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Full Length Research Paper

The protective effect of Thai fermented pigmented rice on urethane induced somatic mutation and recombination in *Drosophila melanogaster*

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This study was aimed to proof that fermentation could increase phenolic and anthocyanin contents, antioxidant activities and antimutagenicity of Thai pigmented rice. Six cultivars of rice (Sung Yod, Mon Poo, Hom Mali Daeng, Hom Nil, Riceberry, and black glutinous rice) were fermented with a mixed culture of yeasts and molds (Look-Pang) at 30°C for 3 days to produce six types of fermented rice (Khao-Mak). Each sample exhibited significantly higher total phenolic and anthocyanin contents as well as antioxidant activities, including 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP), than that of the unfermented one of the same variety. Neither fermented nor unfermented pigmented rice substituted for corn flour in the fly medium was mutagenic in the somatic mutation and recombination test using *Drosophila melanogaster*. Interestingly, the antimutagenicity of fermented rice against urethane in the same testing was proven to be greater than that of its corresponding unfermented rice. This might be due to the higher content of total phenolic compounds, anthocyanins and antioxidant activities in fermented rice probably because of the catalytic action of enzymes produced by the starter organisms during fermentation which are capable of hydrolyzing glucosides of the inactive components to the active aglycones.

Key words: Fermented rice, total phenolic, anthocyanin, antioxidant activities, antimutagenicity.

INTRODUCTION

Rice is a staple food and an economic crop of Thailand. It is not only white rice but also many special rice cultivars that contain color pigments, such as black glutinous rice (black rice), Hom Nil rice (purple rice) and Sung Yod rice (red color). Vichapong et al. (2010) revealed that varieties of Thai pigmented rice such as black glutinous rice and Hom Mali Daeng had higher phenolic compounds, total flavonoid and antioxidant activity than normal white staple rice. Anthocyanins are commonly a group of pigments found in pigmented rice such as purple, black and red rices. These compounds have many biological properties such as scavenging free radicals (Wang and Jiao, 2000), prevention of DNA damage in cancer cells (Hou, 2003), activating glutathione-related enzymes and NAD(P)H:quinone reductase (Shih et al., 2007; Singletary et al., 2007).

Khao-Mak is a traditional fermented rice in Thailand that is made of white glutinous rice fermented with Look-Pang at room temperature for 3 days (Lotong, 1992). Look-Pang is a microbial starter containing a mixed culture of *Aspergillus* species, *Rhizopus* species and *Mucor* species, together with *Saccharomyces cerevisiae* and *Candida* species inoculums in rice flour mixed with herbs such as pepper, garlic and galangal as antibacterial agents (Manosroi et al., 2011). Enzymes from the molds hydrolyze starch in the rice to sugars, which are partially fermented to alcohol by the yeast. Organic acids (e.g. lactic acid) are also produced (Lotong, 1992). The product gives sweet taste, a little alcohol and lactic acid flavor, soft texture, with lumps of cooked glutinous rice and succulent grain. 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 (Sompong et al., 2011). Interestingly, fermented black glutinous rice increased its antimutagenicity against *in vivo* formed nitrosomethylurea in somatic mutation and recombination

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test (SMART) (Vipassanatham et al., 2012) and nitrite treated 1-aminopyrene on *Salmonella typhimurium* TA98 (Sadabpod et al., 2010) compared with that of its corresponding raw and cooked rice.

In this investigation urethane or ethyl carbamate was used as a positive mutagen in the somatic mutation and recombination test as suggested by Abraham and Graf (1996). It is a natural constituent of tobacco leaves and tobacco smoke (Schmeltz et al., 1978) and is also present in fermented food products such as bread, yogurt and cheese (Ough, 1976; Canas et al., 1989; Miller and Miller, 1983). In rodents, urethane was found to produce lymphomas, lung tumors, hepatomas and melanomas (Mori et al., 2000; Mirvish, 1968; International Agency for Research on Cancer (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). IARC classified urethane as possibly carcinogenic to humans (group 2B) (IARC, 1974). Therefore, the objective of this study was to determine the total phenolic content, anthocyanin content and antioxidant activities of fermented pigmented rice as well as the possible antimutagenicity of different types of fermented pigmented rice in the somatic mutation and recombination test.

MATERIALS AND METHODS

Chemicals

Urethane, 2, 4, 6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate and ferrous sulfate heptahydrate were purchased from Sigma Chemical (St. Louis, MO, USA). Diethylether and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Propionic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and Folin-Ciocalteu reagent were supplied from Fluka Chemika (Buchs, Switzerland). Trolox was bought from Aldrich Chemical (Milwaukee, WI, USA). All other chemicals and reagents were of analytical grade.

Preparation of fermented pigmented rice

Six cultivars of pigmented unpolished rice, namely, Sung Yod (red rice), Mon Poo (red rice), Hom Mali Daeng (red rice), Hom Nil (purple rice), Riceberry (purple rice) and black glutinous rice (black rice) were obtained from local markets in Bangkok. A commercial starter called Look-Pang was bought from '100 Year Samchuck Market' in Suphanburi province. Rice was fermented in Thai traditional style as suggested by Lotong (1992) with slight modification as the following. It was mixed with distilled water (1:2 w/v) and cooked in an electric cooker. Cooked rice was cooled at room temperature and fermented with Look-Pang (0.2 g/100 g of raw rice) at 30°C for 3 days in a glass container. The fermented rice was dried at 60°C in a hot air oven for 24 h and was stored under vacuum in a polyethylene bag at 4°C before use.

Antioxidant activities, total phenolic and anthocyanin contents of fermented pigmented rice

Ground sample (1 g) was extracted (both hydrophilic and lipophilic compounds) with dimethylsulphoxide (DMSO) (10 ml) for 30 min in a shaking water bath (30°C) according to the method of Omata et al. (2009). Then, the mixture was centrifuged at 2500 ×g for 10 min and the supernatant was collected. The residue was re-extracted under the same condition. The combined supernatant was determined for its antioxidant activities using the DPPH assay as described by Fukumoto and Mazza (2000), and the ferric reducing antioxidant power (FRAP) assay according to the procedure described by Griffin and Bhagooli (2004). The total phenolic content of the extract was determined according to the method described by Amarowicz et al. (2004). The anthocyanin content of each sample extracted with acidified ethanol was determined according to the method described by Abdel-Aal and Hucl (1999).

SMART

Two strains of *Drosophila melanogaster* were used. Males of *mwh/mwh* and females of *ORR;flr*^{3}*/In(3LR) TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S}*, Ser* were obtained from the Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich. The test was performed as described by Graf et al. (1984). One hundred 3 days old trans-heterozygous larvae (*mwh flr*$^{+}$*/mwh TM3*) were transferred to the *Drosophila* medium (Roberts, 1986) in which each sample was substituted for corn flour (used for the mutagenicity evaluation) or the *Drosophila* medium in which each sample was substituted for corn flour and 20 mM urethane was substituted for water (used for antimutagenicity evaluation). The standard medium was used as the negative control while the medium containing 20 mM urethane was used as the positive control. Each experiment was done twice.

The antimutagenicity of each sample was determined from the percentage of inhibition calculated as follows: percentage of inhibition = ((a-b)/a)x100, where "a" is the frequency of spots induced by urethane alone and b is the frequency of spots induced by urethane in the presence of the sample. It is proposed that percentages of inhibitions less than 20, 20-40, 40-60 and more than 60 are the evidences of negligible, weak, moderate and strong antimutagenicity, respectively as suggested by Kruawan et al. (2012).

Statistical analysis

Total phenolics, anthocyanins and antioxidant activities data were reported as means $\pm$ standard deviations for triplicate analyses of the same sample. One-way analysis of variance (ANOVA) and Schefee's multiple comparison tests were carried out to test for differences among the means of fermented rice using Statistical Package for Social Sciences (SPSS, version 13). The mean values of unfermented rice and those of fermented rice were analyzed by paired-samples t-test. Statistical significance was considered for p less than 0.05. For SMART, the wings of the surviving flies were analyzed for the occurrence of mutant spots and evaluated as described by Frei and Würgler (1988). The estimation of spot frequencies and confidence limits of the estimated mutation frequency were performed with significance level of $\alpha = \beta = 0.05$.

RESULTS AND DISCUSSION

Total phenolic and anthocyanin contents

The total phenolic content of fermented rice was higher than that of its corresponding unfermented one (Figure 1A). Unfermented rice had an average of 431.6 to 1575.6 mg gallic acid equivalent/100 g dry weight, whereas

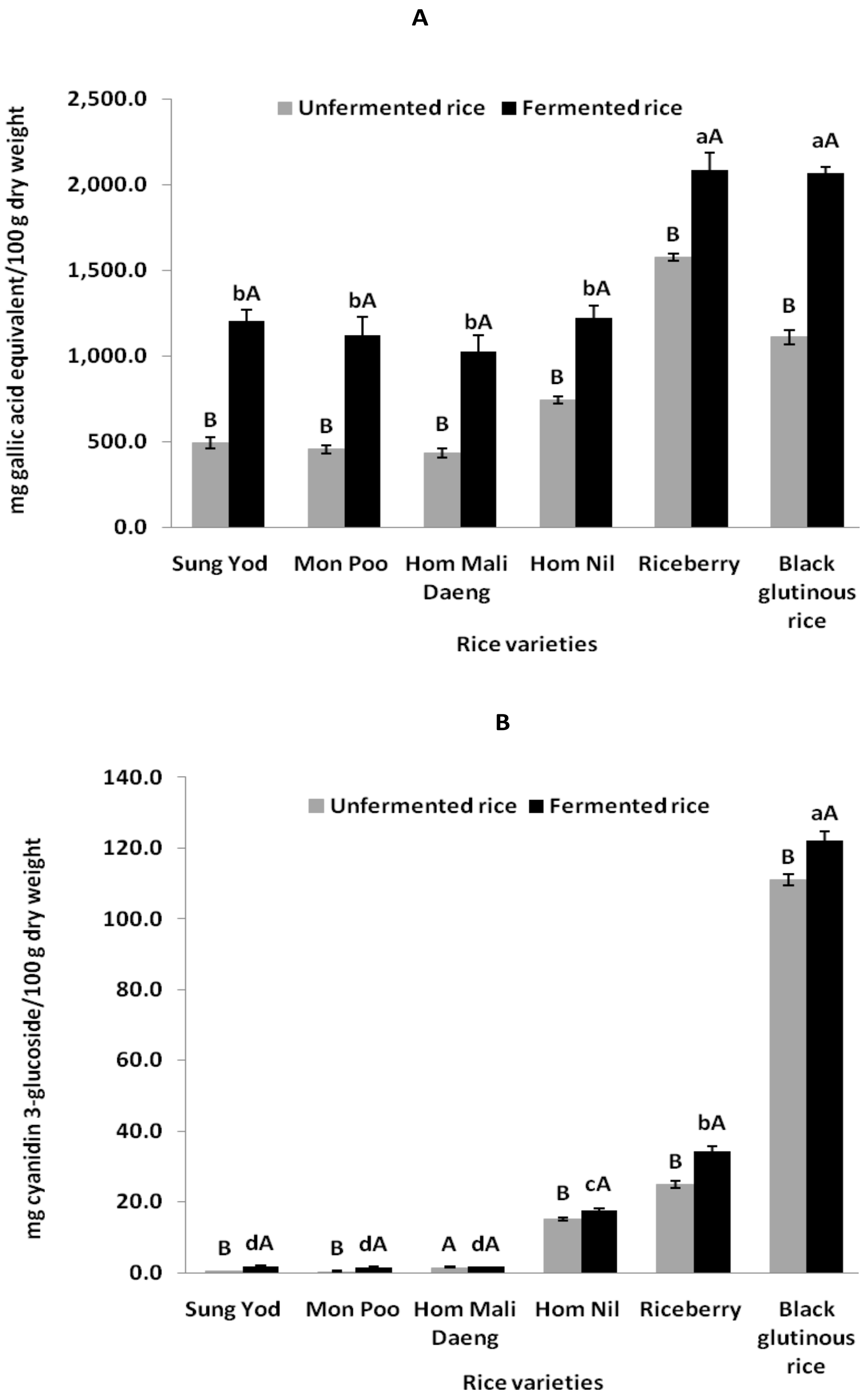

Figure 1. Total phenolic content (A) and anthocyanin content (B) of unfermented and fermented pigmented rice. Values are presented as means ± SD (n = 3). Data with different upper case letters indicate significant differences ($p < 0.05$) between unfermented and fermented rice of the same varieties, while those with different lower case letters indicate significant differences ($p < 0.05$) between fermented rice varieties.

fermented rice had an average of 1026.4 to 2086.9 mg gallic acid equivalent/100 g dry weight. The highest total phenolic content (2086.9 mg gallic acid equivalent/100 g dry weight) belonged to fermented Riceberry. The anthocyanin content of unfermented rice samples of this study ranged from 0.4 to 111.1 mg cyanidin3-glucoside/100 g dry weight, whereas those of the fermented rice samples ranged from 1.5 to 122.2 mg cyanidin 3-glucoside/100 g dry weight (Figure 1B). Fermented black glutinous rice exhibited the highest anthocyanin content with an approximately 122.2 mg cyanidin 3-glucoside /100 g dry weight in this study.

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 fermented soybean paste in Korea, had significantly higher total polyphenol content than that of raw soy bean. Lee et al. (2007, 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 contents 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 *S. cerevisiae*, *Aspergillus* spp. and *Rhizopus* spp. 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 *S. 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 beta-glucoside isoflavone (daidzin, glycitin, and genistin), compared with the unfermented steamed black bean. The increase of aglycone content and the increase of beta-glucosidase activity during the fermentation of this koji showed a similar trend. Therefore, the action of enzyme such as beta-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.

Antioxidant activities of unfermented and fermented rice

The DPPH antioxidant activity expressed as mmol Trolox equivalent/100 g dry weight of each sample as shown in Figure 2A. Unfermented rice had an average of 2.3 to 4.3 mmol Trolox equivalent/100 g dry weight while fermented rice had an average of 3.4 to 5.3 mmol Trolox equivalent/100 g dry weight. The highest DPPH antioxidant activity (5.3 mmol Trolox equivalent/100 g dry weight) was found in fermented black glutinous rice. The FRAP values of unfermented rice ranged from 0.9 to 1.9 mmol Fe(II)/100 g dry weight, whereas those of fermented rice ranged from 1.3 to 2.9 mmol Fe(II)/100 g dry weight (Figure 2B). It was noted that fermented rice had significantly higher reducing abilities than that of unfermented rice of the same variety. The fermented black glutinous rice had the highest FRAP values (2.9 mmol Fe(II)/100 g dry weight).

Our 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* spp. exhibited higher levels of DPPH scavenging activity and Fe^{3+} reducing power than 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. *S. 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).

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 earlier). 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 activity of the anthocyanidins (aglycons) was 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; Sompong et al., 2011) and tocopherols may exhibit antioxidant activity (Aguilar-Garcia et al., 2007; Hyogo et al., 2010; Vichapong et al., 2010; Sompong et al., 2011).

Mutagenicity and antimutagenicity of fermented rice

Substituting each sample for corn flour (50, 75 and 100%) in the *Drosophila* medium for rearing the trans-heterozygous larvae gave adult flies with normal wing hairs indicating that none of the samples contained mutagenic compound (data not shown). Interestingly, the counteracting effect against urethane mutagenicity of unfermented rice and fermented rice on *D. melanogaster*

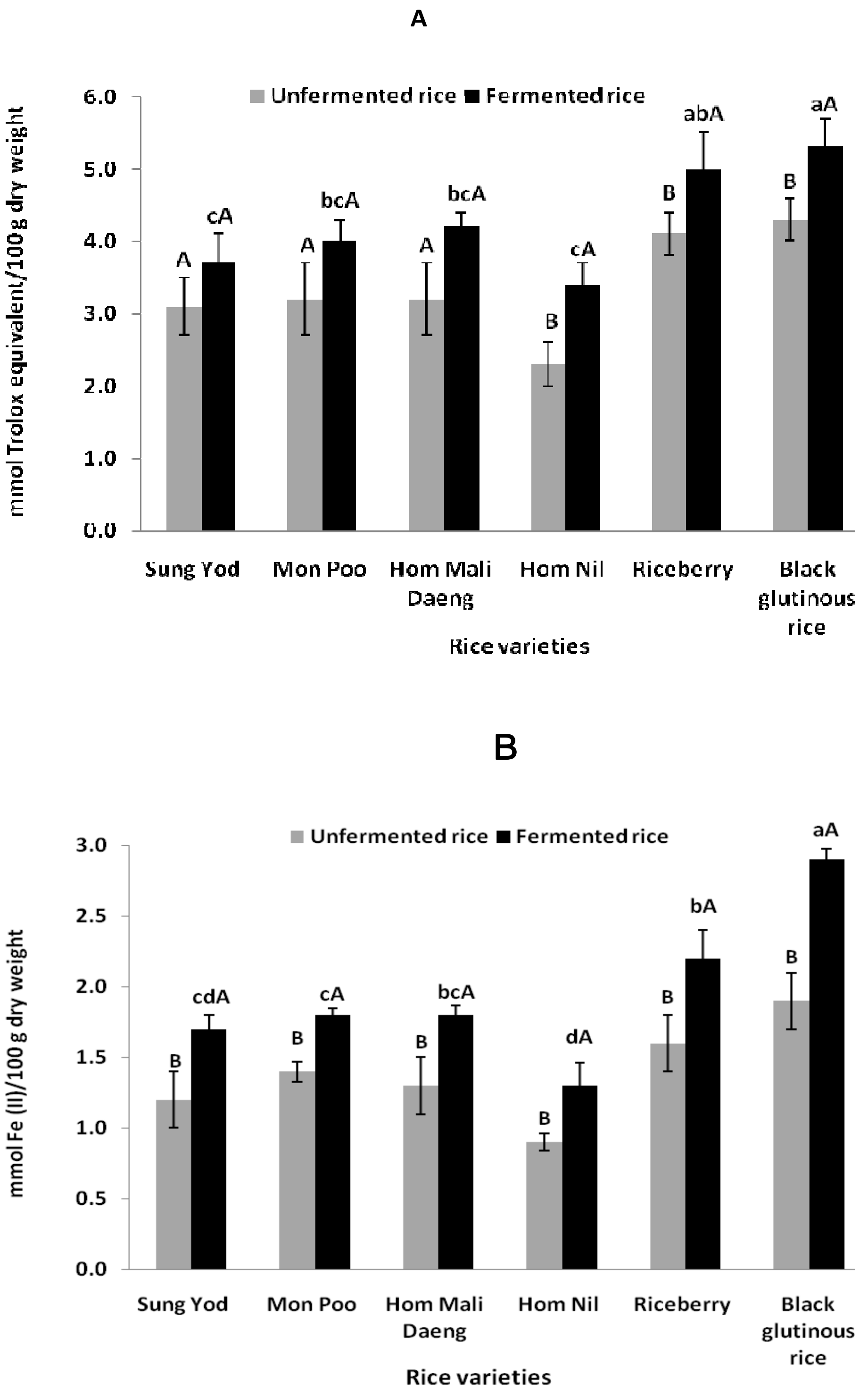

Figure 2. DPPH antioxidant activity (A) and ferric reducing antioxidant power (B) of the unfermented and fermented pigmented rice. Values are presented as means ± SD (n = 3). Data with different upper case letters indicate significant differences ($p < 0.05$) between unfermented and fermented rice of the same varieties, while those with different lower case letters indicate significant differences ($p < 0.05$) between fermented rice varieties.

was obtained (Figure 3). It has shown that fermented rice gave greater inhibitory effect than that of unfermented rice of the same variety. Sung Yod, Mon Poo, Hom Mali Daeng and Hom Nil varieties exhibited negligible

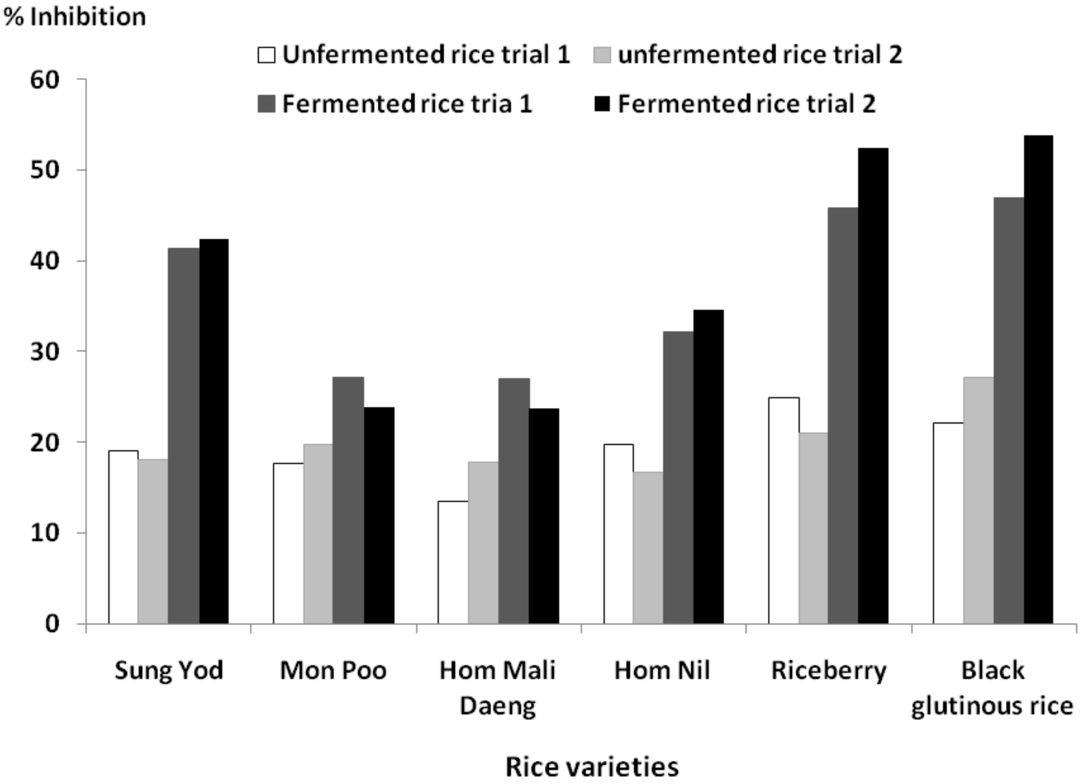

Figure 3. Antimutagenicity of unfermented and fermented pigmented rice against urethane in the somatic mutation and recombination test. It is proposed that percentage of inhibition less than 20, 20–40, 40–60 and higher than 60 indicates negligible, weak, moderate and strong antimutagenicity, respectively (Kruawan *et al,* 2012).

antimutagenicity (< 20% inhibition), while Riceberry and black glutinous rice had weak antimutagenicity (20 to 40% inhibition) in both trials 1 and 2. The antimutagenicity effect of fermented rice increased as Mon Poo, Hom Nil and Hom Mali Daeng turned to be weakly antimutagenic, while Sung Yod, Riceberry and black glutinous rice turned to be moderately antimutagenic (40 to 60% inhibition).

Increasing antimutagenicity of pigmented rice via fermentation, as observed in this study, is compatible with other studies. For example, Sadabpod et al. (2010) revealed that fermented black glutinous rice had higher antimutagenicity against nitrite treated 1-aminopyrene on *S. typhimurium* TA98 than that of its corresponding raw and cooked rice. Vipassanatham et al. (2012) also indicated that black glutinous rice, after being fermented, had higher antimutagenicity against *in vivo* formed nitrosomethylurea in *D. melanogaster* as 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.

Our investigation also suggested that a remarkable increase in phenolic contents such as anthocyanins during fermentation might contribute to the increase of antimutagenicity of fermented rice. Anthocyanins were found to protect N-nitrosomethylbenzylamine (Reen et al., 2006), tert-butyl hydroperoxide (t-BHP) (Hwang et al., 2011a) and dimethylnitrosamine (Hwang et al., 2011b) in inducing hepatic damage in rats. In addition, Choi et al. (2009) showed that anthocyanin obtained from purple-fleshed sweet potato protected 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 the 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. However, other compounds 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) might also contribute to the enhancing antimutagenicity.

This study revealed that fermentation influenced the observed antimutagenicity against urethane and the levels of antimutagenicity depended on rice variety. The high bioactivation (HB) cross of *D. melanogaster* used in this study is highly sensitive to the genotoxic effects of urethane, because of its high constitutive level of cytochrome P-450 activity (Frolich and Würgler, 1990; Graf and van Schaik, 1992); hence, it represents an extreme state of genetic susceptibility to urethane. The pathway of activation of urethane is thought to involve two steps, both catalyzed by the cytochrome P-450 CYP 2E1; these steps are desaturation of urethane to vinyl carbamate, followed by oxidation to vinyl carbamate epoxide (Guengerich et al., 1991) that is supposed to form an adduct to DNA.

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.

Conclusion

The results of this investigation indicated that most types of fermented pigmented rice were safe and might have a health benefit to consumers, because they could counteract the mutagenicity of urethane. However, antimutagenic components of pigmented rice samples need to be characterized for some other possible compounds, besides anthocyanins, that provoke antimutagenic response. Currently, fermented black glutinous rice is being developed as a healthy cereal bar to protect the consumer from some mutagens that have similar bio-characteristics to urethane.

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