CHAPTER VI
DISCUSSION

6.1 Total Phenolic Content, Anthocyanin Content, and Antioxidant Activities of Fermented Pigmented rice

The present study found that fermented rice had higher total phenolic content, anthocyanin content and antioxidant activities than those of corresponding unfermented one. The increases of total phenolic and anthocyanin contents of fermented samples in this study were similar to those found by other investigators who worked on some other plants. Amongst those, Kwak *et al*. (2007) found that Chungkookjang, a Korean fermented soybean paste, had significantly higher total polyphenolic content than that of raw soybean. Lee *et al*. (2007 and 2008) reported that fermented black beans exhibited higher total phenolic and anthocyanin contents than those of unfermented black beans. Furthermore, Liang *et al*. (2009) found that *Phellinus*-fermented rice exhibited higher total phenolic content than that of unfermented rice. Sadabpod *et al.* (2010) also reported that total phenolic content of fermented Hom Nil rice and black glutinous rice were higher than those of both raw rice and cooked rice.

The greater total phenolic and anthocyanin contents of fermented rice may be due to the enzymatic activities of starter organisms in Look Pang such as *Saccharomyces cerevisiae*, *Aspergillus* sp. and *Rhizopus* sp. (Manosroi *et al*., 2011). It was reported that rubi fructus fruit (Ju *et al*., 2009), buckwheat, wheat germ, barley, rye (Đordević *et al*., 2010) and watermelon rind (Erukainure *et al*., 2011) fermented with *Saccharomyces cerevisiae* and koji made from black soybean fermented with *Aspergillus* sp. or *Rhizopus* sp (Lee *et al*., 2008) exhibited higher total phenolic content than those of unfermented ones. It was suggested that the increase of total phenolic content might be due to hydrolysis by a glycoside hydrolase during fermentation (Ju *et al*., 2009). Lee and Chou (2006) revealed that fermentation caused a marked increase in the content of aglycone (daidzein, glycitein, and genistein), the

bioactive isoflavone, and a significant reduction in the content of β-glucoisde isoflavone (daidzin, glycitin, and genistin), compared with the unfermented steamed black bean. The increase of aglycone content and the increase of β-glucosidase activity during the fermentation of this koji showed a similar trend. Therefore, the action of enzyme such as β-glucosidase produced by the starter organism during fermentation might be an important factor contributing to the increase of phenolic and anthocyanin contents of fermented rice.

Enhancing the DPPH scavenging activity and Fe^{3+} reducing power of fermented rice might be due to the increase of total phenolic and anthocyanin contents (as discussed above). Anthocyanins are natural phenolic pigments that were reported to scavenge free radicals such as superoxide (O_2^-), singlet oxygen ($^{\cdot}O_2$), peroxide (ROO^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Wang and Jiao, 2000). Normally, the antioxidant activities of the anthocyanidins (aglycons) were generally greater than those of the corresponding anthocyanins (glycosides) (Wang and Stoner, 2008). It should be noted that other antioxidants commonly found in pigmented rice such as ferulic acid, p-coumaric acid, protocatechuic acid, vanillic acid, caffeic acid (Vichapong *et al*., 2010 and Sompong *et al*., 2011), and tocopherols may exhibit antioxidant activities (Aguilar-Garcia *et al*., 2007; Hyogo *et al*., 2010; Vichapong *et al*., 2010; Sompong *et al*., 2011).

The finding on the benefit of fermentation was consistent with the observations of other researchers. Lee *et al*. (2008) indicated that black bean fermented with *Aspergillus* sp. exhibited higher levels of DPPH scavenging activity and Fe^{3+} reducing power than did the non-fermented one. Moktan *et al*. (2008) found that soybean fermented with *Bacillus subtilis* was more effective in DPPH scavenging activity and Fe^{3+} reducing ability than that of unfermented one. Moreover, Sadabpod *et al.* (2010) reported that DPPH scavenging activity and Fe^{3+} reducing power of fermented Hom Nil rice and black glutinous rice were higher than those of both raw rice and cooked rice while fermented maize (Daker *et al*., 2008) and *Phellinus*-fermented adlay and rice (Liang *et al*., 2009) had better DPPH scavenging effect than that of unfermented ones. The *Saccharomyces cerevisiae* fermented watermelon rind was shown to have higher DPPH scavenging activity and Fe^{3+} reducing power than that of unfermented one (Erukainure *et al*., 2011).

Antioxidants are important in defense systems of the body against free radicals which produced both *in situ* (aerobic respiration, inflammation and other metabolic processes) and exogenous sources (tobacco smoke, pollution, and ionizing radiation (Vijay and Vijayvergia, 2010). In addition, strenuous and prolonged exercise increases oxygen uptake at the body, especially skeleton muscle and heart resulting a large increase free radical formation (Viňa *et al*., 2000; Ji 2003). However, the body does not effectively handle excess free radical formation. Therefore, consumption of food containing antioxidants such as Khao-Mak may be helpful in reducing the hazard created by free radicals.

6.2 Antimutagenicity of Unfermented and Fermented Pigmented Rice

Currently, pigmented rice such as Sung Yod, Mon Poo, Hom Mali Daeng, Hom Nil, Riceberry, and black glutinous rice are widely consumed in Thailand. In addition, black glutinous rice is sometimes substituted for white glutinous rice to produce Khao-Mak which is a traditional dessert in Thailand. Therefore, this study has proved that all pigmented rice are safe to consumer since none of the samples expressed their mutagenicity in the somatic mutation and recombination test.

It is very interesting to find the counteracting effect against urethane mutagenicity on *D. melanogaster* of both unfermented rice and fermented rice. Fermented rice gave greater inhibitory effect than that of unfermented rice of the same variety in both trials. Increasing antimutagenic effect of pigmented rice via fermentation, as observed in the present study, is compatible with that found by other investigators. For example, Sadabpod *et al.* (2010) revealed that the antimutagenicity against nitrite treated 1-aminopyrene on *S. typhimurium* TA98 in the Ames test of fermented black glutinous rice was higher than that of its corresponding raw and cooked rice. Vipassanatham *et al.* (2012) also indicated that fermented black glutinous rice had higher antimutagenicity against *in vivo* formed nitrosomethylurea in *Drosophila melanogaster* in the somatic mutation and recombination test compared to that of raw and cooked rice. In addition, many researchers also found the benefit of

fermentation on other foods such as fermented soymilk (Hsieh and Chou, 2006), soybean koji (Lin and Chou, 2006) and fermented black bean (Hung *et al*., 2007) that could suppress the mutagenicity of 4-nitroquinoline-N-oxide.

A remarkable increase in phenolic content such as anthocyanins during fermentation may also contribute to enhance the antimutagenicity of fermented rice. Anthocyanins were found to counteract N-nitrosomethylbenzylamine (Reen *et al*., 2006), tert-butyl hydroperoxide (t-BHP), (Hwang *et al*., 2011a) and di-methyl nitrosamine (Hwang *et al*., 2011b) in inducing hepatic damage in rats. In addition, Choi *et al*. (2009) showed that anthocyanins obtained from purple-fleshed sweet potato protected experimental rats against acetaminophen-induced hepatotoxicity by blocking CYP2E1-mediated acetaminophen bioactivation. It is suggested that the protective effects of fermented rice against urethane-induced mutagenicity may be due to ability of anthocyanins to inhibit CYP2E1-mediated urethane metabolism since Hoffler *et al*. (2005) reported that urethane-induced micronuclei formation was reduced in CYP2E1-null mice. In addition, other compounds contained in pigmented rice such as ferulic acid, p-coumaric acid, protocatechuic acid, vanillic acid, caffeic acid (Hyogo *et al*., 2010; Vichapong *et al*., 2010; Sompong *et al*., 2011) may also contribute to the enhanced antimutagenicity.

The high bioactivation (HB) *Drosophila melanogaster* strain used in the present study is highly sensitive to the genotoxic effects of urethane because of its high constitutive level of cytochrome P-450 activity (Frölich and Würgler, 1990a; Graf and van Schaik, 1992). Hence, it represents an extreme state of genetic susceptibility to urethane. The present study supported that fermentation influenced the observed antimutagenic properties against urethane. The levels of antimutagenicity also depended on rice variety. Urethane is found in very small quantities in several fermented foods and beverages such as stone-fruit brandies and table wines (Schlatter and Lutz, 1990; Stoewsand *et al*., 1991). This has evoked interest in carrying out investigations to identify fermented pigmented rice that can inhibit the carcinogenic effects of urethane. Although to different extents, the pigmented rice reduced all types of mutations induced by urethane. The differences in antimutagenic effect suggest that the mixture of antimutagenic compounds varied among the varieties of tested pigmented rice. Moreover, the extraction of the available antimutagenic compounds

occurred with different efficiencies depending on fermentation. This suggests that some of the components either must undergo some sort of activation or must induce or activate a biological pathway *in vivo*. This of course does not exclude the concept that some of these components may also react directly with the electrophilic mutagens.

6.3 Selection of Fermented Pigmented Rice for a New Functional Food Product

The results of total phenolic content, anthocyanin content, antioxidant activities, and antimutagenicity of individual fermented rice were extrapolated to be sum of scores which was established as a guideline for the selection of the most beneficial sample. In this study, the fermented black glutinous rice has the highest sum of scores. Therefore, it was selected to be analyzed for its nutritive values as well as used in the formulation of a new functional food product namely, cereal bar.

Being analyzed for nutritive values, it was found that fermented rice contained less amount of carbohydrate than that of unfermented one. The reduction of carbohydrate content of fermented rice should be due to the fact that starch molecules were digested during fermentation to monosaccharides by fungal enzymes (Cheng *et al*., 2008). Then, the molds in Look Pang adjusted the fermenting condition for the flourish growth of yeast by providing the monosaccharides such as glucose, fructose and galactose as the carbon sources to support the growth and metabolism of yeasts (Buglass, 2011). Finally, yeast fermented monosaccharide or sugar to alcohols and acids.

6.4 Formulation of Cereal Bar Containing Dried Fermented Rice

There is no research and development using Khao-Mak as a component of any food product designed to lower the risk concerning dietary mutagens. Therefore, the present study proposed to create a new functional food product namely, cereal bar that had Khao-Mak made of pigmented rice as an ingredient.

Dried fermented black glutinous rice was chosen as an ingredient for developing a cereal bar. It was substitution for corn flakes and crispy rice of the control cereal bar (Table 4.1). Other ingredients were kept constant in order to focus mainly on the effect of dried fermented rice on the quality of cereal bar. The results supported that dried fermented black glutinous rice influenced on general appearance, color, overall acceptability, sweet, odor, and texture scores of the product. The panelists suggested that increasing the amount of added dried fermented black glutinous rice resulted in darker color and softer texture. In addition, the taste was sweeter as the level of dried fermented black glutinous rice increased. This is not surprised since the fungus in Look Pang digested starch molecules of rice to monosaccharides during fermentation process (Cheng *et al*., 2008).

The mean scores of color, sweetness and texture indicated that formula D were not significantly different from score of 3 and had the highest overall acceptability score. Therefore, formulation D, which contained 14.75% dried fermented black glutinous rice, was selected for in-house consumer test and storage evaluation; thus, it was called “new cereal bar”. The result obtained from an in-house consumer test indicated that the odor score of new cereal bar was lower than that of control one; it might be due to the odor of acid produced by yeast in Look Pang during fermenting monosaccharide to alcohols and organic acids (Lotong, 1992).

6.5 Characteristics of the Cereal Bar Containing Fermented Black Glutinous Rice

6.5.1 Physical Properties

The color values (L*, a* and b*) indicated that the higher the dried fermented black glutinous rice content the darker the color. This was similar to the result obtained from sensory test reported above that the higher the amount of added fermented black glutinous rice the lower the overall acceptability of the product; this was possibly due to the contributed by anthocyanins of black glutinous rice.

The water activity of the cereal bar containing dried fermented black glutinous rice was 0.51 (formula D), 0.54 (formula E), and 0.59 (formula F), respectively which was lower than the minimal level of water activity for microbial growth (0.91 for bacteria, 0.88 for yeast, 0.80 for mold, 0.75 for halophilic bacteria, 0.65 for xerophilic fungi and 0.60 for osmophilic yeast) (Beuchat, 1981). It was suggested that the cereal bar containing dried fermented black glutinous rice may not be required any preservative to extend shelf-life of the product.

The texture analysis indicated that the higher the amount of dried fermented black glutinous rice content the softer the texture of the cereal bar. This was similar to the result from the sensory test that the product contained higher amount of dried fermented black glutinous rice resulted in lower acceptability; the panelist evaluated that it was too soft. Dried fermented black glutinous rice should be an important factor that influenced the texture of the product. During fermentation, fungus of Look Pang such as *Aspergillus* sp. and *Rhizopus* sp. produced some enzymes which degraded polymeric components, namely protein, starch and lipid of the rice to be smaller molecules leading to softer texture of the fermented rice (Handoyo and Morita, 2006; Chen *et al*., 2010).

6.5.2 Total Phenolic Content, Anthocyanin Content, and Antioxidant Activities

The new cereal bar had higher total phenolic content, anthocyanin content and antioxidant activities than that of the control cereal bar. The results in this study were in accordance with those found by Sharma *et al*. (2012) who worked on cookies containing dried *Tinospora* leaf powder. They revealed that the percent radical (DPPH) scavenging activity of cookies supplemented with 2.5%, 5.0% and 7.5% dried *Tinospora* leaf increased from 1.21 of control cookie to 8.28, 9.17 and 11.16, respectively. The use of green banana flour, as a functional ingredient, in two types of snacks namely, fish cracker and cassava cracker by Wang *et al*. (2012) increased polyphenol content and antioxidant capacity to varying degrees depending on the substitution level. Moreover, the bakery products supplemented with mango dietary fiber had higher total polyphenol and antioxidant capacity than those of the control formula (Vergara-Valencia *et al*., 2007).

The antioxidant activities of both control cereal bar and new cereal bar might be due to Maillard reaction products occurred during baking. The Maillard reaction is a chemical reaction taking place during food processing and results in a wide variety of Maillard reaction products such as melanoidins which influenced on food quality attribute such as aroma, taste and color (Jaeger *et al*., 2010) as well as antioxidant potential through scavenging oxygen radicals or chelating metals (Rufián-Henares and Morales, 2007; Cheriot *et al*., 2009). Michalska *et al*. (2008) reported that furosine (early indicator of the Maillard reaction) was detected in all bread samples (whole, crumb and crust), showing the highest content in the crust. They also reported that the crust had the highest value of total phenolic compound and antioxidant capacity.

6.5.3 Antimutagenicity

The surviving adult flies brought up on medium containing different percentages of the control cereal bar or new cereal bar are higher than 50%. Each sample was not mutagenic since it did not induce the frequencies of mutant spots of the flies brought up on the experimental medium at any testing concentrations to be higher than that of the flies brought up on negative control medium.

The new cereal bar had greater antimutagenicity than that of the control cereal bar. It may partially be due to total phenolic content such as anthocyanin containing in black glutinous rice (as discussed in section 6.2). Moreover, Maillard reaction products produced during the baking of cereal bar might composed of some compounds posing antimutagenic activity such as malanoidins. Miwa *et al*. (2002) indicated that melanoidins could protect nitric oxide in inducing DNA damage of HL60 (human promyelocytic leukemic cell line).

6.5.4 Nutritive Values

The new cereal bar had 1.4 g total dietary fiber/serving (40 g). This made it is not claimed as a good source of dietary fiber on nutrition fact. According to the regulation number 182 (1998) of the Thai FDA (Ministry of Public Health), the product can be claimed as a good source of fiber if it contains dietary fiber at the amount of 10-19% of Thai RDI or 2.50 to 4.75 g dietary fiber/serving. Therefore, a

new cereal bar can be only the dietary supplement for antioxidants and antimutagenicity.

6.6 Storage Affected on Various Properties of the New Cereal Bar

The new cereal bars were kept in vacuum-sealed laminated aluminum foil bags at ambient temperature (approximately 28°C) for 90 days. The product was determined on the sensory acceptability, physical properties, antioxidant activities, total phenolic content, anthocyanin content, and antimutagenicity after they were stored for 0, 45 and 90 days.

6.6.1 Sensory Acceptability Test and Physical Properties

The scores of general appearance, color, overall acceptability, odor, taste and texture of the product during storage at 0, 45 and 90 days were slightly decreased without significant difference. The results were similar to those found by Singh *et al*. (2011) who reported that the score of sensory attributes of both of the aerobically and vacuum packaged chickens snacks did not decrease much during storage period of 30 days at 30±2°C. This suggested that keeping such product in vacuum-sealed laminated aluminum foil bags at room temperature (approximately 28°C) could maintain it to be accepted for at least 90 days by general consumers. In addition, the color values (L*, a* and b*) and water activity of the new cereal bar were not changed during storage for 90 days. The water activity of the product during storage (0.51-0.52) was still lower than the minimal level of water activity for microbial growth (Beachat, 1981). This might be due to the fact that a vacuum-sealed laminated aluminum foil bag could protect the permeability of the air and light.

It was noted that the hardness of the product increased as the period of storage was extended. This result was similar to that found in cereal bars (Castro, 2005; Lobato *et al*., 2011) and protein bars (Loveday *et al*., 2009). It was suggested that the degree of hardness of cereal bar during storage might be related to the change in structure of sugar in the cereal bar. Castro (2005) and Adams (2008) proposed that the phase transition of the sugars, through crystallization, might also be a cause of bar

hardening; this transition would cause the sugar to lose its ability to hold water. The water originally bound by the sugar then migrates into the protein portion of the bars causing over-hydration and possibly a conformation change in the protein. Moreover, Baier *et al*. (2007) reported that bar hardening during storage was favored by more ordered protein secondary structure and lower surface hydrophobicity of protein particles. Maillard reactions involving protein cross-linking might also be involved in the hardening of protein bars (Loveday et al. 2009).

6.6.2 Total Phenolic Content, Anthocyanin Content, and Antioxidant Activities

The total phenolic content, anthocyanin content, and antioxidant activities were slightly decreased with respect to the period of storage increased; this was similar to the phenomenon observed by Ścibisz and Mitek (2009) who reported that about 18, 68 and 34% of total phenolics, total anthocyanins, and antioxidant activities in low sugar blueberry jams kept in glass jar decreased during the 4-month storage at 22°C. Syamaladevi *et al*. (2012) also found that total anthocyanins, total phenolic compounds and total antioxidant activities decreased up to 86, 69 and 52%, respectively during the 13-month storage of canned blueberry solids. Anthocyanins are very unstable during storage; they degraded by various factors such as acidity, heat, light and oxygen (Mishra *et al*., 2008). Therefore, packaging may be involved in the prevention of degradation of anthocyanin during storage. For example, black soybean koji stored at 25°C with deoxidant and desiccant generally retained a relatively higher anthocyanin residual at the end of storage when compared with that stored under either deoxidant or desiccant alone (Wang *et al*., 2010).

6.6.3 Mutagenicity and Antimutagenicity of the Stored Sample

Storage in vacuum-sealed laminated aluminum foil bags at room temperature (28°C) for 90 days in this experiment did not show any toxic effect toward the testing organism. Such result was similar to that observed on black soybean koji after held at 4°C and 25°C with deoxidant and desiccant for a period of 120 days (Wang *et al*., 2010). In their experiment, the reduction (about 10-20%) of antimutagenicity against 4-NQO on *S. typhimurium* TA 98 in the Ames test of their

product during storage (120 days) was associated with a decrease in polyphenol content namely, anthocyanins. Additionally, it was also noted that the extent of reduction in the antimutagenicity of the koji extract varied with the packaging conditions. Among the various packaging conditions examined, storage with both deoxidant and desiccant generally enabled the koji extract to show the highest residual of antimutagenicity. This idea should be applied to the storage of the new cereal bar since it might keep the antimutagenicity as well as other beneficial factors of the bar constant through out the shelf life of the bar.