CHAPTER III
LITERATURE REVIEWS

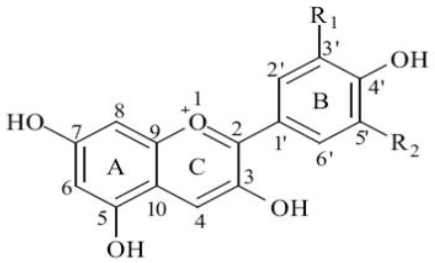
3.1 Pigmented Rice

Humans have consumed rice for almost 5000 years. Rice plays a fundamental role as the staple food for seventeen countries in Asia and the Pacific, nine countries in North and South America and eight countries in Africa (FAO, 2004). Rice is not only the source of dietary energy but also the rich source of vitamins e.g. thiamine, riboflavin and niacin and amino acids e.g. glutamic acid and aspartic acid (FAO, 2004). Commercially, either polished or unpolished rice is consumed. Unpolished rice is an important source of bioactive compounds; its grain contains more nutritional components, such as γ-oryzanol, dietary fibers, phytic acids, vitamin E, vitamin B and γ-amino butyric acid than the ordinary polished rice grain. These bio-functional components exist in the germ and bran layers which are removed during grain polishing (Champagne *et al*., 2004).

Besides white rice is widely consumed, there are many special cultivars of rice that contain color pigments, such as black rice, red rice and brown rice. Their names refer to the kernel color (black, red or purple) which are anthocyanins in different layers of the pericarp, seed coat and aleurone (Chaudhary, 2003). Many varieties of pigmented rice are reported as potent sources of antioxidants; therefore, encouragements as viable sources of antioxidants for functional foods were made (Yawadio *et al*., 2007). Black rice has a number of nutritional advantages over common white rice, such as a higher content of protein, vitamins and minerals, although the latter varies with cultivar and production location (Suzuki *et al*., 2004).

3.2 Anthocyanins in Pigmented Rice

Anthocyanins occur ubiquitously in the plants and confer the bright red, blue and purple colors to fruits and vegetables such as berries, grapes, apples, purple cabbage and corn (Wang and Stoner, 2008). They belong to a large group of compounds collectively known as flavonoids, which are a subgroup of an even larger group of compounds known as polyphenolics (McGhie and Walton, 2007). Chemically, anthocyanins are glycosylated, polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium and contain two benzoyl rings (A and B) separated by a heterocyclic (C) ring (Figure 3.1) (Mazza and Miniati, 1993). The structural variations of anthocyanins are due to differences in the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature and number of the sugar moiety attached to the phenolic (aglycone) molecule and the position of the attachment, as well as the nature and number of aliphatic or aromatic acids attached to the sugars (Mazza and Miniati, 1993). Anthocyanins most frequently occur as 3-monosides, 3-biosides and 3-triosides as well as 3,5-diglycosides and more rarely 3,7-diglycosides associated with the sugars glucose, galactose, rhamnose, arabinose, and xylose (Mazza and Miniati, 1993). The de-glycosylated or aglycone forms of anthocyanins are known as anthocyanidins. The six most common anthocyanidin skeletons are cyanidin, delphinidin, pelarogonidin, malvidin, petunidin, and peonidin (Figure 3.1). The sugar components of anthocyanins are usually conjugated to the anthocyanidin skeleton via the C3 hydroxyl group in ring C. Several hundred anthocyanins are known varying in the basic anthocyanidin skeleton and the position and extent to which the glycosides are attached to the skeleton (Harborne *et al*., 1988).



Name	R1	R2
Delphinidin	OH	OH
Petunidin	OCH_3	H
Cyanidin	OH	H
Pelargonidin	H	H
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3

Figure 3.1 Chemical structure of anthocyanidins (Hou *et al*., 2004).

Cyanidin-3-glucoside and peonidin-3-glucoside are two major anthocyanins in black rice. Hu *et al*. (2003) investigated the capacity of the anthocyanins extract of black rice to neutralize reactive nitrogen and oxygen species (free radicals) in model cell cultures. These researchers demonstrated that, as a consequence of a marked antioxidant activity and a capacity to capture free radicals in vitro, the pigmented fraction of black rice prevents DNA scission and the deterioration of human LDL induced by reactive oxygen species. The extract of black rice suppressed the formation of nitric oxide in activated macrophage without producing cell toxicity. Chen *et al*. (2006) determined the effect of cyanidin 3-glucoside and peonidin 3-glucoside, major anthocyanins extracted from black rice (*Oryza sativa* L. indica), on cell invasion, motility, and adhesion, matrix metalloproteinase (MMP)-9 and urokinase-type plasminogen activator (u-PA) expression, as well as the DNA binding activity and the nuclear translocation of AP-1 on SKHep (human hepatocellular carcinoma). They found that peonidin 3-glucoside and cyanidin 3-

glucoside inhibited invasion and motility of SKHep-1 cells; the effect was associated with a reduced expression of MMP-9 and u-PA. In the same investigation, peonidin 3-glucoside and cyanidin 3-glucoside also exerted an inhibitory effect on the DNA binding activity and the nuclear translocation of AP-1. Furthermore, these compounds also exerted an inhibitory effect of cell invasion on various cancer cells (SCC-4, Huh-7, and HeLa). Interestingly, anthocyanins from *O. sativa* L. indica (OAs) were evidenced by its inhibition on the growth of SKHep-1 cells *in vivo*. Moreover, Hyun and Chung (2004) showed that cyanidin and malvidin isolated from black rice have exerted cytotoxicity against human monocytic leukemia cells by arrest of G(2)/M phase and induction of apoptosis. Ichikawa *et al*. (2001) reported that purple black rice containing cyanidin 3-O-β-D-glucoside contributed to the antioxidative activity of the rice extract through scavenging of superoxide anions. Anthocyanidins isolated from red rice was effective on the suppression of HCT-15 cancer cells growth (Koide *et al*., 1996).

The influence of natural red or black rice consumption on atherosclerotic plaque formation or development induced by high cholesterol diet feeding in rabbits was investigated by Ling *et al*. (2001). They reported that consumption of red or black rice reduced or retarded the progression of atherosclerotic plaque development induced by dietary cholesterol. The enhanced serum HDL cholesterol and apo A-I concentrations, and the increased antioxidant and decreased oxidative status may be due to the antiatherogenic effect of red or black rice. Xia *et al*. (2003) demonstrated that the presence of black rice pigment fraction in a diet fed to apoE-deficient mice was effective in decreasing atherosclerotic plaque development in the aortic sinus. This cardioprotective effect was related to several mechanisms that corresponded to lowering serum total cholesterol concentration and LDL/HDL ratios, decreasing cholesterol accumulation in aortic arterial tissue and reducing LDL oxidation and $CD4^{+}$T lymphocytes in aortic tissue. Zawistowski *et al*. (2009) evaluated the efficacy of an anthocyanin pigmented rice (e.g. black rice) to mitigate the onset of hypercholesterolemia in rats-fed atherogenic diets. The results of the present study indicated that supplementing diets with black rice extract decreased lipid levels in the plasma, heart and liver, as well as plasma LDL, total cholesterol and triglycerides in Wistar Kyoto rats. Sangkitikomol *et al*. (2010) found that anthocyanins extract from

Thai pigmented rice inhibited hemolysis induced by 2,2'-azobis(2-amidinopropane) dihydrochloride and Heinz body formation induced by *N*-acetylphenylhydrazine. Moreover, 200 - 1000 mg/L of ARE (antioxidant response element) was able to inhibit hydrogen peroxide induced genotoxicity in mononuclear leukocytes in a dose-dependent manner. Black rice pigment fraction could exert cardioprotective effects on patients with coronary heart disease by improving plasma antioxidant status and inhibiting inflammatory factors [soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble CD40 ligand (sCD40L) and high sensitive C-reactive protein (hs-CRP)] (Wang *et al*., 2007). Prior and Wu, (2006) suggested that consumption of anthocyanins lowers the risk of cardiovascular disease, diabetes, arthritis and cancer due, at least in part, to their anti-inflammatory and antioxidant activities.

The antioxidant effects of anthocyanins *in vitro* were demonstrated using several cell culture systems including colon (Renis *et al*., 2008; Parry *et al*., 2006), endothelial (Bagchi *et al*., 2004), liver (Shih *et al*., 2007; Meyers *et al*., 2003), breast (Singletary *et al*., 2007; Olsson *et al*., 2004) and leukemic cells (Feng *et al*., 2007) and keratinocytes (Afaq *et al*., 2007).The antioxidant activity of anthocyanins is due largely to the presence of hydroxyl groups in position 3 of ring C and also in the 3', 4' and 5' positions in ring B of the molecule. In general, the radical scavenging activity of the anthocyanidins (aglycons) is superior to their respective anthocyanins (glycosides), and it decreases as the number of sugar moieties increase (Wang *et al*., 2008). Treatment of rat liver clone 9 cells with 50 μM anthocyanins (Shih *et al*., 2007) and non-cancerous breast cells with 10–20 μg/ml anthocyanins (Singletary *et al*., 2007) enhanced their antioxidant capacity by activating glutathione-related enzymes (glutathione reductase, glutathione peroxidase, and glutathione S-transferase) as well as the activity of NAD(P)H: quinone reductase. The mechanism by which anthocyanins exhibited these effects was through activation of the ARE upstream of genes that code for these enzymes. Shih *et al*. (2007) concluded that the promoting effect of anthocyanins on ARE-regulated phase II enzyme expression was critical for defending cells against oxidative stress.

Anthocyanin glycosides was rapidly absorbed from the stomach after ingestion by a process that may involve bilitranslocase; they enter the systemic circulation after passing through the liver and excreted in urine as the intact form

(McGhie and Walton, 2007). Anthocyanins are widespread in plants and they are consumed as part of normal diet. Average anthocyanin consumption was in the order of 82 mg/day in Finland and 12.5 mg/day in the United States (Wu *et al*., 2006) revealed.

3.3 Fermented Rice (Khao-Mak)

Khao-Mak is a traditional fermented rice in Thailand that is made of white glutinous rice. It is soaked overnight, streamed, washed, drained to remove excess starch and cooled at room temperature. Then, the streamed glutinous rice is fermented with a Look-Pang at room temperature for 3 days (Lotong, 1992). Look-Pang is a microbial starter containing a mixed culture of *Aspergillus* sp., *Rhizopus* sp. and *Mucor* sp., together with *Saccharomyces cerevisiae* and *Candida* sp. inocculum in rice flour mixed with herbs such as pepper, garlic and galanga as antibacterial agents (Manosroi *et al*., 2011). In the fermentation, rice starch is firstly fermented to monosaccharides by molds (Cheng *et al*., 2008). The molds in Look Pang provide monosaccharides such as glucose, fructose and galactose as the carbon sources to support the growth and metabolism of yeasts (Buglass, 2011). Finally, yeast ferments monosaccharide or sugar to alcohols and organic acids (Lotong, 1992). The ethanol (up to 10% v/v) serves as a source of calories and helps prevent growth of disease- or toxin-producing microorganisms in the products (Aidoo *et al*., 2006). The product gives sweet taste, a little alcohol, lactic acid flavor, soft texture, with lumps of cooked glutinous rice, and succulent grain (Wongpiyachon, 1995).

The fermentation of Khao-Mak uses mold and yeast such as *Saccharomyces cerevisiae* strain which had been approved to be probiotics (de Llanos *et al*., 2006). It also increases and maintains a healthy bacterial gut flora by providing an increasing amount of food for these bacteria (Manosroi *et al*., 2011). In Thai folklore wisdom, Khao-Mak has been considered to promote the growth development of malnutritioned children, activate bacterial activity, and used as a dietary supplement (Pitiporn, 2008). It is a probiotic consisting of live microorganisms that have a health benefit on consumers when it is administered in adequate amounts.

Pigmented rice, especially black glutinous rice, is sometimes substituted for white glutinous rice to produce Khao-Mak since it is a rich source of phytochemicals such as anthocyanins, cyanidin-3-glucoside and peonidin-3-glucoside, (Hu *et al*., 2003, Abdel-Aal *et al*., 2006, Chen *et al*., 2006; Sompong *et al*., 2011). Interestingly, antioxidant activity and total phenolic content of fermented Hom-Nil rice and black glutinous rice extract were higher than those of both raw rice extract and the cooked rice extract (Sadabpod *et al*., 2010). The greater total phenolic content of fermented rice may be due to the enzymatic activities of starter organisms in Look Pang such as *Aspergillus* sp. and *Rhizopus* sp. (Manosroi *et al*., 2011). Lee and Chou (2006) reported that *Aspergillus* sp. and *Rhizopus* sp. can produce β-glucosidase enzyme which is capable of hydrolyzing glucoside isoflavones in back bean koji with the formation of aglycones. In addition, Lee and Chou (2006) found that black soybean koji fermented with *Aspergillus* sp. or *Rhizopus* sp. had a significant increase in the aglycone contents (daidzein, glycitein, and genistein) and β-glucoside isoflavone contents (daidzin, glycitin, and genistin) compared with those of unfermented steamed black bean. Normally, the antioxidant activity of the anthocyanidins (aglycons) was generally greater than those of the corresponding anthocyanins (glycosides) (Wang and Stoner, 2008). Therefore, the action of β-glucosidase produced by the starter organism during fermentation might be an important factor contributing to the increase of phenolic content such as anthocyanins of fermented rice. Enhancing the antioxidant activity of fermented rice might be due to the increase of total phenolic.

The antimutagenicity against *in vivo* formed nitrosomethylurea in the somatic mutation and recombination test of fermented black glutinous rice was higher than that of its corresponding raw or cooked rice (Vipassanatham *et al*., 2012) and nitrite treated 1-aminopyrene on *S. typhimurium* TA98 (Sadabpod *et al*, 2010). It is suggested that a remarkable increase in phenolic contents such as anthocyanins during fermentation might contribute to enhancing of antimutagenicity of fermented rice.

3.4 Free Radicals and Antioxidants

A free radical is any atom that there is at least one unpaired electron in the outermost shell (Gutteridge and Mitchell, 1999). These uncoupled electrons are very reactive with adjacent molecules such as lipids, proteins, and carbohydrates and can cause cellular damage (Kuhn, 2003). Free radicals play an important role in a number of biological processes. Some of these are necessary for life, such as the intracellular killing of bacteria by phagocytic cells such as granulocytes and macrophages. Researchers have also implicated free radicals in certain cell signaling processes (Pacher *et al.*, 2007). This is dubbed redox signaling.

Karthikeyan *et al*. (2011) suggested that excessive amounts of these free radicals can lead to cell injury and death, which may contribute to many diseases such as cancer, stroke, myocardial infarction, diabetes, and major disorders. Many forms of cancer are thought to be the result of reactions between free radicals and DNA that can adversely affect the cell cycle and potentially lead to malignancy (Mukherjee *et al*., 2004).

Free radicals may also be involved in Parkinson's disease, senile and drug-induced deafness, schizophrenia, and Alzheimer's (Floyd, 1999). The classic free-radical syndrome, the iron-storage disease hemochromatosis, is typically associated with a constellation of free-radical-related symptoms including movement disorder, psychosis, skin pigmentary melanin abnormalities, deafness, arthritis, and diabetes mellitus. The free radical theory of aging proposes that free radicals underlie the aging process itself. Similarly, the process of mitohormesis suggests that repeated exposure to free radicals may extend life span. Because free radicals are necessary for life, the body has a number of mechanisms to minimize free-radical-induced damage and to repair damage that occurs, such as the enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (Scheibmeir *et al*., 2005).

Antioxidants are substances which counteract free radicals and prevent the damage caused by them (Azzi *et al*., 2004). Antioxidants are divided into two classes based on mechanism of action: (1) preventive antioxidants and (2) chain-breaking antioxidants (Scheibmeir *et al*., 2005). Preventive antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase are enzymes that scavenge initiating radicals before they start an oxidation chain (Guo *et al*., 2003).

Chain-breaking antioxidants obtained from dietary sources break the chain of free radical formation by donating an electron to stabilize an existing free radical. Therefore, dietary intake of natural antioxidants such as polyphenols, vitamins, carotenoids, organosulfural compounds, and minerals (Liu, 2004; Ratnam *et al*., 2006) may be an important factor in the body's defense mechanism against free radicals and many antioxidants are identified as anticarcinogens. Furthermore, there is good evidence that bilirubin and uric acid, compounds formed in living organisms, can act as antioxidants to help neutralize certain free radicals. Bilirubin comes from the breakdown of red blood cells' contents, while uric acid is a breakdown product of purines. Too much bilirubin, though, can lead to jaundice, which could eventually damage the central nervous system, while too much uric acid causes gout (Rhodes, 2000).

3.5 Maillard Reaction

The Maillard reaction is a chemical reaction taking place during food processing and results in a wide variety of Maillard reaction products (MRP) such as melanoidins which influenced on food quality attribute such as aroma, taste and color (Jaeger *et al*., 2010). The antioxidant properties of melanoidins or MRPs from different model systems (Hayase *et al*., 2006; Jing and Kitts, 2000) and melanoidin fractions in foods such as coffee (Daglia *et al*., 2008), bread crust (Somoza *et al*., 2005), biscuit (Martin *et al*., 2009), roasted barley (Papetti *et al*., 2006), and roasted cocoa (Summa *et al*., 2008) were investigated.

The brown chromophoric MRPs, independent of the reaction conditions, displayed the *in vitro* antioxidant protective effects on lipids (Verzelloni *et al*., 2010; Wang *et al*., 2010), rat microsomes (Daglia *et al*., 2008), hepatocytes (Valls-Bellș *et al*., 2004), HepG2 cells (Martin *et al*., 2009) and human lymphocytes (Wang *et al*., 2010) against oxidation challenge. An *in vivo* study (Somoza *et al*., 2005) showed that bread crust, caraffa malt, or pronyl bovine serum albumin (which contained pyrrolinone reductonyl-lysine, a food melanoidin) decreased oxidative stress levels and thiobarbituric acid reactive substances levels, and increased tocopherol in the

plasma of rats. Although the specific components responsible for the protective antioxidant behavior observed for melanoidins are not yet known, melanoidins are thought to be important in preventing oxidative damage and diseases related to free radicals (Wang *et al*., 2011). In addition, Wen *et al*. (2005) found that MRPs of brewed coffee decreased zinc-chelating activity. It is assumed that the action mechanism of MRPs is based on the ability for trapping positively charged electrophilic species; scavenge oxygen radicals or metal chelation to form inactive complexes.

MRPs might be composed of some compounds posing antimutagenic activity. Miwa *et al*. (2002) indicated that melanoidins could protect nitric oxide in inducing DNA damage of HL60 (human promyelocytic leukemic cell line). Hwang *et al*. (2011) found that MRPs showed an antiproliferative effect on human colon cancer cells. In addition, melanoidin fractions of bread crust increased glutathione S-transferase (GST) activity (Faist *et al*., 2002; Lindenmeier *et al*., 2002) but decreased phase I NADPH cytochrome C reductase (CCR) activity in Caco-2 cells (Lindenmeier *et al*., 2002). A decrease in phase I enzyme activity associated with an increase in phase II enzyme activity is effective in contributing to chemopreventive potential (Wilkinson and Clapper, 1997).

3.6 Effect of Storage on Antioxidant Activity, Total Phenolic Content, Anthocyanin Content, and Antimutagenicity

Storage of foods has some negative effects on antioxidant activity, total phenolic and anthocyanin contents. Gliszczynska-Swiglo and Tyrakowska (2003) found a decrease (6-14%) in the total phenolic content of apple juice stored at room temperature for 11 -month. Yang *et al*. (2007) noted that noni juice stored for 1 month at 24°C, 4°C, and -18°C lost 80%, 30%, and 10% of initial free-radical scavenging activity (DPPH), respectively; and after 3 months it lost 90%, 55%, and 15%, respectively. Noni powder stored at 24°C, 4°C, and -18°C for 21 days significantly decreased free-radical-scavenging activity by 20–30%. The effect of storage on the total phenolic content and antioxidant capacity of ketchups and tomato juices was

studied by Vallverdú-Queralt *et al*. (2011). They reported that the phenolic content and antioxidant capacity of the hydrophilic fraction decreased by about 12% and 10-12%, respectively for tomato juices and 7% and 7- 9 %, respectively for ketchups after 9-months storage. Ścibisz and Mitek (2009) reported that about 34, 18 and 68% of antioxidant activities, total phenolic and total anthocyanin contents in low sugar blueberry jams kept in glass jar decreased during the 4-month storage at 22°C. Syamaladevi *et al*. (2011) also found that total antioxidant activities, total phenolic compounds, and total anthocyanins decreased up to 52, 69 and 86%, respectively during the 13-month storage of canned blueberry solids. However, storage periods did not affect the antioxidant activity or anti-proliferation effect of the juice on different cancer cell lines. Akim *et al*. (2011) evaluated the antioxidative capacity of commercialized Roselle juice at three storage periods (one week, one month and one year) at 4°C and its antiproliferative effect on breast (MCF-7 and MDA-MB-231), ovarian (Caov-3), and cervical (HeLa) cancer cell lines. They found that **c**ommercial Roselle juice at different storage periods exhibited fairly strong antioxidant activity; this activity was not significantly different between different samples ($p>0.05$). The juice also showed significant anti-proliferative activity on Caov-3, HeLa, MDA-MB-231, and MCF-7 cells, with the juice being most selective towards MCF-7 cells. However, the difference in IC50 values between samples and cell lines was not significant ($p>0.05$).

Food packaging is sometimes the variable factor for the lost of natural components. Trošt *et al.* (2008) reported that after 207-day of storage at 30°C in the dark, only 11% of total anthocyanins were still present in the nectar filled in glass. After 183-day of storage, 9% of total anthocyanins were still present in carton packaging. These data show that the destruction of anthocyanins occurs faster in carton packaging than in glass packaging. Wang *et al*. (2010) investigated the effect of storage temperature and packaging condition on the changes in antimutagenicity and anthyocyanin content of black soybean koji. They found that black soybean koji contained an initial anthocyanin content of 0.81 mg cyanidin-3-glucoside/g dry koji before storage. While the anthocyanin content reduced to 0.68, 0.69 and 0.71 cyanidin-3-glucoside mg/g dry koji, respectively, after the koji was stored 120-day at 25°C with deoxidant, desiccant and both deoxidant and desiccant, respectively. The

extract of koji before storage exhibited an inhibition rate of 81.07% against the mutagenesis induced by 4-Nitroquinoline-N-oxide in *S. Typhimurium* TA 100. While the extract of koji stored at 25°C with deoxidant, desiccant and both deoxidant and desiccant for 120 days exhibited an antimutagenicity of 62.72, 59.33 and 71.18 %, respectively.

3.7 Somatic Mutation and Recombination Test (SMART)

The fruit fly *Drosophila melanogaster* was shown to have a good capacity for describing mutagenic and carcinogenic activity of various chemical compounds (Rodriguez and Tellez, 2002). The extensive knowledge of the genetics of *Drosophila melanogaster* and the long experimental experience with this organism has made it useful in genetic toxicology (Hamss *et al*., 2003).

Somatic mutation and recombination tests (SMART) of *Drosophila*, the rapid, inexpensive and sensitive tests, are increasingly interest because of its well-known array of genotoxicity test systems. These assays are able to detect a wide spectrum of genetic end points, such as point mutations, deletions, certain types of chromosome aberrations as well as mitotic recombination and gene conversion (Graf *et al*., 1984; Vogel and Zijlstra, 1987; Würgler and Vogel, 1986). This eukaryote, namely *Drosophila melanogaster*, presents several advantages. The main points are: it is a eukaryotic organism with a short generation time (approx. 10 d at 25°C); it has easily detectable genetically controlled morphological characters; large numbers of mutants and genetically characterized strains are available; culture media are inexpensive and allow the breeding of large numbers of animals using simple facilities; it is capable of activating enzymatically promutagens and procarcinogens *in vivo* (Sarikaya and Cakir, 2005). It is well established that *Drosophila* possesses a versatile system for the metabolism of xenobiotics (Baars, 1980; Hallstrom *et al*., 1984). The SMART provides a suitable substitute or at least a complementary *in vivo* method to mammalian *in vivo* investigation. *Drosophila* has detoxification-activating systems in many respects closely resembling the corresponding systems in mammals, which makes it possible to extrapolate data to mammals. The used of SMART assays

is based on the treatment of larvae, and besides the number of mutated spots appearing in the adult flies, indicating the frequency of genetic events, the size of the spots indicates the time of action during embryogenesis.

Two different SMART systems are vastly investigated, namely the wing spot test and the eye spot test. Both are based on the fact that during early embryonic development, groups of cells (imaginal discs) are set apart (Graf *et al*., 1998). They proliferate mitotically during the larval development until they differentiate during metamorphosis into structures of the body of the adult fly (eyes, wings, etc.). The somatic assays take advantage of the possibility to expose such large populations of mitotically growing cells in the imaginal discs of larvae. If a genetic alteration occurs in one of these imaginal disc cells, this alteration will be present in all the descendant cells and will form a clone of mutant cells. If the alteration causes a visible change in the phenotype, the mutant cell clone can be detected as a spot of mutant cells on the body surface of the adult flies. The use of improved high-bioactivation (HB) strains of *Drosophila melanogaster* which are characterized by increased cytochrome P-450-dependent bioactivation capacity facilitates the detection of promutagens of different chemical classes (Graf and Singer, 1989; Graf and Van Schaik, 1992). The SMART assays were developed to detect the loss of heterozygosity of suitable gene markers which determine detectable phenotypes expressed on the eyes or the wings of the flies. The somatic assays can be performed in only one fly generation in contrast to the classical test for sex-linked recessive lethals in germ cells which need at least one month for completion (Vogel, 1987). Owing to these advantages, the SMART assays have become a very suitable approach for genotoxicity testing of chemical and physical agents (Vogel and Nivard, 1993; Würgler and Vogel, 1986).

3.8 Urethane: A Standard Mutagen

Urethane ($NH_2COOCH_2CH_3$), also known as ethyl carbamate, is the ethyl ester of carbamic acid (NH_2COOH). Urethane may occur as a colorless, odorless crystal or white, granular powers. It is slightly soluble in olive oil and soluble in water, ethane, ether, glycerol, chloroform and ethyl ether. Urethane is used as both an animal

anesthetic (Kotanidou *et al*., 1996; Norlen *et al*., 2000) and an industrial chemical (Crout, 1976). The major source of human exposure to urethane is from fermented food products (bread, cheese and yoghurt) and alcoholic beverages (beer and white wine) (IARC, 1974; Ough, 1976; Miller and Miller, 1983; Canas *et al*., 1989).

In rodents, urethane was found to produce lymphomas, lung tumors, hepatomas, and melanomas (Mori *et al*., 2000; Mirvish, 1968; IARC, 1974) and was also found to induce point mutation, gene conversion, intrachromosomal recombination, chromosomal aberration, and sister chromatid exchanges in yeast, plant system, and mammalian cells (Schlatter and Lutz, 1990; Uggla and Busk, 1992). The International Agency for Research on Cancer (IARC) classified urethane as possibly carcinogenic to humans (group 2B) (IARC, 1974). Urethane is generally used as positive mutagen in evaluation the mutagenicity of the unknown in the somatic mutation and recombination test (Abraham and Graf, 1996). Inducing genotoxicity in *Drosophila melanogaster* was reported by Zimmerli *et al*. (1991).

Urethane is not commercialized. Since this chemical requires metabolic activation to express its mutagenic activity (Frolich and Würgler, 1990b). It exerts its carcinogenic effect following bioactivation to vinyl carbamate epoxide which forms RNA and DNA adducts and initiates tumorigenesis (Dahl *et al*., 1978; Leithauser *et al*., 1990). The activation of urethane is important in exerting its carcinogenic effect. The two step oxidation of urethane to the active vinyl canbamate epoxide is catalyzed primarily by cytochrome P-450 subtype 2E1 (Guengerich *et al*., 1991). Vinyl carbamate epoxide is a major strong ultimate reactive electrophilic, mutagenic and carcinogenic metabolite of urethane and vinyl carbamate in mouse (Park *et al*., 1993). Generation of the electrophilic vinyl carbamate epoxide leads to the formation of RNA and DNA adducts and the initiation of tumorigenesis (Leithauser *et al*., 1990). Park *et al*. (1993) showed that vinyl carbamate and the oxide metabolite produced guanine adducts in both mouse and rat liver DNA.

The major detoxification pathway of urethane is through hydrolysis to ethanol, ammonia and carbon dioxide (IARC, 1974), a reaction mediated by CYP2E1 (Hoffler *et al*., 2003). In rats, mice and humans, CYP2E1 is induced five to 20-fold by ethanol (Lieber, 1988 and 1990; Kurata *et al*., 1991a; Ingelman-Sundberg *et al*., 1993) which suggests that chronic ethanol exposure could increase the oxidation of urethane

to its epoxide derivative. On the other hand, ethanol has been reported to decrease the metabolism of urethane, presumably by acting as a competitive substrate (Waddell *et al*., 1987; Yamamoto *et al*., 1988; Kurata *et al*., 1991b). Kemper *et al*. (1995) investigated the role of glutathione in protection against vinyl carbamate epoxide-mediated adduct formation and the involvement of glutathione-*S*-transferase (GST) in detoxification of vinyl carbamate epoxide. They reported that glutathione inhibited formation of ethenoadenosine in a concentration-dependent manner ranging from 1 to 8 mM. This effect was significantly enhanced by addition of rat liver GST. In addition, De flora *et al*. (1986) reported that *N*-acetylcysteine (NAC), a precursor of intracellular glutathione, efficiently prevented the induction of lung tumors in Swiss albino mice when supplemented to the diet both before and after and i.p. injection of the carcinogen urethane. Irrespective of urethane administration, NAC also significantly enhanced GST activity in liver preparations of the same animals. Investigation on the change in GST activity in relation to the observed *in vivo* antigenotoxicity of fresh vegetables, spices, tea and coffee was done by Abraham *et al*. (1998). This experiment showed that treatment with urethane alone resulted in inhibition of GST activity. Co-administration of urethane with extracts of vegetables, coffee and spices resulted in dosed- related attenuation of the inhibitory effect of urethane on GST activity. However, tea had no effect on inhibition of GST activity by urethane. Hence and association between antigenotoxicity and GST activity could not be established. Furthermore, Abraham and Graf (1996) investigated the protective effects of coffee against somatic mutation and mitotic recombination induced by urethane were evaluated in the standard (ST) and high bioactivation (HB) crosses of the wing spot test in *Drosophila melanogaster*. The results showed high sensitivity of the HB cross to urethane. Co-administration of instant coffee was effective exerting significant dose-related inhibitory effects on the genotoxicity of urethane in the ST and genetically susceptible HB cross. Pretreatment of 2-day-old HB larvae with coffee for 24 h followed by treatment with urethane was also effective insignificantly reducing the induction of mutation and recombination. The magnitude of the protective effects of coffee against the genotoxin (urethane) was independent of the genotype of the larvae used for treatment. A dose-dependent increase in the genotoxic activity of urethane was observed in SMART (Frölich and Würgler, 1990b). The frequency of

induction of mutation in the modification strain with increased cytochrome P450 enzyme activities was increased by about one order of magnitude compared with the standard strain. The frequencies of spots per wing in high bioactivation cross were higher than those of standard cross (Frölich and Würgler, 1990a). This might result from the constitutive expression of the enzymes required for the transformation of urethane into ultimate genotoxic metabolites.

3.9 Functional Food and Health Benefits

Using foods to provide benefits beyond preventing deficiency diseases is a logical extension of traditional nutritional interventions. The combination of consumer desires, advances in food technology, and new evidence-based science linking diet to disease and disease prevention has created an unprecedented opportunity to address public health issues through diet and lifestyle. It is interested in selection foods that possibly promote health resulted in the use of the term "functional foods." Generally, foods can be considered "functional," if they have been proved to provide specific health benefits beyond basic nutrition such as retarding bone loss, alteration of immunological defense, increasing antioxidant status etc.

The term functional food is nonetheless useful because it might convey to the consumer both the unique characteristics of the food and the associated health benefits. Today's science and technology can provide many additional functional foods that promise a greater range of health benefits for consumers. Functional foods might provide some health benefits by reducing the risk of chronic disease and enhancing the ability to manage chronic disease, thus improving the quality of life. Functional foods might alter growth and development of human body and enhance physical performance as well as preventing internal formation of free radical during exercise as indicated by Mergener *et al.* (2009).

Food sciences and improved nutrition play crucial roles in the increase in life expectancy over the past 200 years; however, the impact of diet on health is much broader than basic nutrition. A growing body of evidence documents positive health benefits from food components not considered nutrients in the traditional definition

(Liu, 2007). The advances in health sciences allow researchers to better characterize the biological basis of disease states, understand the metabolism of food at the cellular level as well as identify the role of bioactive components in food and assess their impact on metabolic processes. Well-planned experimental designs can enable scientists to unlock the biological functions of numbers of food components and their role in disease prevention and health promotion.

Functional foods can be designed in many forms. Some may be conventional foods with bioactive components that can now be identified and linked to positive health outcomes e.g. antimutagenesis, antioxidant, prevention of DNA damage etc. Some specific fortified foods are specifically created to reduce disease risk for a certain group of people such as the victims of obesity (Gonzalez-Castejon and Rodriguez-Casado 2011)

Foods may be developed to promote the expression of specific metabolites, reducing or preventing common diseases that afflict consumers with a specific genotype. Consumers might select functional foods and tailor their diets to meet changing health goals and different requirements at different ages. Future benefits might include functional foods for increased energy, mental alertness, better sleep and possible reduced degenerative diseases.

Consumers can select their desired functional food from a wide spectrum of foods that contain specific components either inherently (e.g., soy protein, cranberries) or via fortification (e.g., folate-fortified foods). Health benefits may result from increasing the consumption of substances already part of an individual's diet or from adding new substances to an individual's diet. Adding identified bioactive components to normal foods, the opportunities for developing functional foods will be broad (O'Donnell, 2003). Foods that naturally provide a bioactive substance may be enhanced to increase the level present in the food e.g., eggs with increased levels of omega-3 fatty acids. In addition, a particular component with health benefit can be fortified to food that do not naturally contain a substance significantly provides consumers with a broader selection of food sources for e.g., calcium-fortified orange juice.

Areas for research include better understanding the role and optimal levels of traditional nutrients for specific segments of the population, as well as identifying

bioactive substances present in foods and establishing optimal levels. Early researches in nutrition and food sciences focused on food containing the ranges of vitamins and minerals necessary to prevent deficiencies. Now, researchers are investigating the optimum intake levels for both traditional and untraditional nutrients in various subpopulations. Similar research is needed to identify the role of other bioactive food components, an area of research that is still in its infancy. Only recently, several government agencies have begun developing a standard definition for "bioactive" food components (HHS/OS/OPHS, 2004). Research has proven that food and isolated food components might reduce the risk of disease. The successfulness of increase vitamin A in chickne eggs (Mendonc *et al*., 2002) might reduce number of malnourished children with blindness. Some examples of foods that may be considered functional foods include calcium-fortified orange juice, phytosterol/stanol-fortified spreads and juices, folate-enriched foods, soluble oat fiber, cranberry, and soy.

Research currently underway at academic, industry and government facilities will reveal how natural substances can be used as functional food components. For instances, at the Institute of Nutrition, Mahidol University the water extracts of ten natural color sources, namely *Aegle marmelos* Corr. (Bael fruit), *Oryza sativa* L. indica (Black rice), *Clitoria ternatea* Linn. (Butterfly pea flower), *Daucus carota* Linn. (Carrot), *Chrysanthemum indicum* (Chrysanthemum flower), *Curcuma longa* Linn. (Turmeric), *Hylocereus polyrhizus* (Dragon Fruit), *Pandanus odorus* Ridl. (Fragrant screw pine), *Hibiscus sabdariffa* Linn. (Roselle flower) and *Carthamus tinctorius* Linn. (Safflower flower) were incorporated into Khanomjeen (Thai Rice Noodles) and express their antimutagenicity against urethane in the somatic mutation and recombination test (Kraiket, 2007). Although additional research is necessary to validate efficacy and establish appropriate dietary levels, researchers have identified functional food components that may improve or reduce some degenerative diseases and provide other benefits typically associated with drugs. In addition, new technologies will provide opportunities to produce bioactive food components from nontraditional sources. For example, Abbadi *et al*. (2004) developed transgenic plant oils enriched with very long chain polyunsaturated fatty acids. Other research has produced stearidonic acid (a precursor for eicosapentaenoic acid) in canola seeds to

provide another source of omega-3 fatty acids in the diet (James *et al*., 2003; Ursin, 2003).

Nowadays, a lot of consumers realize the ability to manage their health by improving their present health and/or counteracting against aging and degenerative diseases. They create a wish for food items with enhanced characteristics and associated health benefits. In one study, 93% of consumers believed certain foods have health benefits that may reduce the risk of disease or other health concerns. In addition, 85% expressed interest in learning more about the health benefits offered by functional foods (IFIC, 2002). Developing a new functional food is an expensive process. Food companies in the western countries have traditionally funded research for new food product formulations but for functional foods, the stakes are higher-for both food companies and consumers.