CHAPTER II

LITERATURE REVIEW

1. Scientific name: *Glycine max* (L.) Merr.

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Subfamily: Faboideae

Genus: *Glycine*

Species: *G. max*

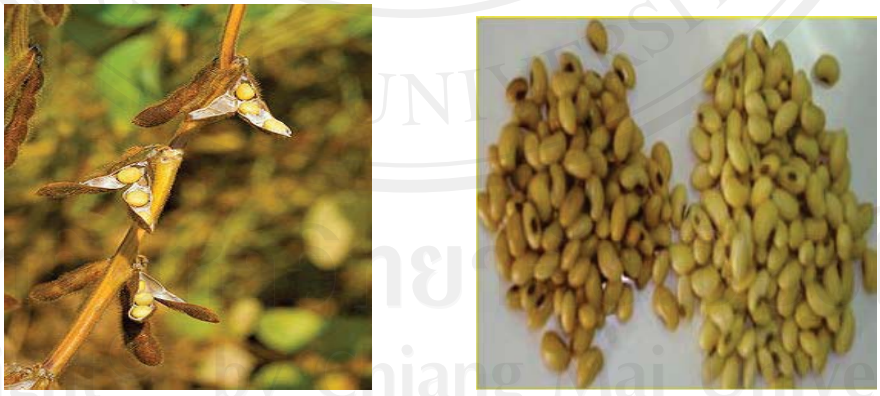


Figure 1. Leaves and seed of soybean

(From : www. mof.or.th/rai/soybean/soybean.html)

1.1 Description

The soybean (U.S.) or soya bean (UK) (*Glycine max*) is a species of legume native to East Asia. The plant is classed as an oilseed rather than a pulse. It is an annual plant that has been used in China for 5,000 years as a food and a component of drugs. Soy contains significant amounts of all the essential amino acids for humans, and so is a good source of protein. Soybeans are the primary ingredient in many processed foods, including dairy product substitutes. The plant is sometimes referred to as greater bean or edamame, though the latter is more commonly used in English when referring to a specific dish. The English word soy is derived from the Japanese pronunciation of shoyu, the Japanese word for Soya sauce; soya comes from the Dutch adaptation of the same word.

Soybeans are an important source of vegetable oil and protein world wide. Soy products are the main ingredients in many meat and dairy substitutes. They are also used to make soy sauce, and the oil is used in many industrial applications. The main producers of soy are the United States, Brazil, Argentina, China and India. The beans contain significant amounts of alpha-linolenic acid, omega-6 fatty acid, and the isoflavones genistein and daidzein.

1.2 Chemical composition of the seed

Together, oil and protein content account for about 60% of dry soybeans by weight; protein at 40% and oil at 20%. The remainder consists of 35% carbohydrate and about 5% ash. Soybean cultivars comprise approximately 8% seed coat or hull, 90% cotyledons and 2% hypocotyl axis or germ. The majority of soy protein is a relatively heat-stable storage protein. This heat stability enables soy food products requiring high temperature cooking, such as tofu, soymilk and textured vegetable

protein (soy flour) to be made. The principal soluble carbohydrates, saccharides, of mature soybeans are the disaccharide sucrose (range 2.5–8.2%), the trisaccharide raffinose (0.1–1.0%) composed of one sucrose molecule connected to one molecule of galactose, and the tetrasaccharide stachyose (1.4 to 4.1%) composed of one sucrose connected to two molecules of galactose. While the oligosaccharides raffinose and stachyose protect the viability of the soy bean seed from desiccation. They are not digestible sugars and therefore contribute to flatulence and abdominal discomfort in humans and other monogastric animals; compare to the disaccharide trehalose. Undigested oligosaccharides are broken down in the intestine by native microbes producing gases such as carbon dioxide, hydrogen, methane, etc. Since soluble soy carbohydrates are found in the whey and are broken down during fermentation, soy concentrate, soy protein isolates, tofu, soy sauce, and sprouted soy beans are without flatus activity. On the other hand, there may be some beneficial effects to ingesting oligosaccharides such as raffinose and stachyose, namely, encouraging indigenous bifidobacteria in the colon against putrefactive bacteria. The insoluble carbohydrates in soybeans consist of the complex polysaccharides cellulose, hemicellulose, and pectin. The majority of soybean carbohydrates can be classed as belonging to dietary fiber.

1.3 Nutrition

The most important point regarding the use of soybeans for human nutrition is the absolute necessity to cook the soybean with "wet" heat in order to destroy the trypsin inhibitors; serine protease inhibitors. It is not advisable to eat raw soybeans. Soybeans are considered by many agencies, including the US Food and Drug Administration, to be a source of complete protein. (USFDA, 2000). A complete

protein is one that contains significant amounts of all the essential amino acids that must be provided to the human body because of the body's inability to synthesize them. For this reason, soy is a good source of protein, amongst many others, for many vegetarians and vegans or for people who cannot afford meat.

According to the FDA, Soy protein products can be good substitutes for animal products because, unlike some other beans, soy offers a complete protein profile. Soybeans contain all the amino acids essential to human nutrition, which must be supplied in the diet because they cannot be synthesized by the human body. Soy protein products can replace animal-based foods--which also have complete proteins but tend to contain more fat, especially saturated fat without requiring major adjustments elsewhere in the diet. Because soybeans contain no starch, they are a good source of protein for diabetics (USFDA, 2000). However, as with any dietary health claim, there are opposing viewpoints on the health benefits of soybeans (James, 1998). The gold standard for measuring protein quality, since 1990, is the Protein Digestibility Corrected Amino Acid Score (PDCAAS) and by this criterion soy protein is the nutritional equivalent of meat and eggs for human growth and health. Soybean protein isolate has a Biological Value of 74, whole soybeans 96, soybean milk 91, and eggs 97 (FAO, 1991). Soy protein is essentially identical to that of other legume pulses seeds (Derbyshire, 1976). Moreover, it has the highest yield per square meter of growing area, and is the least expensive source of dietary protein. Consumption of soy may also reduce the risk of colon cancer, possibly due to the presence of sphingolipids (Symolon et al., 2004).

Table 1. The amount of essential amino acid in soybean compare with FAO/WHO recommendation.

Amino acid	FAO/WHO mg/g protein	Soybean mg/g protein
Isoleucine	40	37
Leucine	70	74
Lysine	55	59
Methionine + Cystine	35	22
Phenylalanine + tyrosine	60	64
Threonine	40	42
Tryptophan	10	15
Valine	50	50

Table 2. The amount of fatty acid in soybean oil (Shil, 1994).

Fatty acid	Soy milk (%)
Saturated fatty acid	
Palmitic acid (C 16 : 0)	11
Stearic acid (C 18 : 0)	4
Unsaturated fatty acid	
Oleic acid (C 18 : 1)	23
Linoleic acid (C 18 : 2 omega-6)	51
Linolenic acid (C 18 : 3 omega-3)	7

Table 3. Proximate composition of whole seeds of 6 soybean varieties.

Variety	Content* (%, dry wt basis)				
	Protein	Lipid	Carbohydrates	Crude fiber	Ash
Chiangmai 1	37.59 ± 0.30c	20.82 ± 0.28a	36.28 ± 0.56c	4.90 ± 0.21c	5.31 ± 0.01
Chaingmai 60	37.62 ± 0.04c	18.00 ± 0.62d	38.90 ± 0.77b	8.02 ± 0.44a	5.49 ± 0.09
Rajamangala	37.61 ± 0.36c	14.71 ± 0.10e	42.24 ± 0.17a	6.11 ± 0.40b	5.44 ± 0.10
Sor Jor 4	38.54 ± 0.35b	20.34 ± 0.06b	35.79 ± 0.41c	5.91 ± 0.61b	5.34 ± 0.09
Sor Jor 2	39.55 ± 0.18a	19.19 ± 0.19c	35.91 ± 0.25c	5.09 ± 0.50c	5.46 ± 0.01
Sor Jor 5	39.49 ± 0.33a	18.54 ± 0.12d	36.48 ± 0.46c	5.32 ± 0.18c	5.49 ± 0.12

*Mean ± SD of triplicate determinations. Means within a column followed by different letters are significantly different ($P<0.05$).

Table 4. Moisture contents of soybeans varieties.

variety	Moisture (%)
Chiangmai 1	9.71
Chaingmai 60	10.58
Rajamangala	8.03
Sor Jor 4	11.49
Sor Jor 2	11.48
Sor Jor 5	10.07

The proximate compositions of soybeans of 6 varieties commonly grown in Thailand are shown in Table 3. Moisture contents of soybeans tested (Table 4) were in the range of 8.0 to 11.5 %. As the water content of soybeans may vary according to storage conditions, composition data are expressed on moisture-free basis. Significant differences were observed among these varieties concerning their contents of protein lipid, carbohydrates and crude fiber. Sor Jor varieties generally contained higher content of protein. Glycinin and β-conglycinin are the major storage proteins in soybeans (Cai and Chang, 1999). In addition soy proteins contain a number of bioactive components that are of interests for human health benefits (Potter, 1995). Isoflavones are a class of flavonoids that are common constituents of soybean. During processing of soybeans, most of the isoflavones remain with the protein fraction. The isoflavone concentration in soy proteins varies widely due to differences in levels in the soybeans. However, soy protein can contain up to about 4-5 mg of isoflavones per g of protein (Hodgeson, 2003). The lipid levels are between 18 and 20% except for Rajamangala variety. The lipids of soybeans consist typically of triglycerides, phospholipids, unsaponifiables, free fatty acids and minute amounts of carotenoid pigments. Rajamangala had the highest carbohydrate content. Usually soybeans contain about 30% carbohydrates. These carbohydrates can be divided into two groups including soluble sugars (such as sucrose, stachyose, and raffinose) and insoluble fiber comprising with complex mixture of cell wall carbohydrates. Among varieties tested, Chaingmai 60 contained the highest crude fiber content, followed by Rajmangala, and Sor Jor 4, respectively. However, no significant differences in ash content were observed ($P>0.05$). Berk (1992) reported that the major mineral constituents are potassium, calcium and magnesium. The minor constituents comprise trace elements of nutritional importance, such as iron, zinc, copper etc.

1.4 Role of soyfoods in disease prevention

1.4.1 Omega-3 fatty acids

Omega-3 fatty acids, for example, alpha-linolenic acid C18-3, all cis, 9,12,15 octadecatrienoic acid (where the omega-3 refers to carbon number 3 counting from the hydrocarbon tail whereas C-15 refers to carbon number 15 counting from the carboxyl acid head) are special fat components that benefit many body functions. However, the effects which are beneficial to health are associated mainly with the longer-chain, more unsaturated fatty acids eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) found in fish oil and oily fish. For instance, EPA and DHA, inhibit blood clotting, while there is no evidence that alpha-linolenic acid (aLNA) can do this. Soybean oil is one of the few common vegetable oils that contains a significant amount of aLNA; others include canola, walnut, hemp, and flax. However, soybean oil does not contain EPA or DHA. Soybean oil does contain significantly greater amount of omega-6 fatty acids in the oil: 100g of soybean oil contains 7g of omega-3 fatty acids to 51g of omega-6: a ratio of 1:7. Flaxseed, in comparison, has an omega-3:omega-6 ratio of 3:1.

1.4.2 Isoflavones

Soybeans also contain the isoflavones genistein and daidzein, types of phytoestrogen, that are considered by some nutritionists and physicians to be useful in the prevention of cancer and by others to be carcinogenic and endocrine disruptive. Soy's content of isoflavones are as much as 3 mg/g dry weight. Isoflavones are polyphenol compounds, produced primarily by beans and other legumes, including peanuts and chickpeas. Isoflavones are closely related to the antioxidant flavonoids found in other plants, vegetables and flowers. Isoflavones such

as genistein and daidzein are found in only some plant families, because most plants do not have an enzyme, chalcone isomerase which converts a flavone precursor into an isoflavone. In contradiction to well known benefits of isoflavones, Genistein acts as an oxidant (stimulating nitrate synthesis) (Gottstein et al., 2003) as well as it blocks formation of new blood vessels (antiangiogenic effects) (Sasamura et al., 2004). Some studies show Genistein to act as inhibitor of the activity of substances in the body that regulate cell division and cell survival (growth factors).

1.4.3 Claims of cholesterol reduction

The dramatic increase in soyfood sales is largely credited to the Food and Drug Administration's (FDA) approval of soy as an official cholesterol-lowering food, along with other heart and health benefits. A 2001 literature review argued that these health benefits were poorly supported by the available evidence, and noted that disturbing data on soy's effect on the cognitive function of the elderly existed (Sirtori, 2001). In 2008, an epidemiological study of 719 Japanese men found that tofu intake was associated with worse memory, but tempeh (a fermented soy product) intake was associated with better memory (Hogervorst, 2008). This study replicated other studies. From 1992 to 2003, sales have experienced a 15% compound annual growth rate, increasing from $300 million to $3.9 billion over 11 years, as new soyfood categories have been introduced, soyfoods have been repositioned in the market place, thanks to a better emphasis on marketing nutrition. In 1995, the New England Journal of Medicine (Vol. 333, No. 5) published a meta-analysis financed by DuPont Protein Technologies International (PTI), which produces and markets soy through The Solae Company. The meta-analysis concluded that soy protein is correlated with significant decreases in serum cholesterol, LDL (bad cholesterol) and triglycerides.

However, HDL (good cholesterol) did not increase by a significant amount. Soy phytoestrogens (isoflavones: genistein and daidzein) adsorbed onto the soy protein were suggested as the agent reducing serum cholesterol levels. On the basis of this research PTI filed a petition with FDA in 1998 for a health claim that soy protein may reduce cholesterol and the risk of heart disease. The FDA granted the following health claim for soy: "25 grams of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease (Henkel, 2008). One serving, (1 cup or 240 mL) of soy milk, for instance, contains 6 or 7 grams of soy protein. Solae resubmitted their original petition, asking for a more vague health claim, after their original was challenged and highly criticized. Solae also submitted a petition for a health claim that soy can help prevent cancer. They quickly withdrew the petition for lack of evidence and after more than 1,000 letters of protest were received. In February 18, 2008 Weston A. Price Foundation submitted a petition for removal of this health claim. (In January 2006 an American Heart Association review (in the journal "Circulation") of a decade long study of soy protein benefits casts doubt on the FDA allowed "Heart Healthy" claim for soy protein and did not recommend isoflavone supplementation. The review panel also found that soy isoflavones have not been shown to reduce post menopause "hot flashes" in women and the efficacy and safety of isoflavones to help prevent cancers of the breast, uterus or prostate is in question (Sack et al., 2006).

1.4.4 Phytoestrogen

Soybeans contain isoflavones called genistein and daidzein, which are one source of phytoestrogens in the human diet. Because most naturally occurring estrogenic substances show weak activity, normal consumption of foods that contain

these phytoestrogens should not provide sufficient amounts to elicit a physiological response in humans. Plant lignans associated with high fiber foods such as cereal brans and beans are the principal precursor to mammalian lignans which have an ability to bind to human estrogen sites. Soybeans are a significant source of mammalian lignan precursor secoisolariciresinol containing 13–273 μg/100 g dry weight (Adlercreutz et al., 2000). Another phytoestrogen in the human diet with estrogen activity is coumestans, which are found in beans, split-peas, with the best sources being alfalfa, clover, and soybean sprouts. Coumestrol, an isoflavone coumarin derivative is the only coumestan in foods (De Kleijn et al., 2002). Soybeans and processed soy foods do not contain the highest "total phytoestrogen" content of foods. A study in which data were presented on an as-is (wet) basis per 100 g and per serving found that food groups from highest to lowest levels of total phytoestrogens per 100 g are nuts and oilseeds, soy products, cereals and breads, legumes, meat products, various processed foods that may contain soy, vegetables, and fruits (Thompson et al., 2006). In women, a 2001 literature review suggested that women with current or past breast cancer should be aware of the risks of potential tumor growth when taking soy products, based on the effect of phytoestrogens to promote breast cancer cell growth in animals (De lemos et al., 2001). A 2006 commentary reviewed the relationship with soy and breast cancer. They stated that soy may prevent breast cancer, but cautioned that the impact of isoflavones on breast tissue needs to be evaluated at the cellular level in women at high risk for breast cancer (Messina et al., 2006). A high consumption of omega-6 polyunsaturated fatty acids, which are found in most types of vegetable oil including soybean oil, may increase the likelihood that postmenopausal women will develop

breast cancer (Emily et al., 2008). Other analysis suggested an inverse association between total polyunsaturated fatty acids and breast cancer risk (Valeria et al., 2001).

In Men**,** because of the phytoestrogen content, some studies, but not all, have suggested that there is an inverse correlation between soybean ingestion and testosterone in men (Dillingham et al., 2005). For this reason, they may protect against the development of prostate cancer (Heald et al., 2007). A theoretical decrease in the risk of prostate cancer should, however, be weighed against the possible side-effects of decreased testosterone, which are still unclear. The popular fear that soybeans might cause reduced libido and even feminine characteristics in men has not been indicated by any study; the popularity of the notion seems to be based on the simplistic misapprehension that estrogen and testosterone have a simple, inverse relationship in sexual hormone systems and sex-related behaviour. Their interplay is very complicated and largely still unknown (Maskarinec et al., 2006). A study published in April 2008 concluded that soy food intake has an inverse association with sperm concentration in fertility-deficient men. The same study found that soy intake does not affect sperm motility, morphology or ejaculate volume (Chavarro et al., 2008).

1.5 Promotion as health food

Soy consumption has been promoted by natural food companies and the soy industry's aggressive marketing campaign in various magazines, television ads and in health food markets. Research has been conducted examining the validity of the beneficial health claims with regard to the increase in consumption of soybeans which mimic hormonal activity. A practice guideline published in the journal *Circulation* questions the efficacy and safety of soy isoflavones for preventing or treating cancer

of the breast, endometrium, and prostate (although the same study also concludes that soy in some foods should be beneficial to cardiovascular and overall health) and does not recommend usage of isoflavone supplements in food or pills (Sacks, 2006). A review of the available studies by the United States' Health and Human Services' Agency for Healthcare Research and Quality (AHRQ) found little evidence of substantial health improvements and no adverse effects, but also noted that there was no long-term safety data on estrogenic effects from soy consumption.

Estrogen helps protect and repair the brain during and after injury (Eberling et al., 2003). The mimicry of estrogen by the phytoestrogens in soy has introduced a controversy over whether such a replacement is harmful or helpful to the brain. Several studies have found soy to be harmful for rats (File et al., 2003). Nevertheless the cited study was based on rats fed with concentrated phytoestrogens and not common soybeans. The common amounts of phytoestrogens in soy beans are not to be compared to concentrated estrogen. One study followed over 3000 Japanese men between 1965 and 1999, and that showed a positive correlation between brain atrophy and consumption of tofu (White et al., 2000). A study on elderly Indonesian men and women found that tempeh consumption was independently related to better memory (Hogervorst et al., 2008).

Raw soy flour is known to cause pancreatic cancer in rats (Dethloff et al., 2000). Whether this is also true in humans is unknown because no studies comparing cases of pancreatic cancer and soy intake in humans have yet been conducted, and the doses used to induce pancreatic cancer in rats are said to be larger than humans would normally consume. Heated soy flour may not be carcinogenic in rats. Existing cancer patients are being warned to avoid foods rich in soy because they can accelerate the growth of tumours (Roebuck et al., 1987).

2. Fermented soyfoods

Some microorganisms, especially fungi and yeasts, are known to produce antioxidative substances (Hayashi et al., 1995). *Aspergillus* strains used in traditional manufacturing of fermented foods and beverages are safe since those microbes have been eaten in safety by people over a long term of years (Barbesgaard et al., 1992). In oriental countries, filamentous fungi such as *Aspergillus* and *Rhizopus*, are usually inoculated into the solid culture of steamed soybean, rice or barley in koji preparation. The prepared koji was then used to prepare traditional fermented food products (Wang et al., 2004). The antioxidative activity of fermented soybean products such as miso, tempeh and natto, inoculated with *Aspergillus oryzae*, *Rhizopus oligosporum* and *Bacillus natto*, respectively, was significantly higher than in non-fermented steamed soybean.

2.1 Characteristic of *Aspergillus*

Taxonomic Classification

Kingdom: Fungi

Phylum: Ascomycota

Class: Ascomycetes

Order: Eurotiales

Family: Trichocomaceae

Genus: *Aspergillus*

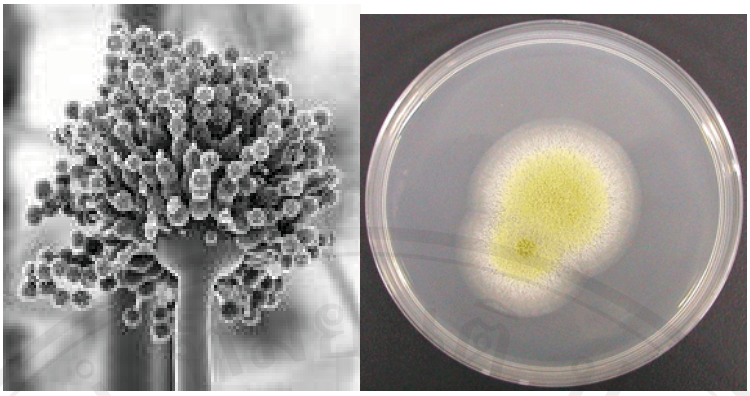


Figure 2. *Aspergillus* conidium head and colony on agar medium (From : www. biomedcentral.com)

2.2 Description and Natural Habitats

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. While a teleomorphic state has been described only for some of the *Aspergillus* spp., others are accepted to be mitosporic, without any known sexual spore production.

2.3 Growth and distribution

Aspergillus species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on the surface of a substrate, as a result of the high oxygen tension. Commonly, fungi grow on carbon-rich substrates such as monosaccharides (such as glucose) and polysaccharides (such as amylose). *Aspergillus* species are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees (Reverberi et al., 2008).

In addition to growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key

nutrients. *A. niger* is a prime example of this; it can be found growing on damp walls, as a major component of mildew.

2.4 Commercial importance

Species of *Aspergillus* are important medically and commercially. Some species can cause infection in humans and other animals. Some infections found in animals have been studied for years. Some species found in animals have been described as new and specific to the investigated disease and others have been known as names already in use for organisms such as saprophytes. More than 60 *Aspergillus* species are medically relevant pathogens (Thom and church, 1926). For humans there is a range of diseases such as infection to the external ear, skin lesions, and ulcers classed as mycetomas.

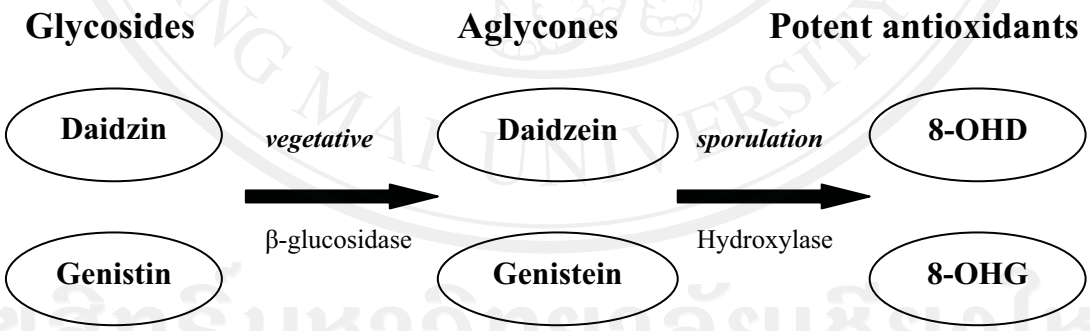
Other species are important in commercial microbial fermentations. For example, alcoholic beverages such as Japanese sake are often made from rice or other starchy ingredients (like manioc), rather than from grapes or malted barley. Typical microorganisms used to make alcohol, such as yeasts of the genus *Saccharomyces*, cannot ferment these starches, and so koji mold such as *Aspergillus oryzae* is used to break down the starches into simpler sugars.

Aspergillus oryzae is a filamentous fungus (a mold). It is used in Chinese and Japanese cuisine to ferment soybeans to produce soy sauce and miso. It is also used to saccharify rice, other grains, and potatoes in the making of alcoholic beverages such as huangjiu, *sake*, and shochu. Also, the fungus is used for the production of rice vinegars. The protease enzymes produced by this species are marketed by the company Novozymes under the name Flavourzyme. The importance of *A. oryzae* has led to its recognition as Japan's national micro-organism (kokkin),

just as the sakura cherry blossom is Japan's national flower (Bozkurt et al., 2008; Du et al., 2008).

2.5 *Aspergillus* strains used in fermented foods

- *A. oryzae*
- *A. sojae*
- *A. niger*
- *A. terricola*
- *A. flavas*
- *A. ornatus*
- *A. awamori*
- *A. kawachii*
- *A. japonicus*



Scheme 1. Proposed scheme for the formation of potent antioxidative substances by fermented with *Aspergillus.*

Daidzin and genistin , which each have β-glucosidic linkage, were gradually hydrolyzed into the corresponding aglycones, daidzein and genistein, respectively, by β-glucosidase produced from the *Aspergillus* fermentation. This hydrolysis tended to

proceed slowly during the stage of vegetative hypha growth because of its poor production of β-glucosidase. During this stage, no 8-OHD or 8-OHG was detectable. β-glucosidase activities were greatly enhanced at the stage of sporulation. Therefore, daidzin and genistin were found to decrease and their aglycones to increase during this stage. The resulting daidzein and genistein were then hydroxylated to produce the potent antioxidant, 8-OHD and 8-OHG, respectively, by the hydroxylase produced from *Aspergillus* fermentation during stage of sporulation (Esaki et al., 1999).

2.6 The examples of well known food products made from fermented soybeans

2.6.1 Miso

Miso is a traditional Japanese seasoning produced by fermenting rice, barley and/or soybeans, with salt and the filamentous fungi (*Aspergillus*), the most typical miso being made with soy. The result is a thick paste used for sauces and spreads, pickling vegetables or meats, and mixing with dashi soup stock to serve as miso soup called Misoshiru , a Japanese culinary staple. High in protein and rich in vitamins and minerals, miso played an important nutritional role in feudal Japan. Miso is still very widely used in Japan, both in traditional and modern cooking, and has been gaining world-wide interest. Miso is typically salty, but its flavor and aroma depend on various factors in the ingredients and fermentation process. Different varieties of miso have been described as salty, sweet, earthy, fruity, and savory, and there is an extremely wide variety of miso available (Katz, 2003).

The nutritional benefits of miso have been widely touted by commercial enterprises and home cooks alike. However, claims that miso is high in vitamin B_{12} have been contradicted in some studie. Part of the confusion may stem from the fact

that some soy products are high in B vitamins (though not necessarily B_{12}), and some, such as soy milk, may be fortified with vitamin B_{12}. Some, especially proponents of healthy eating, suggest that miso can help treat radiation sickness, citing cases in Japan and Russia where people have been fed miso after the Chernobyl nuclear disaster and the atomic bombings of Hiroshima and Nagasaki. Notably, Japanese doctor Shinichiro Akizuki, director of Saint Francis Hospital in Nagasaki during World War II, theorized that miso helps protect against radiation sickness. Also some experts suggest that miso is a source of Lactobacillus acidophilus (Farnworth, 2003).

2.6.2 Koji

Koji is steamed rice that has had koji-kin, or koji mold spores, cultivated onto it, which is a grain of rice cultivated with koji mold. This magical mold, for which the official scientific name is *Aspergillus Oryzae*, creates several enzymes as it propagates, and these are what break the starches in rice into sugars that can be fermented by the yeast cells, which then give off carbon dioxide and alcohol. Without koji, there is no sake. For what it is worth, sake is not the only beverage in the world using koji. There are a couple of others throughout Asia. But the brewing methodologies are vastly different.

In general, the koji-making process takes 40 to 45 hours. During this time, the developing koji is checked and mixed constantly to ensure proper temperature and moisture, as well as an even distribution of both. As the koji mold works its way into the center of the steamed rice grains, heat is generated. Different temperatures are ideal at different stages of the process. Not only that, but these ideals will change depending on the sought-after flavor profile. The type of rice, pH and mineral content of the water, and a myriad of other things affect the way koji is made as well.

These variables compound to create a process that is more art and intuition than science. When koji is ready for use, it looks like rice with a small amount of white frosting on each grain. The smell and taste are slightly sweet, as might be expected. There is a characteristic light chestnut-like aroma that wafts wonderfully up (Fujita et al., 1993).

2.6.3 Natto

Natto is made from soybeans, typically a special type called natto soybeans. Smaller beans are preferred, as the fermentation process will be able to reach the center of the bean more easily. The beans are washed and soaked in water for 12 to 20 hours. This will increase the size of the beans. Next, the soybeans are steamed for 6 hours, although a pressure cooker can be used to reduce the time. The beans are mixed with the bacterium *Bacillus subtilis natto*, known as natto-kin in Japanese. From this point on, care has to be taken to keep the ingredients away from impurities and other bacteria. The mixture is fermented at 40°C for up to 24 hours. Afterwards the natto is cooled, then aged in a refrigerator for up to one week to add stringiness. During the aging process at a temperature of about 0°C, the Bacilli develop spores, and enzymatic peptidases break down the soybean protein into its constituent amino acids. Historically, natto was made by storing the steamed soy beans in rice straw, which naturally contains *B. subtilis natto*. The soy beans were packed in straw and then left to ferment. The fermentation was done either while the beans were buried underground underneath a fire or stored in a warm place in the house as for example under the kotatsu.

It is often said in Japan that natto is good for one's health, and these claims can be backed by medical research. One example is pyrazine: Pyrazine is a compound that, in addition to giving natto its distinct smell, reduces the likelihood of blood

clotting. It also contains a serine protease type enzyme called *nattokinase* which may also reduce blood clotting both by direct fibrinolysis of clots, and inhibition of the plasma protein plasminogen activator inhibitor 1 (Fujita et al., 1993). This may help to avoid thrombosis, as for example in heart attacks, pulmonary embolism, or strokes. An extract from natto containing nattokinase is available as a dietary supplement. Studies have shown that oral administration of nattokinase in enteric capsules leads to a mild enhancement of fibrinolytic activity in rats and dogs (Fujita et al., 1995). It is therefore plausible to hypothesize that nattokinase might reduce blood clots in humans, although clinical trials have not been conducted. Another study suggests the FAS in natto is the substance which initiates fibrinolysis of clots, which accelerates the activity of not only nattokinase but urokinase (Hiroyuki, 2000).

A 2009 study in Taiwan indicated that the nattokinase in natto has the ability to degrade amyloid fibrils suggesting that it might be a preventative or a treatment for amyloid-type diseases such as Alzheimer's. Natto contains large amounts of Vitamin K, which is involved in the formation of calcium-binding groups in proteins, assisting the formation of bone and preventing osteoporosis. Vitamin K1 is found naturally in seaweed, liver, and some vegetables, while vitamin K2 is found in fermented food products such as cheese and miso. Natto has very large amounts of vitamin K2, approximately 870 micrograms per 100 grams of natto.

According to a study, fermented soybeans, such as nattō, contains Vitamin PQQ, which is very important for the skin (Kumazawa et al., 1995). PQQ in human tissues is derived mainly from diet. According to recent studies, polyamine suppresses excessive immune reactions, and natto has a much larger amount of it than any other food.[6] Dietary supplements containing the substances extracted from natto such as polyamine, nattokinase, FAS and vitamin K2 are available. Natto contains many

chemicals alleged to prevent cancer, for example, daidzein, genistein, isoflavone, phytoestrogen, and the chemical element selenium. However, most of these chemicals can also be found in other soy bean products, and their effect on cancer prevention is uncertain at best.

Recent studies show natto may have a cholesterol-lowering effect (Tomakusa, 2006). Natto is also said to have an antibiotic effect, and its use as medicine against dysentery was researched by the Imperial Japanese Navy before World War II. Natto is claimed to prevent obesity, possibly due to a low calorie content of approximately 90 calories per 7–8 grams of protein in an average serving. Unverified claims include improved digestion, reduced effects of aging, and the reversal of hair loss in men due to its phytoestrogen content, which can lower testosterone that can cause baldness. These conjectured physiological effects of eating natto are based on biochemically active contents of natto, and have not been confirmed by human study. Natto is also sometimes used as an ingredient of pet food, and it is claimed that this improves the health of the pets (Kuniyasu et al., 2005).

2.6.4 Soy sauce

Soy sauce (US), soya sauce (Commonwealth) is a fermented sauce made from soybeans (soya beans), roasted grain, water and salt. Soy sauce was invented in China, where it has been used as a condiment for close to 2,500 years. In its various forms, it is widely used in East and Southeast Asian cuisines and increasingly appears in Western cuisine and prepared foods. Authentic soy sauces are made by mixing the grain and/or soybeans with yeast or koji (the mold *Aspergillus oryzae* or *A. sojae*) and other related microorganisms. Traditionally soy sauces were fermented under natural conditions, such as in giant urns and under the sun, which was believed to contribute

to additional flavours. Today, most of the commercially-produced counterparts are instead fermented under machine-controlled environments.

In 2001 the United Kingdom Food Standards Agency found in tests of various low-grade soy sauces (those made from hydrolyzed soy protein, rather than being naturally fermented) that some 22% of samples contained a chemical called 3-MCPD (3-monochloropropane-1,2-diol) at levels considerably higher than those deemed safe by the European Union. About two-thirds of these samples also contained a second chemical called 1,3-DCP (1,3-dichloropropane-2-ol) which experts advise should not be present at any levels in food. Both chemicals have the potential to cause cancer and the Agency recommended that the affected products be withdrawn from shelves and avoided (Tanaka, 2000). Furthermore, the latter unregulated chemical can cause genetic damage to be passed on to offspring who never consumed the sauces (Kobayashi, 2005).

Britain's Food Standards Agency (FSA) issued a Public Health Advice leaflet in June 2001 to warn against a small number of soy sauce products having been shown to contain high levels of potentially cancer-causing chemicals (Tanasupawat et al., 2002). The leaflet singled out brands and products (some by batch numbers) imported from Thailand, China, Hong Kong and Taiwan. Although the leaflet primarily looked at soy sauce, the leaflet does include oyster sauce, marinades and other types of sauces, that affected the brands Golden Mountain, King Imperial, Pearl River Bridge, Jammy Chai, Lee Kum Kee, Golden Mark, Kimlan, Golden Swan, Sinsin, Tung Chun and Wanjasham. Despite these being small in number in the UK, they are the dominant brands in their respective nations.

In Vietnam, 3-MCPD was found in toxic levels (In 2004, the HCM City Institute of Hygiene and Public Health found 33 of 41 sample of soya sauce with high rates of

3-MCPD, including six samples with up to 11,000 to 18,000 times more 3-MPCD than permitted, an increase over 23 to 5,644 times in 2001) in soy sauces there in 2007, along with formaldehyde in the national dish Pho, and banned pesticides in vegetables and fruits. A prominent newspaper Thanh Nien Daily commented: "Health agencies have known that Vietnamese soy sauce, the country's second most popular sauce after fish sauce, has been chock full of cancer agents since at least 2001**.** In March 2008, some Australian soya sauces were found to contain carcinogens and consumers were advised to avoid consumption (Bamford, 2005).

2.6.5 Tempeh

Tempeh, or tempe in Japanese, is made by a natural culturing and controlled fermentation process that binds soybeans into a cake form. It is especially popular on the island of Java, where it is a staple source of protein. Like tofu, tempeh is made from soybeans, but tempeh is a whole soybean product with different nutritional characteristics and textural qualities. Tempeh's fermentation process and its retention of the whole bean give it a higher content of protein, dietary fiber and vitamins compared to tofu, as well as firmer texture and stronger flavor. Tofu, by contrast, is said to be more versatile in dishes. Because of its nutritional value, tempeh is used worldwide in vegetarian cuisine; some consider it to be a meat analogue. Even long before Westerners found and realized its rich nutritional value, tempeh was referred to as “Japanese meat.”

Tempeh begins with whole soybeans, which are softened by soaking and dehulled, then partly cooked. Specialty tempehs may be made from other types of beans, wheat, or may include a mixture of beans and whole grains. A mild acidulent, usually vinegar, may be added in order to lower the pH and create a

selective environment that favors the growth of the tempeh mold over competitors. A fermentation starter containing the spores of fungus *Rhizopus oligosporus* is mixed in. The beans are spread into a thin layer and are allowed to ferment for 24 to 36 hours at a temperature around 30°C (86°F). In good tempeh, the beans are knitted together by a mat of white mycelia. Under conditions of lower temperature, or higher ventilation, gray or black patches of spores may form on the surface, this is not harmful, and should not affect the flavor or quality of the tempeh. This sporulation is normal on fully mature tempeh. A mild ammonia smell may accompany good tempeh as it ferments, but it should not be overpowering. In Indonesia, ripe tempeh (two or more days old) is considered a delicacy.

The soy protein in tempeh becomes more digestible as a result of the fermentation process. In particular, the oligosaccharides that are associated with gas and indigestion are greatly reduced by the *Rhizopus* culture. In traditional tempeh making shops, the starter culture often contains beneficial bacteria that produce vitamins such as B12 (though it is uncertain whether this B12 is always present and bioavailable) (Liem et al., 1977; Delores et al., 1987; Allison, 2000). In western countries, it is more common to use a pure culture containing only *Rhizopus oligosporus* which makes very little B12 and could be missing *Klebsiella pneumoniae* which has been shown to produce significant levels of B12 analogs in tempeh when present. Whether these analogs are true, bioavailable B12, hasn't been thoroughly studied yet (Shurtleff et al., 1989).

3. Antioxidant activity of fermented soybeans

The fermented soybeans have been developed as the result of the finding potential antioxidant phytochemicals. They also possess several naturally-occuring

especially isoflavone substances, daidzien and genistein, that exert an antioxidative effect (Hanasaki et al., 1994). The isoflavones are compounds found in fruits, vegetables, and certain beverages that have diverse beneficial biochemical and antioxidant effects. Their dietary intake is quite high compared to other dietary antioxidants like vitamins C and E. The antioxidative activity of isoflavones depends on their molecular structure and, surprisingly, is found considerably in soybeans. The position of hydroxyl groups and other features in the chemical structure of isoflavones are important for their antioxidative and free radical scavenging activities (Thomas et al., 2001).

3.1 Free radical and reactive oxygen species (ROS)

A free radical can be defined as any species capable of independent existence that contain one or more unpaired electrons, and unpaired electron being one that is alone in an orbit. Most biological molecules are non-radicals, containing only paired electrons (Halliwell, 1991). A radical can react with both radicals and non-radical molecules in several ways and may donate its unpaired electron to a non-radical molecule or it might take an electron from another molecule in order to form a paired electron. Free radicals include hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O2^{\bullet-}$), nitric oxide ($NO^{\bullet}$) and peroxyl radical ($RO2^{\bullet}$). Whereas peroxylnitrite (HNOO), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($1\Delta gO2$, $1\Sigma g^{+}O2$) and ozone (O_3) are not free radicals but can easily lead to free radical reaction. The term “Reactive oxygen species (ROS)” is often used to include both the radical and non-radical species as shown in Table 5 (Strain and Benzie, 1999).

Table 5. Reactive oxygen species found in vivo

Name	Singlet/formula	Radical(R) or non-radical (NR)
Molecular oxygen	O_2	NR
Nitric oxide	$NO^{\bullet}$	R
Nitrogen dioxide	$NO_2^{\bullet}$	R
Superoxide	$O_2^{\bullet-}$	R
Peroxyl	$ROO^{\bullet}$	R
Singlet oxygen	$1\Delta gO_2$	NR
	$1\Sigma g^{+}O_2$	R
Hydrogen peroxide	H_2O_2	NR
Hydroperoxyl	$HOO^{\bullet}$	R
Alkoxyl	$RO^{\bullet}$	R
Hypochlorous acid	HOCl	NR
Peroxynitrite	HNOO	NR
Hydroxyl	$OH^{\bullet}$	R

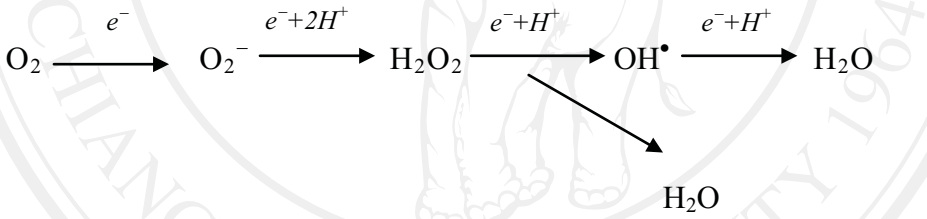
Reactive oxygen species are potentially very toxic to cells. Due to their highly reactive nature, they can readily combine with other molecules, such as enzymes, receptors, and ion pumps, causing oxidation directly, and inactivating or inhibiting their normal functions. Some of the products of reactive oxygen species attack of other molecules can interfere with nucleic acid function, generating alterations in the

base sequence with the potential for mutations. Changes in normal proteins and other structures by free radical species can also generate novel immunogenic structure.

3.2 Sources of free radicals

3.2.1 Reduction of O_2

The biological reduction of O2 to water, especially in respiratory cells, usually refers to a univalent pathway of reduction and the sequential release of toxic intermediates such as $O2^{-}$, H_2O_2, and $OH^{\bullet}$ (Fridovich, 1978). Thus, molecular O_2 can be reduced by 1 electron giving rise to superoxide radical (O_2) which can be further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) and finally to water as shown below (Sjodin, 1990).



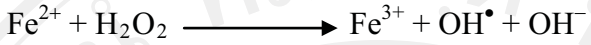
Scheme 2. The univalent pathway of O_2 reduction.

3.2.2 Transition metals catalyzed reactions

It is generally accepted that much of the toxicity of O_2^{-} and H_2O_2 in living organisms is due to their conversion into $OH^{\bullet}$ and into reactive-radical-metal ion complexes. These processes are often referred to as either the ion-catalyzed Haber-Weiss reaction, or the superoxide-driven Fenton reaction (Aruoma and Halliwell, 1995; Aruoma, 1994).

Haber-Weiss

Fenton



Scheme 3. Haber-Weiss reaction and Fenton reaction.

3.2.3 Radiation

The impact of ionizing radiation on biological material can generate a wide range of radicals, including $OH^{\bullet}$, O_2- and hydrogen radical (H^{-}) (Slater, 1984). When ionizing radiation interacts with water, three free radicals and two non-radical products are formed:

Scheme 4. Ionizing radiation interacts with water.

Then, $e^{-}aq$ can react with molecular O_2 in water and form O_2^{-}. The processes by which these products are formed involved both ionization and excitation (Halliwell and Gutteridge, 1999).

3.2.4 Microbial killing by phagocytic cell

Some of the O_2^- production in vivo may be accidental, but much is functional. Thus activated phagocytic cells generate O_2^-, as has been shown for monocytes, neutrophills, eosinophills, and many types of macrophages. Radical production is important in allowing phagocytes to kill some of the bacterial strains that they can engulf (Halliwell, 1994).

3.2.5 A group of cellular enzymes metabolism

Cellular enzymes are involved in catalyzing oxidation reactions, such as xanthine oxidase, flavin dehydrogenases, peroxidases, which can produce free radical species. The scavenger enzyme system can also produce oxygen free radical species. For example, superoxide dismutase (SOD) can dismutate O_2^- to form H_2O_2 (Halliwell and Gutteridge, 1999).

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

3.3 Oxidative stress and human diseases

Oxidative stress is the amount of free radical damage that is being produced in an organelle / cell / organ / organism. A free radical is a very reactive atom with an unpaired electron, which can be in a reduced or oxidized state. The majority of free radicals that damage biological systems are oxygen radicals. What determines the amount of free radicals being produced depends on the balance of many different factors, some of which are shown in Figure 3 (Culter and Rodriguez, 2003).

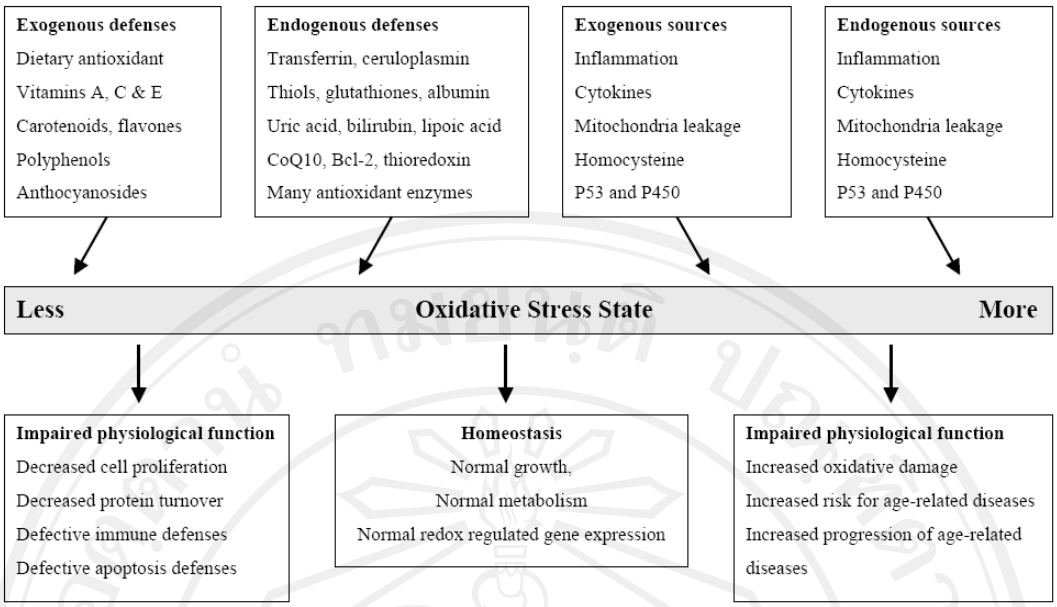


Figure 3. Oxidative stress and human diseases (Ames, 1993).

Oxidative stress occurs when there is an imbalance between pro-oxidant and antioxidant owing to a relative or absolute deficiency of antioxidant. An antioxidant shift will promote damaging-oxidative change to important cellular constituents and this may lead to dysfunction and disease (Strain and Benzie, 1999). Oxidative stress causes changes to carbohydrate, DNA, proteins and lipids. Such changes may lead to a wide range of disorders such as a DNA break, mutagenesis, protein inactivation, mitochondria damage, and receptor malfunction and membrane disruption. The mechanism for DNA damage, leading to mutation is explained by the attack of hydroxyl radicals on guanine in DNA, then guanine is converted into 8-hydroxyguanine which is easily oxidized and reduced as seen in Figure 3 (Halliwell and Gutteride, 1994)

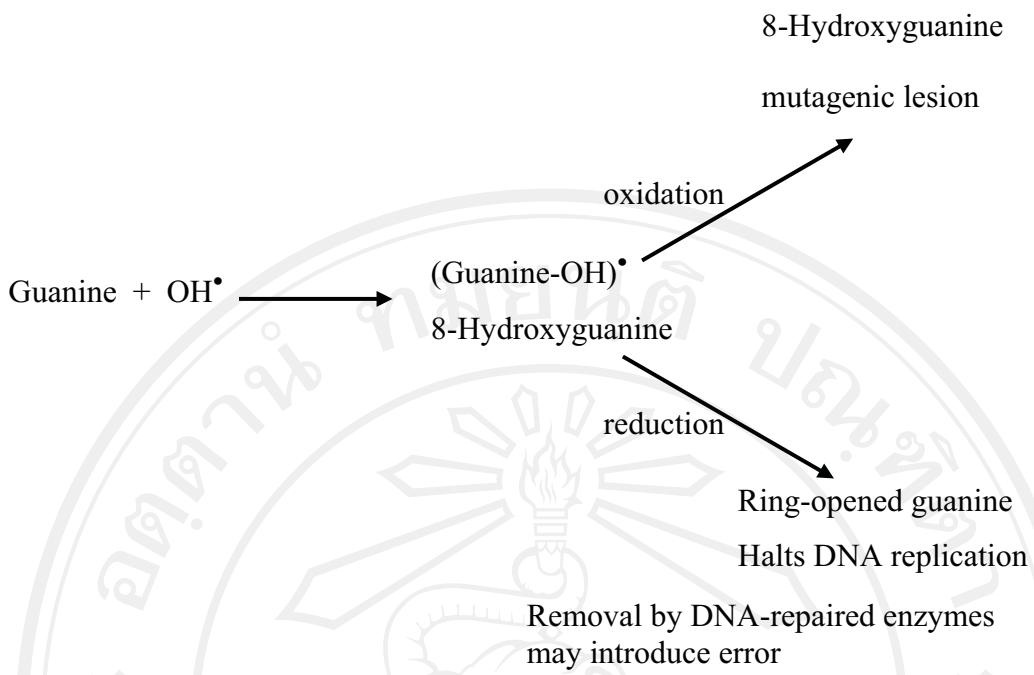


Figure 4. The attack of hydroxyl radical on guanine in DNA which leads to mutation (Thomas, 1994).

Oxidative stress occurs in most, if not all, human diseases, for example, cancer, Alzheimer's disease, cataracts, arthritis, diabetes, Parkinson's disease, ischemic-reperfusion injury and cardiovascular disease (Thomas, 1994).

3.4 Oxidative stress defense systems

An antioxidant has been defined as "any substance that, when present at low concentration compared to a dose of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate". Antioxidants can act at different levels in the oxidative sequence. As far as lipid peroxidation is concerned, they could act by:

1. Decreasing localized O_2 concentrations (e.g. sealing of food stuffs under nitrogen).

2. Preventing first-chain initiation by scavenging initiating radicals such as $OH^{\bullet}$.

3. Binding metal ions in a form that will not generate such initiating species as $OH^{\bullet}$, ferryl, or Fe^{3+} / Fe^{2+} / O_2 and / or will not decompose lipid peroxides to peroxyl or alkoxyl radicals.

4. Decomposing peroxide by converting them to non-radical products, such as alcohol.

5. Chain-breaking, i.e. scavenging intermediate radicals such as peroxyl and alkoxyl radicals to prevent continued hydrogen abstraction. Chain-breakers are often phenols and amines (Halliwell and Gutteridge, 1989; Punchard and Kelly, 1996).

Antioxidants acting by mechanisms 1, 2 and 3 can be called preventive antioxidants. Those acting by mechanism 3 are not usually consumed in the course of the reactions. Antioxidants of the fourth type are also preventive antioxidants, but they may or may not be consumed during the reaction, depending on their chemical behavior (e.g. gluthathione peroxidase acts by this mechanism and being an enzyme, is a catalyst and is not consumed). Chain-breaking antioxidants, acting by combining with the intermediate radicals, will be consumed, as will antioxidants of type 2 as shown above. It should be stressed that many antioxidants have multiple mechanisms of action.

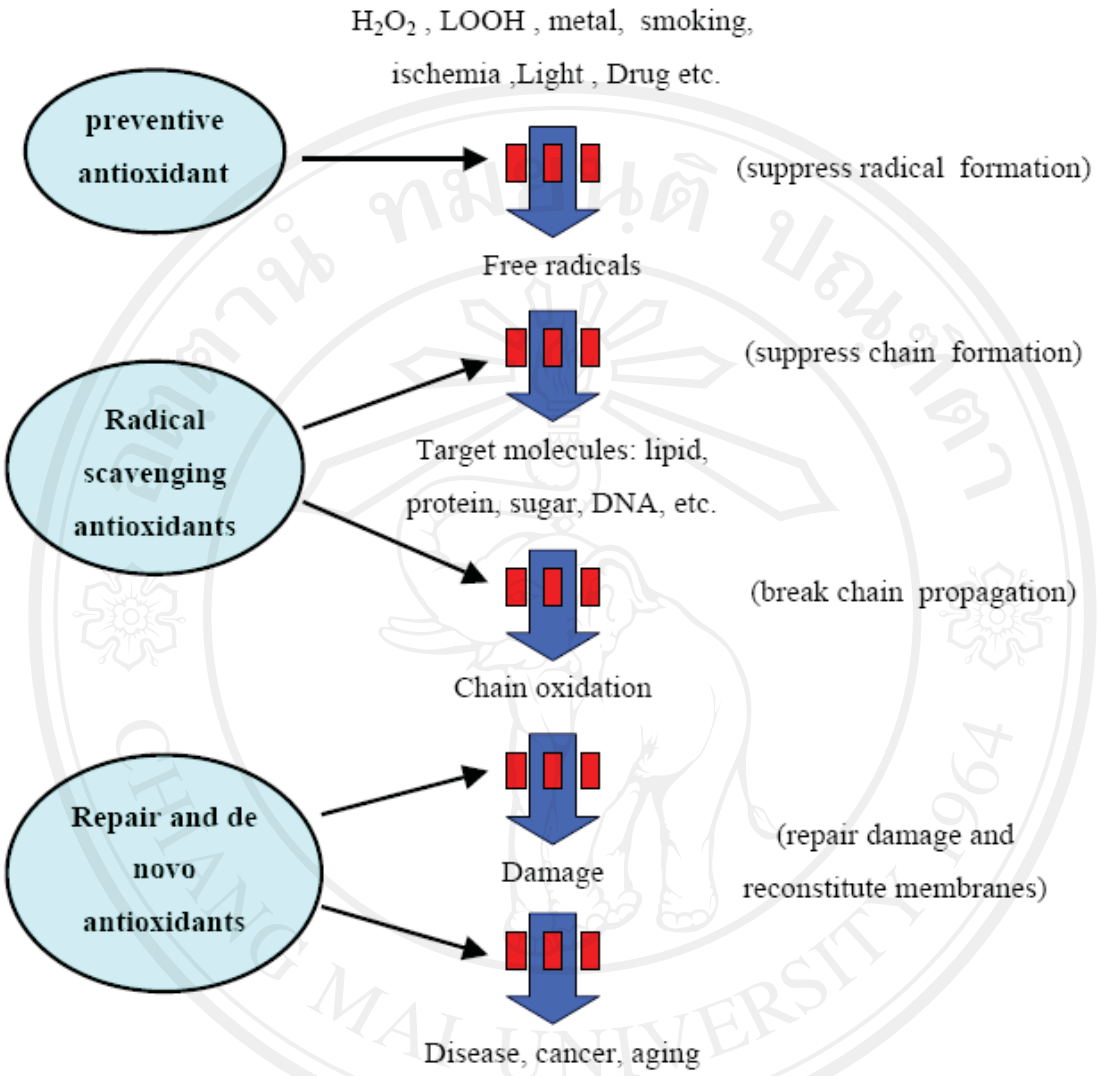


Figure 5. Defense systems in vivo against oxidative damage (Aruoma, 1994).

Table 6. Defenses systems against oxidative damage.

1. Preventive antioxidants: suppress the formation of free radicals
   (a) Non-radical decomposition of hydroperoxides and hydrogen peroxide

catalase	decomposition of hydrogen peroxide $2H_2O_2 \longrightarrow 2H_2O + O_2$
glutathione peroxidase(cellular)	decomposition of hydrogen peroxide and fatty acid hydroperoxide $H_2O_2+2GSH \longrightarrow 2H_2O+2GSSG$ $LOOH+2GSH \longrightarrow LOH+H_2O+GSSG$
glutathione peroxidase(plasma)	decomposition of hydrogen peroxide and phospholipid hydroperoxides $PLOOH+2GSH \longrightarrow PLOH+H_2O+GSSG$
phospholipid hydroperoxide glutathione peroxidase	decomposition of phospholipid hydroperoxide
peroxidase	decomposition of hydrogen peroxide and lipid hydroperoxide $LOOH+AH_2 \longrightarrow LOH+H_2O+A$ $H_2O+ AH_2 \longrightarrow 2H_2O+A$

glutathione-S-transferese	decomposition of lipid hydroperoxides

(b) Sequestration of metal by chelation

tranferrin, lactoferrin	sequestration of iron
haptoglobin	sequestration of hemoglobin
hemopexin	stabilization of heme
ceruloplasmin, albumin	sequestration of copper

(c) Quenching of active oxygens

superoxide dismutase(SOD)	disproportionation of superoxide
	$2O_2^{\bullet-}+2H^+ \longrightarrow H_2O_2+O_2$
carotenoids	Quenching of singlet oxygen

2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation.

hydrophilic: vitamin C, uric acid, bilirubin, albumin

lipophilic: vitamin E, ubiquinol, carotenoids

3. Repair and *de novo* enzymes: repair the damage and reconstitute membranes lipase, protease, DNA repair enzymes, transferase.

4. Adaptation: generate appropriate antioxidant enzymes and transferase them to the right site at the right time and in the right concentration.

All aerobic organisms, including human beings, utilize a series of primary antioxidant defenses in an attempt to protect against oxidant damage, and numerous damage removal and repair enzymes to remove and/or repair molecules that do get damaged. Those antioxidants concentrate on the non-enzymic and enzymic primary antioxidant defenses.

Such antioxidants include vitamin C, which acts as a cytosolic antioxidant; vitamin E, which acts as a membrane antioxidant; and glutathione, which acts to protect both cytosol and membranes against free radical attacks. Also present are the glutathione-dependent enzymes, glutathione peroxidase (GSH-Px), glutathione reductase, glutathione transferase, and catalase, which break down H_2O_2 to oxygen and water, and enzyme superoxide dismutase (SOD), which converts $O_2^{\bullet}-$ into H_2O_2. Some enzymes exist in several different forms. Membrane, cytosolic, and plasma forms of GSH-Px have been reported. Similarly, there are mitochondrial, cytosolic, and extracellular forms of SOD. Other important antioxidants include carotenoid, ubiquinones, bilirubin and uric acid.

Table 7. Examples of some disorder and diseases associated with free-radical pathology.

Cancers
Coronary heart disease/atherosclerosis
Diabetes
Cataract
Adverse drug reactions
Toxic liver injuries
- CCl_4 and other halogenoalkanes
- Bromobenzene
- Allyl alcohol
- Iron overload
- Paracetamol
- Alcohol
Redox cycling mechanisms
- Quinones
- Nitroimidazoles
Arthritis
Immune hypersensitivity
Inflammatory disorders
Reperfusion injuries
- Thrombosis
- Organ storage
- Transplantation

Neurological degeneration

- Aging
- Traumatic inflammation

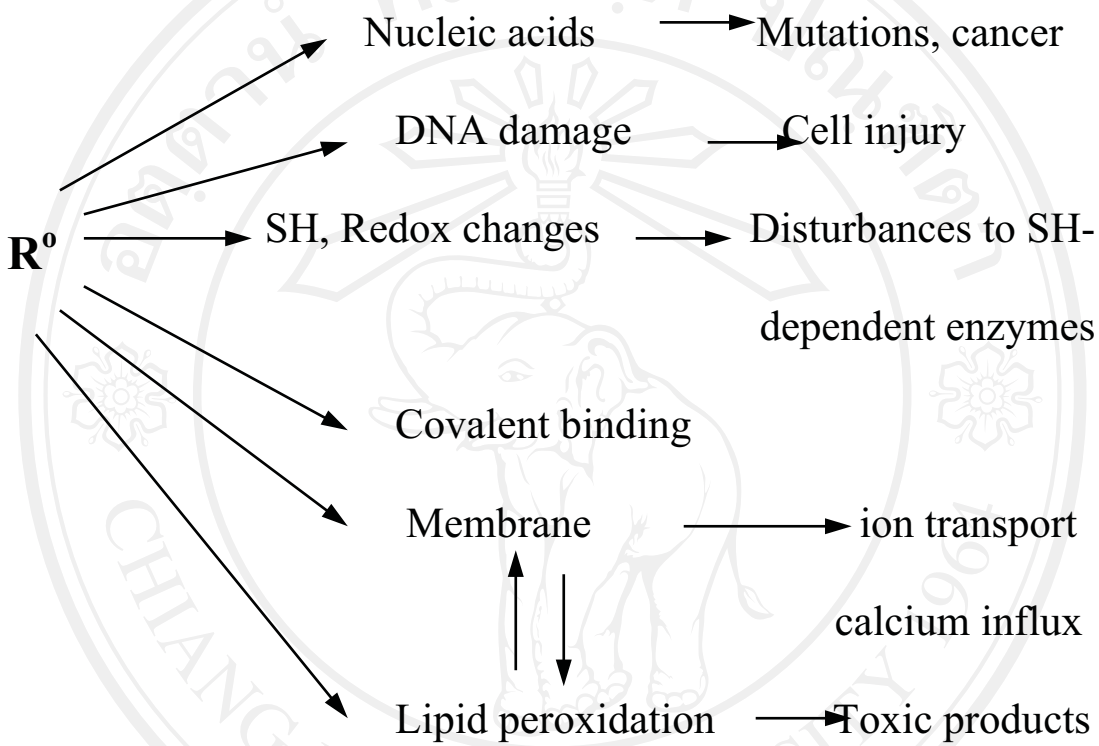


Figure 6. A representation of a free-radical-mediated disturbance producing a diverging set of metabolic perturbations, with some or al of these repaired or prevented by effective cell defenses involving free radical scavengers and antioxidants.

3.5 Antioxidant related to fermented soybeans

The supplements from soybeans have been developed as the result of the finding potential antioxidant phytochemicals such as phenolics or polyphenols. They also possess several naturally-occuring phenolic compounds and flavonoids (especially isoflavone substances, daidzin and genistein), that exert an antioxidative effect (Hanasaki et al., 1994). Flavonoids are compounds found in fruits, vegetables, and certain beverages that have diverse beneficial biochemical and antioxidant effects. Their dietary intake is quite high compared to other dietary antioxidants like vitamins C and E. The antioxidative activity of flavonoids depends on their molecular structure and, surprisingly, is found considerably in soybeans. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidative and free radical scavenging activities (Tomas et al., 2001).

Phenolic compounds are plant-derived antioxidants that possess metal-chelating capabilities and radical-scavenging properties (Bors and Saran, 1987; Lopes et al., 1999). In plants, phenolic compound are usually found in conjugated forms through hydroxyl groups with sugar as glycosides (Robbins, 1980). Soybean and soybean products, containing various amounts of phenolic compounds, have been revealed to possess antioxidative ability. The increased total phenolic content in soybean after fermentation suggests that β-glucosidase, produced by fungi, catalyses the release of aglycones from the soybean substrate and thereby increases their phenolic content. (Vattem and Shetty, 2002).

Flavonoids and phenolic acid can act as antioxidant by a number of potential pathways. The most important is likely to be by free radical scavenging in which the polyphenol can break the free radical chain reaction. A number of studies have been

carried out on the structure-antioxidant activity relationships of the flavonoids. The main structural feutures of flavonoids required for efficient radical scavenging could be summarized as follows :

1. An ortho-dihydroxy (catechol) structure in the B ring for electron delocalization.
2. A double bond (2,3) in conjugation with a 4-keto function, provides electron delocalization from the B ring.
3. Hydroxyl groups at position 3 and 5, providing hydrogen bonding to the keto

The phenolic acid may also be good antioxidants, paricularly those possessing the catechol-type structure such as caffeic acid. Recent studies have indicated that simple cell-derived phenolic acid such as hydroxyanthranilic acid may also be efficient co-antioxidants for α - tocopherol, able to inhibit lipoprotein and plasma lipid peroxidation in humans. The possible interaction between flavonoid and phenolic acids with other physiological antioxidants such as ascorbate or tocopherol is another possible antioxidant pathway for these compounds. The synergistic interaction of these antioxidants may be exemplificd by the enhancement of the antiproliferative effect of quercetin by ascorbic acid, possibly due to its ability to protect the polyphenol from oxidative degradation. In a similar manner, coincubation of low-density lipoprotein (LDL) with ascorbate and caffeic or coumaric acid resulted in a synergistic protection from oxidation promoted by apparent antioxidant action.

Another pathway of apparent antioxidant action of the flavonoids, particularly in oxidation systems using transition metal ions such as copper or iron, is the chelation of the metal ions. Chelations of the catalytic metal ions may prevent their involvement in Fenton-type reactions which can generate highly reactive hydroxyl radicals.

Other biological actions of phenolic compounds have been noted which may be relevant to their effects on human health. For example, caffeic acid may have cytoprotective effects on endothelial cells related not only to its antioxidant action but also to its ability to block the rise in intracellular calcium in response to oxidized lipoproteins. Some phenolic compounds may also inhibit platelet aggregation. The ability of phenolic compounds to trap mutagenic electrophiles such as reactive nitrogen species may also protect biological molecules from damage (Randhir et al., 2004).

3.5.1 Reaction of phenolic antioxidants

Phenolic phytochemicals are important aromatic secondary metabolites in plants. Phenols are chemically characterized by molecules in which a hydroxyl-functional group (-OH) is bonded directly to an aromatic or benzene ring. Compounds containing two or more hydroxyl groups on the aromatic rings are called polyphenols and are widely in plants. The reaction of antioxidants with radicals as follow;

$$R^{o} + AH \longrightarrow RH + A^{o}$$

$$RO^{o} + AH \longrightarrow ROH + A^{o}$$

$$ROO^{o} + AH \longrightarrow ROOH + A^{o}$$

$$R^{o} + A^{o} \longrightarrow RA$$

$$RO^{o} + A^{o} \longrightarrow ROA$$

$$ROO^{o} + A^{o} \longrightarrow ROOA$$

Where AH is any antioxidants ; A is a free radical for antioxi The important reason phenolic compounds are antioxidant because they induce the stable resonance radicals which have low energy, does not continue propagation reaction and the activation energies are lower than another molecules of RH so they can react with

other molecule more quickly than unsaturated fatty acid. The main classes of polyphenols are defined according to the nature of carbon skeleton : phenolic acids, flavonoids and less common stillbenes and lignan. Flavonoids represent the vast majority of plant phenolics. They are found in almost all parts of the plants and numerous quantity have appeared on leaf part (Madhavi et al., 1996). Flavonoids are the most abundant polyphenols in diet foods sush as fruits and vegetables. They can be devided into several classes based on very few core structures. Their multitude derives mainly from the various hydroxylation pattern (Bors et al., 2003) such as flavonols, flavones, isoflavones and anthocyanidin. The main flavonoids constituents are flavonol aglycones such as quercetin, myricetin (Miean & Mohamed, 2001).

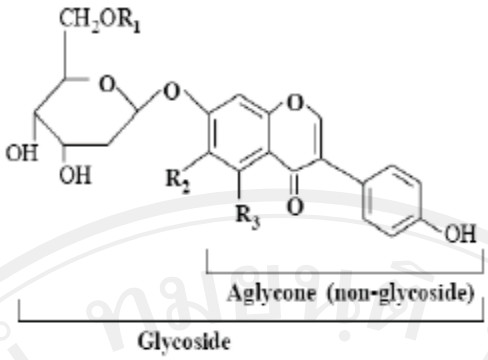
3.5.2 Soybean isoflavones

Fermented soyfoods are the most important source of dietary isoflavones which have been found to have important secondary compounds with many chemical actions. Examples include antioxidative actions, and functions as anticancer agents (Kim et al., 2005). Soybean isoflavones have phenol hydroxyl groups which can scavenge free radicals in vivo and in vitro. Generally, the more hydroxyl groups, the greater the antioxidant capacity (Cao et al., 1997), suggesting that these groups are the chemical basis for the antioxidant properties of isoflavones.

Genistein and daidzein, the most abundant dietary isoflavones, are the potent antioxidants because they have the structural features that suit for free radical scavenging activity. Although isoflavones are not essential nutrients, they seem to play an important role in health maintenance. Soybean isoflavones are composed of two major forms, isoflavone glucosides (genistin,daidzin and glycitin) and aglycones (genistein, daidzein and glycitein), which are known to be the antioxidative

components. The liberation of lipophilic aglycones of isoflavone glucosides such as daidzein and genistein by the catalytic action of β-glucosidase enzyme which are produced from filamentous fungi during fermentation (Esaki, 1998) results in the increased antioxidative activity of miso and tempeh. The fermented soyfoods such as tempeh and miso contain greater amounts of aglycones because of enzymatic hydrolysis (β-glucosidase) during fermentation, while non-fermented soyfoods contain greater level of glucosides (Zheng et al., 1997). Furthermore, the use of diadzein and genistein during the fermentation of Japanese soybean has yielded o-hydroxyisoflavones, a potent antioxidant (Esaki, et al., 1999).

According to many studies, isoflavones are essential for preventing certain forms of cancer, as well as reducing the risk of cardiovascular diseases (Anderson, 1995). Recent literature studies indicate that isoflavones have benefits against cardiovascular diseases and cancers, and function by acting as anti-oestrogens (Cassidy, 1996). More specifically, two forms of isoflavones, genistein and daidzein, have been found to be the major isoflavones in fermented soybeans. They have the ability to control or inhibit the growth of human breast cancer cell lines in culture. In particular, genistein possesses the strongest antioxidative properties (Choi et al., 1998).



Name	R_1	R_2	R_3
[Glycoside]			
Daidzin	H	H	H
Glycitin	H	OCH_3	H
Genistin	H	H	OH
6″-O-malonyldaizin	$COCH_2COOH$	H	H
6″-O-malonylglycitin	$COCH_2COOH$	OCH_3	H
6″-O-malonylgenistin	$COCH_2COOH$	H	OH
6″-O-acetyldaizin	$COCH_3$	H	H
6″-O-acetylglycitin	$COCH_3$	OCH_3	H
6″-O-acetylgenistin	$COCH_3$	H	OH
[Non-glycoside]			
Daidzein	OH	H	H
Glycitein	OH	OCH_3	H
Genistein	OH	H	OH

Figure 7. The chemical structure of soybean isoflavones.

(From : www. journals.prous.com)

Soybean isoflavones have phenol hydroxyl groups which can scavenge free radicals in vivo and in vitro. Generally, the more hydroxyl groups, the greater the antioxidant capacity (Cao et al., 1997), suggesting that these groups are the chemical basis for the antioxidant properties of isoflavones.

3.5.3 Antioxidant activity of soybean isoflavones: in vitro and ex vivo studies

There are many reports dealing with the antioxidant activity of soybean isoflavones in vitro, at the level of the cell, in enzyme systems and in non-biological

systems. Isoflavones can significantly inhibit lipid peroxidation of rat liver microsomes stimulated by the Fe^{2+}-ADP–NADPH complex (Jha et al., 1985). The IC50 of genistein, daidzein and glycitein is 1.8 10 4, 6.0 10 4 and 1.610 3 mol l 1, respectively. Using this method to measure total antioxidant activity assay, Ruiz-Larrea et al. (1997) determined the order of antioxidant activity of several isoflavones to be as follows: genistein > daidzein = genistin = biochanin A = daidzin > formononetin = ononin. However, using a similar method to that used above but measuring production of thiobarbituric acid-reactive substances (TBARS), Mitchell et al. (1998) observed only weak antioxidant activity by genistein and daidzein. Soybean extract rich in isoflavones can inhibit lipid peroxidation formation in ox brain phospholipid stimulated by Fe^{3+}/vitamin C (Wiseman et al., 1996).

Genistein, in a dose-dependent manner, inhibits the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) of calf thymus activated by ultraviolet (UV) radiation or by the Fenton reaction ($H_2O_2/FeCl_2$) (Wei et al., 1996). The IC50 is 0.25 10 6 mol l 1 for UV radiation and 25 10 6 mol l 1 for the Fenton reaction. It was found that 8-OHdG is increased in cancerous tissues and is responsible for DNA base mutation. 8-OHdG may activate certain oncogenes such as H-ras and K-ras. Inhibition of 8-OHdG formation by isoflavones suggests a potential anticarcinogenic action against photocarcinogenesis.

H_2O_2 is present in normal tissues and induces damage if the concentration rises because it generates hydroxyl radicals which are the most reactive free radicals found in the body. Isoflavones can inhibit the formation of active oxygen induced by some chemicals. Wei et al. (1995) used 12-O-tetradecanoylphorbol-13-acetate (TPA, a tumour promoter) to stimulate HL-60 cells to produce H_2O_2. Both genistein and, to a lesser extent, daidzein inhibited H_2O_2 formation in this system. Wei et al. (1995) also

showed that various isoflavones are able to suppress $O_2^{\bullet}$ production by xanthine/ xanthine oxidase. Genistein almost completely inhibited the production of $O_2^{\bullet}$, while daidzein inhibited it by 80% at the same concentration.

Genistein can also prevent some of the damage done by H_2O_2 to cells. Genistein, like the hydroxyl scavenger dimethylthiourea, can reduce the induction of adenylyl cyclase activity by H_2O_2 in A10 cells (a murine vascular smooth muscle cell line) (Tan et al., 1995) and prevent haemolysis of sheep red blood cells by dialuric acid or H_2O_2 in vitro (Pratt et al., 1981).

Collagen can induce platelet aggregation and produce reactive oxygen species (ROS). Schoene and Guidry (1996) found that a collagen-stimulated rat blood sample will produce a much lower concentration of ROS and platelet aggregation if pretreated with genistein. Gaudette and Holub (1990) previously reported that genistein possesses anti-platelet activity. It is not clear whether this comes from its inhibiting ROS formation in platelets.

Chait (1996) reported that low-density lipoprotein (LDL) oxidation stimulated by copper ions was prevented by genistein and daidzein in a concentration-dependent manner. It was also shown that genistein suppresses the oxidation of LDL induced by copper ions or free radicals (superoxide/nitric oxide radicals) (Kapiotis et al., 1997).

Kanazawa et al. (1995) reported that when patients with cerebro- vascular disease were given a soybean supplement in their diets for 6 months, their lipoproteins were less susceptible to peroxidation by copper ions than lipoproteins from unsupplemented subjects. There was a much greater suppression of oxidation of very-low-density lipoproteins and LDL than of high-density lipoproteins. In a similar study, healthy subjects were given a supplement containing 12 mg genistein and 7 mg daidzein daily for 2 weeks. This increased the resistance of their LDL to oxidation

(Tikkanen et al., 1998).

3.5.4 Antioxidant activity of soybean isoflavones : in vivo studies

Cai and Wei (1996) observed the effects of genistein on the activities of antioxidant enzymes in mice. They fed Sencar mice with a diet containing 0, 50 and 250 mg kg 1 genistein for 1 month. Results showed that although some antioxidant enzymes (superoxide dismutase (SOD), chloramphenicol acetyl transferase, glutathione peroxidase (GSH-Px), glutathione reductase and glutathione-S-transferase) were increased in some tissues (liver, kidney, lung, small intestine and skin), this was generally by no more than 10–20%. Another report showed that genistein and daidzein can inhibit sister- chromatid exchanges of bone marrow cells and DNA adduct formation in mouse liver induced by 7,12-dimethylbenz anthracene, a carcinogen (Giri and Lu, 1995). The relatively low antioxidant activity of soybean isoflavones in normal animals is not consistent with its potent activity in vitro. This possibly results from using normal, young rats possessing relatively high levels of antioxidant enzymes, which are resistant to further increase. Moreover, the normal tissue levels of free radicals are essential to physiological and immune functioning. For these reasons we designed an experiment to measure the antioxidant activity of soybean isoflavones extract (SIE) in animal models of oxidative stress. This extract contains 41% genistein, 52% daidzein and very little glucoside (genistin and daidzin). Adriamycin (ADR) is a drug used for treating cancer. Its usage is often limited by severe cardiac toxicity which is generally considered to be a result of oxidative damage. In our study we used ADR to produce oxidative stress in mice and we then monitored the effect of treatment with SIE. Mice were treated with ADR (20 mg/kg intraperitoneally as four doses over 12 days). It induced a decrease in the levels of two antioxidant enzymes (SOD and GSH-Px) in red blood cells, liver and heart tissues and

an increase in LPO in these tissues. The toxicity of ADR appears to be unrelated to its anticancer action as indicated by the fact that antioxidants, namely vitamin E and probucal, can prevent ADR-induced cardiomyopathy without reducing its antitumour properties (Perez Ripoll et al., 1986; Siveski- Iliskovic et al., 1995). SIE at doses of total isoflavone of 10 and 40 mg/kg body weight daily for 2 weeks significantly increased the activities of the two antioxidant enzymes and lowered LPO in comparison with ADR-treated mice not given SIE. Effects were generally more pronounced with the higher dose of SIE. The results therefore indicate that SIE helped to reverse the effects of ADR. Moreover, SIE also prevented myocardial damage by ADR. The results demonstrate the antioxidant action of soybean isoflavones.

3.5.5 Potential of soybean isoflavones in the prevention of cardiovascular disease and cancer

Animal experiments have revealed the anticarcinogenic properties of soybean isoflavones and soybean products (Messina et al., 1994). Genistein arrested the cell cycle at the G2–M phase while daidzein did so at the G1 phase. Proposed mechanisms include oestrogenic and anti-oestrogenic effects, induction of cancer cell differentiation, inhibition of tyrosine kinase in cell signaling and DNA topoisomerases in DNA replication and suppression of angiogenesis. But, as stated earlier, soybean isoflavones can protect DNA from oxidative damage caused by UV or $H_2O_2/FeCl_2$ and inhibit the H_2O_2 formation induced in cancer cell lines by the tumour promoter, TPA. These data demonstrate that the antioxidant activity of soybean isoflavones may play an important role in their anticancer activity. It must be

stressed that soybeans contain a variety of compounds in addition to soybean isoflavones which may have anticarcinogenic activity (Kennedy, 1995).

LDL oxidation is a critical event in atherogenesis; it promotes the formation of foam cells and proliferation of smooth muscle cells. This suggests that suppression of LDL oxidation by soybean isoflavones may help prevent atherosclerosis. This possibility is supported by recent reports indicating that soybean isoflavones can reduce the levels of blood lipids including total cholesterol, LDL and apolipoprotein B (Anthony et al., 1996). However, there are as yet no reports demonstrating that soybean isoflavones prevent atherosclerosis in vivo.

Research in recent years has supported the concept linking free radicals with many diseases and symptoms, such as cancer, atherosclerosis, inflammation, impaired mental functioning and ageing. Experimental studies have supported the potential value of antioxidant treatment. For example, flavone extracts of Ginkgo biloba have been used in the treatment of cardiovascular diseases for several decades. In this regard natural antioxidants may be preferable, as synthetic agents often have side effects. Soybean isoflavones may prove to be a family of compounds well worthy of further investigation.

4. Antioxidant activity determination

The antioxidant capacity of many compounds, beverages and foods has been evaluated by various methods. Several methods were developed for measuring the total antioxidant capacity of food and beverage. These assays differ in their chemistry (generation of different radicals and/or target molecules) and the way end points are measured because different antioxidant compounds may act in different mechanisms. No single method can fully evaluate the total antioxidant capacity of food. The

methods of detecting and quantitatively estimating synthetic and natural antioxidants have been detected by color reaction to semiquantitative and quantitative methods such as spectrophotometry ; voltametry ; polarography ; and chromatographic methods like paper, thin-layer, and column chromatography and the more advanced gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC).

In food related systems, antioxidant activity means chain breaking inhibition which effects with adding antioxidant in food system by determining the products (malonaldehyde, hexanol or conjugated diene hydroperoxides) which produced while occurs in lipid oxidation. But this result can not classify type of antioxidant (primary or secondary antioxidant). Therefore, the antioxidant activity can and must be evaluated with different tests for different mechanisms. The most frequently measured products are conjugated diene hydroperoxides for primary product and volatile compounds (TBARS, hexanol) for secondary product. Besides, the peroxide value is applicable for following peroxide, the main initial product of oxidation, at the early stage of oxidation, it is, nevertheless, highly empirical. The accuracy is questionable, the results vary with details of the procedure used and the test is extremely sensitive to temperature changes. During the course of oxidation, peroxide value reach a peak and then decline. Substrate of the protective action toward lipid oxidation has been frequently used vegetable oils, fish oils or lard. Marine oils are rich in polyunsaturated fatty acids (PUFA) which are highly sensitive to oxidative deterioration and have also been used to test natural antioxidants (Pokorny et al., 2001). Method for determining food antioxidants are list in Table 8.

Table 8. Analytical method for determining antioxidant activity

Analytical method	Substrate	Plants
Oxidation in fats		
-Peroxide value	Linoleic acid	180 varities of oriental herbs
-Conjugated diene, hydroperoxide, Hexanol	Safflower oil	Evening primrose seed
-Peroxide value	Lard	180 varities of oriental herbs
-Volatile acids with rancimat apparatus	Chicken fat,soya oil,sunflower oil, lard	Rosemary,sage,thyme,oregano, ginger,turmeric,Cayenne pepper
Oxidation in food system		
-TBARS	Model meat system	Aloe vera, fenugreek, ginseng, mustard, rosemary, sage, soya
-TBARS	Pork patties	protein, tea
Oxidation in emulsion		
-ßcarotene bleaching method	Linoleic acid emulsion	28 varities of fruits,vegetables and grains
-ßcarotene bleaching method	Linoleic acid emulsion	Fresh pepper
-ßcarotene bleaching method	Linoleic acide emulsion	Oat
-Conjugated diene hydroperoxide, Hexanol	Corn oil emulsion	16 varities of Spanish wines Rosemary and sage extracts
Scavenging of radicals		
-Antiradical activeity	DPPH	39 varities of seashore plants
-H-donor activity	DPPH	Anthriscus cerefolium L.
-Radical scavenging effect	DPPH	3 varieties of roasted cereals

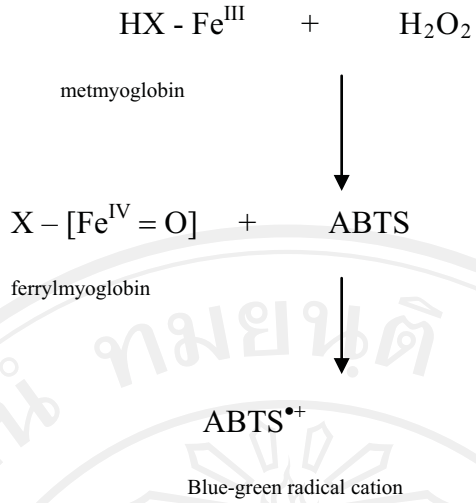
Table 8. (continue)

Analytical method	Substrate	Plants
-DPPH redical scavenging effect	DPPH	9 varieties of berries
-Free radical scavenging measurement	DPPH	4 cultivars of raspberries
-Radical scavenging activity	DPPH	virgin olive oil
-The oxygen radical absorbance capacity (ORAC)	AAPH	4 species of *Vaccinium* sp.
-Automated ORAC assay	AAPH	22 varieties of common vegetables
-Automated ORAC assay	AAPH	12 varities of fruits
-Myoglobin assay	ABTS	7 cultivars of cereal grains
-Antioxidant capacity	ABTS	17 varieties of blueberries and blackberries

There are many methods for the determination antioxidant activity depending on what we want to measure such as free radicals free radical products, antioxidant agents, metal chelating activity. We can choose the stable free radicals for studying the antioxidant activity using the following method.

4.1 Total antioxidant determination by ABTS - metmyoglobin method

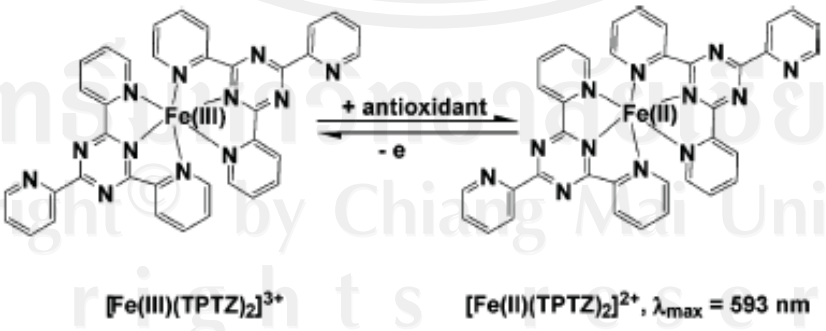
The ABTS-metmyoglobin assay for measuring total antioxidant activity is a measure of the collective hydrogen-donating abilities of the antioxidants in sample. The relative abilities of antioxidants to scavenge the radical cation $ABTS^{+}$, produced by the ferrylmyoglobin radical generated from metmyoglobin and hydrogen peroxide, is a blue-green chromogen, and the absorbance change at 734 nm monitored at 30°c, determined relative to Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman -2- carboxylic acid, a water soluble vitamin E analogue) antioxidant standard.

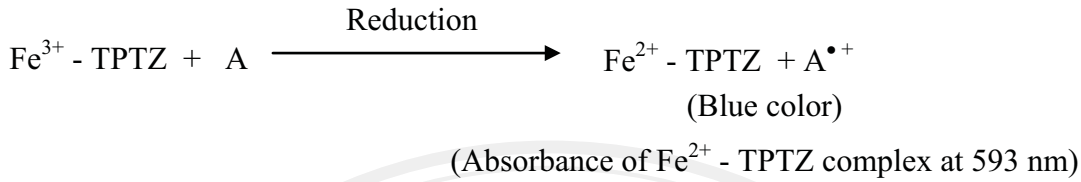


Scheme 5. Formation of $ABTS^{\bullet+}$ radical cation from activated myoglobin.

4.2 Ferric reducing ability power assay (FRAP)

The ferric reducing ability power assay is a simple measurement of reducing ability of antioxidants. The FRAP assay is a method for assessing antioxidant power. Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. Fe^{2+}-TPTZ has intensive blue color and can be monitored at 593 nm (Benzie and Strain, 1996).

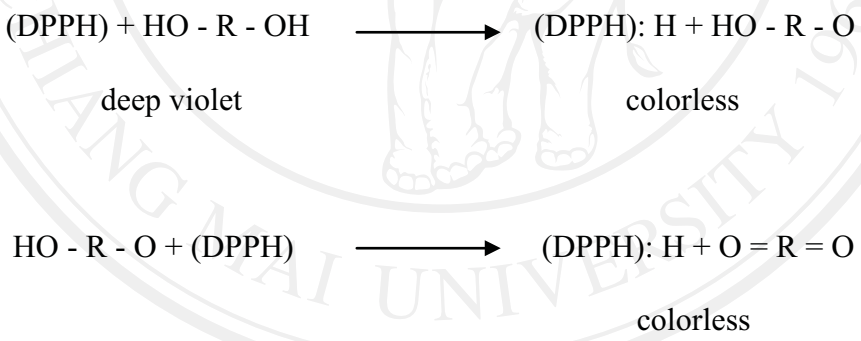




Scheme 6. Formation of ferrous tripyridyltriazine complex by ferric reduction.

4.3 DPPH scavenging assay

1,1- Dipheny1-2-picrylhydrazyl (DPPH) radical is a very stable radical with a deep violet color. When DPPH radical receives a proton from the antioxidant then it converts to a colorless protonated DPPH molecule. The mechanism of the reaction of antioxidant with a DPPH radical showed in scheme 7.

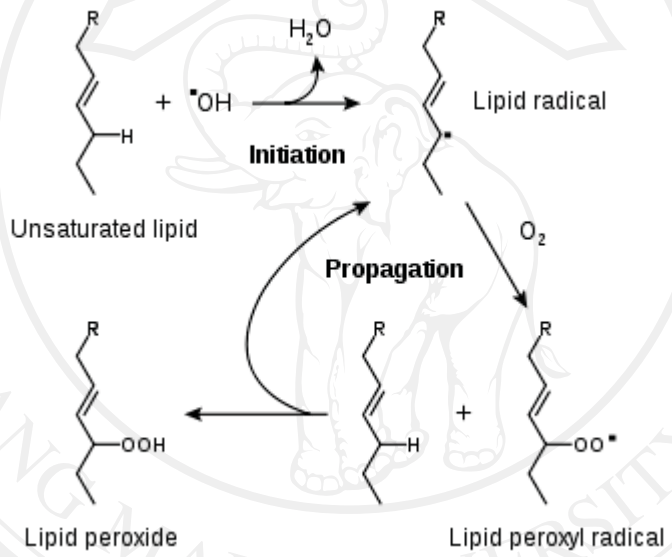


Scheme 7. The mechanism of the reaction of antioxidant with a DPPH radical

4.4 Lipid peroxidation assay

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double

bonds in between which lie methylene -CH2- groups that possess especially reactive hydrogens. As with any radical reaction the reaction consists of three major steps: initiation, propagation and termination. Certain diagnostic tests are available for the quantification of the end products of lipid peroxidation, specifically malondialdehyde (MDA). The most commonly used test is called a TBARS Assay.

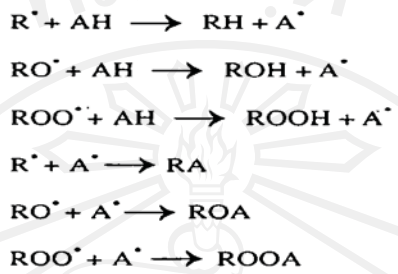


Scheme 8. Mechanism of lipid peroxidation (Wiseman et al., 1996)

4.5 Total phenolic and total flavonoid assay

Phenolics are a group of chemical substances found in plants, characterized by the presence of more than one phenol unit or building block per molecule. Phenolics are generally divided into hydrolyzable tannins (gallic acid esters of glucose and other sugars) and phenylpropanoids, such as lignins, flavonoids, and condensed tannins. The largest and best studied phenolics are the flavonoids, which include several thousand compounds, among them

the flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. Folin – ciocalteu and aluminum chloride colorimetric method are widely used to measure total phenolics and total flavonoid content, respectively. (Zhishen, 1999; Marinova et al., 2005).



Scheme 9. Reaction of phenolic antioxidants (Zhishen, 1999)

4.6 DNA relaxation assay

DNA oxidation is the process of oxidative damage on Deoxyribonucleic Acid. It occurs most readily at guanine residues due to the high oxidation potential of this base relative to cytosine, thymine, and adenine. Oxidative damage of DNA caused by a variety of chemical and physical agents appears to be linked to cancer. However, it is becoming increasingly clear that endogenous generation of oxidants, such as hydroxyl radical and peroxynitrite, lead to oxidation of DNA, and this may cause cancer in individuals where no obvious exposure to chemical or physical agents known to be carcinogenic has occurred.

4.7 Protein oxidation inhibition assay

Protein oxidation plays an important pathophysiological role and may affect protein function in normal and pathological processes, as well as during normal aging. In proteins, mainly methionine amino acid residues are oxidized, but oxidation of

cysteines is becoming increasingly of interest for its physiological and pathophysiological role.

Oxidation DNA bases and protein oxidation can be identified and quantified by using several techniques such as gas chromatography, mass spectroscopy with selected ion monitoring. In this study, the technique of electrophoresis was used to determine the DNA relaxation and protein oxidation. Free radicals can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals. The hydroxyl radical ($HO^{\cdot}$), or a closely related species, is probably the final mediator of most free radical induced tissue damage (Lloyd, 1997). All of the reactive oxygen species (ROS) exert most of their pathological effects by giving rise to hydroxyl radical formation. The reason for this is that the hydroxyl radical reacts, with extremely high rate constants, with almost every type of molecule found in living cells including sugars, amino acids, lipids, and nucleotides. Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is likely to be the transition metal catalysed decomposition of superoxide ($O2^{\cdot -}$) and hydrogen peroxide (H_2O_2) (Stohs, 1995). All elements in the first row of the d-block of the periodic table are classified as transition metals. In general, they contain one or more unpaired electrons and are therefore themselves radicals when in the elemental state. The most important transition metals in human disease are iron and copper. These elements play a key role in the production of hydroxyl radicals in vivo. Hydrogen peroxide can react with iron to generate the hydroxyl radical, a reaction first described by Fenton in 1894:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\cdot} + OH^{-}$$

This reaction can occur in vivo, but the situation is complicated by the fact that superoxide (the major source of hydrogen peroxide in vivo) will normally also be present. Superoxide and hydrogen peroxide can react together directly to produce the hydroxyl radical, but the rate constant for this reaction in aqueous solution is virtually zero. However, if transition metal ions are present a reaction sequence is established that can proceed at a rapid rate:

$$Fe3^{+} + O_2^{\cdot -} \longrightarrow Fe2^{+} + O_2$$

$$Fe2^{+} + H_2O_2 \longrightarrow Fe3^{+} + HO^{\cdot} + OH-$$

net result:

$$O2^{\cdot -} + H_2O_2 \longrightarrow OH^{-} + HO^{\cdot} + O_2$$

The net result of the reaction sequence illustrated above is known as the Haber-Weiss reaction. The resulting hydroxyl radicals have the potential to damage cellular nucleic acids, proteins, lipids and carbohydrates

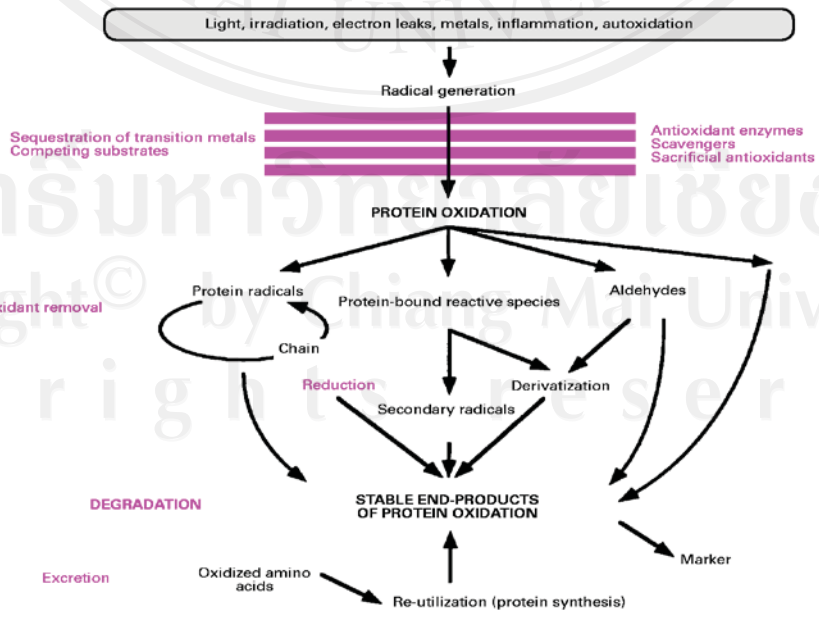


Figure 8. Postulated mechanisms of protein oxidation *in vivo*

(From : www.biochemj.org)