Recorder, integrator, or data acquisition system. Compatible with detector used.
2. Balance, analytical, capable of weighing to 0.00001 g.
3. Balance, analytical, capable of weighing to 0.001 g.
4. Dispenser, capable of dispensing 50 ± 0.5 ml 80% methanol.
5. Pipettes, capable of dispensing 1–5 ml; with disposable tips.
6. Water bath, capable of maintaining 65°; equipped with shaker.
7. Orbital platform shaker, equipped to hold 250-ml Erlenmeyer flasks.
8. Filter paper, 15 cm, quantitative grade, medium porosity, and fan folded.
9. Centrifuge, capable of centrifuging 1 ml fluid at 7000 × *g*.
10. Microfuge tube, 1.5 ml disposable.
11. Extraction flasks, Erlenmeyer flasks, 250 ml, glass stoppered.
12. Beakers, 250 ml.
13. Filter funnels, 75 mm.
14. Graduated cylinders, glass stoppered, 10 and 50 ml capacity.

Determination of Isoflavones in Soy and Selected Foods Containing Soy by Extraction, Saponification, and Liquid Chromatography (continued)

15. Volumetric flasks, low actinic, 50 ml; volumetric flasks, 50, 100, 200, and 1000 ml.

16. Glass vials for LC autosampler, equipped with Teflon-lined septa.

Reagents

1. Stock standard solutions. Using an analytical balance capable of weighing to 0.00001 g, weigh 5 mg daidzin, 5 mg genistin, 20 mg daidzein, 20 mg genistein, and 5 mg glycitein into five separate 50-ml low-actinic volumetric flasks. Quantitatively transfer contents of a 2-mg vial of glycitin into a 50-ml low-actinic volumetric flask, rinsing vial repeatedly with methanol and adding to the volumetric flask. Dissolve in methanol and dilute to volume. Stopper each flask and mix well by repeated inversion. Store at room temperature in low-actinic glass flasks for up to 6 months.

2. Working standard solutions. Prepare five levels of working standards by combining the volume of each stock standard shown in Table I in the size volumetric flask indicated in Table I. Add the volume of water shown in Table I and dilute to volume with 50% methanol. The approximate concentration of each isoflavone is shown in Table II. For standards below 99+% purity, adjust values

TABLE I
Working Standard Dilutions from Stock Standard Solution

Working Standard	Each Stock Standard (ml)	Water (ml)	Final Volume (ml)
1	1.0	6.0	200
2	1.0	6.0	100
3	2.0	12.0	100
4	4.0	24.0	100
5	4.0	24.0	50

TABLE II
Approximate Concentrations of Each Working Standard Solution

Working Standard	Daidzin Stock Standard (µg/g)	Glycitin Stock Standard (µg/g)	Genistin Stock Standard (µg/g)	Daidzein Stock Standard (µg/g)	Glycitein Stock Standard (µg/g)	Genistein Stock Standard (µg/g)
1	0.5	0.02	0.5	2.0	0.5	2.0
2	1.0	0.04	1.0	4.0	1.0	4.0
3	2.0	0.08	2.0	8.0	2.0	8.0
4	4.0	0.16	4.0	16.0	4.0	16.0
5	8.0	0.32	8.0	32.0	8.0	32.0

Determination of Isoflavones in Soy and Selected Foods Containing Soy by Extraction, Saponification, and Liquid Chromatography (continued)

for the standard purity accordingly. Store solutions at room temperature in low-actinic glass flasks for up to 6 months.

3. Methanol, LC grade.

4. Hexane, LC grade.

5. Acetic acid, glacial.

6. Extraction solution, 80% methanol. Add 800 ml methanol to a 1000-liter volumetric flask. Add 200 ml of water (do not dilute to volume), stopper, and mix well with inversion.

7. Methanol, 50%. Combine 250 ml methanol with 250 ml water, mix well, and filter, using vacuum through a 0.45-µm filter.

8. Mobile phase A, H_2O + methanol + acetic acid (88 + 10 + 2). Combine 3520 ml water, 400 ml methanol, and 80 ml glacial acetic acid. Mix well and filter, using vacuum through a 0.45-µm filter.

9. Mobile phase B, methanol + acetic acid (98 + 2). Add 3920 ml methanol to a 6-liter Erlenmeyer flask. Add 80 ml glacial acetic acid and mix well. Filter through a 0.45-µm filter disk with vacuum.

10. Sodium hydroxide, 2*M*. Weigh 80 g sodium hydroxide into 1-liter volumetric flask, dissolve in water, allow to cool to ambient temperature, and dilute to volume with water.

Procedure

Extraction

1. Accurately weigh an amount of test sample that would contain ~1 g protein, but not exceeding 5 g test sample, into a 250-ml Erlenmeyer flask with ground glass stopper. Add 40 ml extraction solution and place stopper on flask. Cover stopper and neck of flask with aluminum foil and shake in a 65°C water bath for 2 hr.

2. Cool to room temperature.

Saponification

1. Add 3 ml 2*M* NaOH. Replace aluminum foil and shake at room temperature on orbital shaker for 10 min. Remove flasks from shaker, and add 1 ml glacial acetic acid.

2. Swirl to suspend test portion and pour into a 50-ml graduated cylinder equipped with ground glass stopper. Dilute to 50 ml with extraction solution and mix well.

3. Filter test portion solutions through quantitative-grade filter paper into a 250-ml beaker. Pipet 5 ml filtrate into a 10-ml graduated cylinder equipped with

Determination of Isoflavones in Soy and Selected Foods Containing Soy by Extraction, Saponification, and Liquid Chromatography (continued)

ground glass stopper. Add 4.0 ml water and dilute to 10 ml with methanol. Stopper graduated cylinder and invert repeatedly to mix.

4. Transfer ~1 ml extract to a 1.5-ml centrifuge tube and centrifuge for 5 min at 7000 × *g*. Transfer clear supernatant to LC sample vials. Do not filter supernatant through membrane filter.

LC determination

1. Set LC system to 0.4-ml flow rate for 2.1-mm i.d. columns and initial mobile phase composition shown in Table III. If using 4.6-mm i.d. column, set flow rate to 1.5 ml/min and use same gradient. Set detector wavelength to 260 nm. Allow system to equilibrate by running one complete gradient with no injection.

2. Verify system performance by injecting 20 µl working standard 3, and analyze using gradient conditions in Table III. Verify baseline separation of daidzein and glycitein peaks.

3. Tailing factor for any peak should be ≤1.5. Adjust either %B or gradient times, as needed, to obtain required separation of all six components. Typical relative retention times (in min) are as follows: daidzin, 0.53; glycitin, 0.58; genistin, 0.66; daidzein, 0.89; glycitein, 0.92; and genistein, 1.00. Retention times will vary with age and condition of column. See Figure 1 for chromatogram of standard solution 3.

4. Inject all working standards and each test sample extract. Determine area of each isoflavone peak.

Integration

Peak width, threshold settings, and other integration parameters should be chosen to ensure consistent integration of both samples and standards. Carefully

TABLE III
Liquid Chromatography Pump Gradient for Each Run

Step	Start Time (min)	End Time (min)	Mobile Phase Composition at End Time[a]	
			%A	%B
Initial	0	0.1	90	10
2	0.1	30	40	60
3	31	31.5	0	100
4	37	37.5	90	10
5	44.5	Stop run	90	10

[a] All gradients are linear.

Determination of Isoflavones in Soy and Selected Foods Containing Soy by Extraction, Saponification, and Liquid Chromatography (continued)

monitor integrator baseline selection to be sure that it is appropriate. Peak area may be used for quantification.

Calculation

1. Determine response for each isoflavone by calculating the slope (m) and intercept (b), using linear regression analysis of area counts vs. response for five levels of isoflavone standards. Calculate concentration of each isoflavone in test samples using the following equation:

$$\text{Isoflavone, } \mu\text{g/g} = \frac{[(A_s \times m) + b] \times 50 \times 10}{W_s \times 5}$$

where A_s = area of isoflavone peak in test sample; m = slope from linear regression for standard response; b = intercept from linear regression for standard response; W_s = weight of test sample, g; 50 = dilution volume; 10 = second dilution volume; 5 = aliquot volume.

2. Convert the isoflavone glucosides genistin, glycitin, and daidzin concentrations to aglycon equivalents using the following equation:

$$C_{ae} = \left(\frac{MW_a}{MW_g}\right) \times C_g$$

where C_{ae} = concentration of isoflavone aglycon equivalent (µg/g); MW_a = molecular weight of aglycon (see Table IV); MW_g = molecular weight of

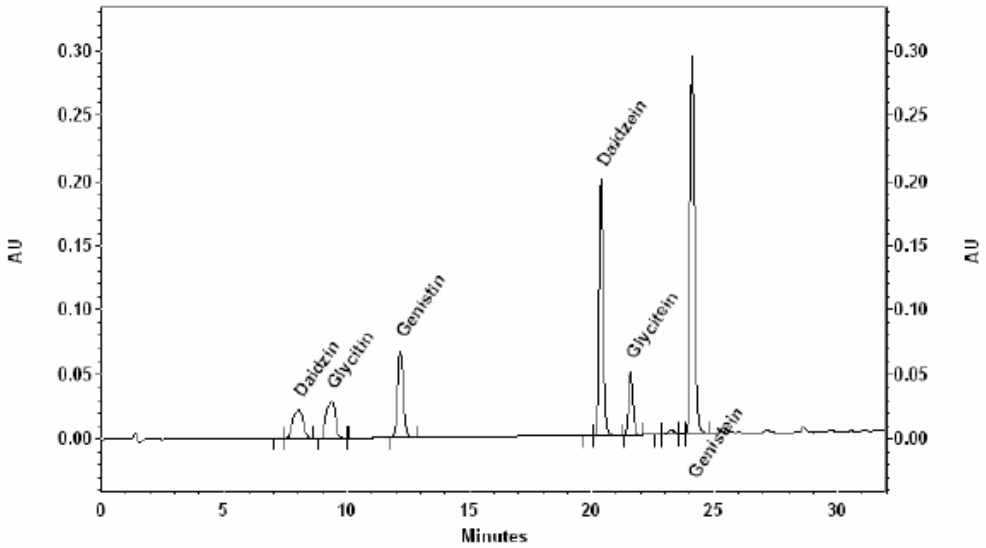

Fig. 1. Chromatogram of isoflavone standard solution 3.

Determination of Isoflavones in Soy and Selected Foods Containing Soy by Extraction, Saponification, and Liquid Chromatography (continued)

TABLE IV
Aglycon Conversion Factors

Isoflavone Glucoside	MW_a	MW_g	MW_a/MW_g
Genistin	270	432	0.625
Glycitin	284	446	0.637
Daidzin	254	416	0.611

glucoside (see Table IV); and C_g = concentration of genistin, glycitin, or daidzin (µg/g).

3. Calculate total isoflavones, µg/g aglycon equivalent, by summing the concentrations of daidzein, glycitein, and genistein and adding to the sum of the concentrations of the aglycon equivalents daidzin, glycitin, and genistin.

$$T_a = C_a\,(\text{daidzein}) + C_a\,(\text{glycitein}) + C_a\,(\text{genistein})$$
$$T_{ae} = C_{ae}\,(\text{daidzin}) + C_{ae}\,(\text{glycitin}) + C_{ae}\,(\text{genistin})$$

where T_a = sum of concentrations of aglucons, and T_{ae} = sum of concentrations of aglycon equivalents of glucosides.

$$\text{Total isoflavones, µg/g aclycon equivalent} = T_a + T_{ae}$$

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