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Antioxidant Activity of Rice Bran Protein Extract, its Enzymatic Hydrolysates and its Combination with Commercial Antioxidants

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Abstract: Rice bran protein extracted from defatted Khao Dawk Mali (KDML 105) rice bran using alkali extraction and isoelectric precipitation (RBPE) was prepared. RBPE was hydrolyzed with Alcalase 2.4 L and antioxidant activity of the hydrolysates were determined by 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and Ferric Reducing Ability Power (FRAP) assay at hydrolysis time of 0, 30, 60, 90 and 120 min. The DPPH free radicals scavenging activity and FRAP value of the alcalase RBPE hydrolysates were 32.1-35.5% and 951-1,018 μmol $FeSO_4$/mL of hydrolysate, respectively and were not significantly different ($p>0.05$) at different hydrolysis times. RBPE was freeze-dried (FD-RBPE) and it was found that freeze-drying did not affect antioxidant activity of RBPE. DPPH radical scavenging activity and FRAP of the FD-RBPE was 41.9% and 92.6 μmol $FeSO_4$/g protein, respectively. FD-RBPE was combined with butyrate hydroxyanisole (BHA) and their antioxidant activities were evaluated. It showed that FD-RBPE did not affect antioxidant activity of BHA. Based on DPPH radical-scavenging activity, 106 μg protein of FD-RBPE can replace 1 μg of BHA. It suggested that FD-RBPE can replace BHA or be combined with BHA as antioxidant in food products.

Key words: Antioxidant activity, rice bran protein extract, combination effect

INTRODUCTION

Lipid oxidation plays an important role in the food industry since it causes undesirable off-flavor development and potentially toxic reaction products. It results in quality deterioration and shortens the shelf-life of food products and may cause diseases after consumption of those toxic products (Kim *et al*., 2007). In addition, oxidation can generate free radicals and these free radicals are known as major factors causing various diseases, such as cancer, multiple sclerosis, inflammation, coronary heart, cardiovascular disorders and atherosclerosis (Willcox *et al*., 2004). Therefore, it is important to retard lipid oxidation and formation of free radicals occurring in food products to prevent foods from deterioration.

Antioxidants have been defined as any substances which significantly delay or inhibit oxidative damage to a target molecule. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) may be added to food products to retard lipid oxidation. However, their use is restricted due to their potential health risks (Ito *et al*., 1985). In recent years, there is a great interest to identify antioxidants from many natural sources to replace the use of synthetic antioxidants. There are numerous proteins and peptides derived from hydrolyzed food proteins that have been found to possess significant antioxidant activity against lipid peroxidation, such as fish (Bougatef *et al*., 2009), milk casein (Blanca *et al*., 2007), soybean (Li *et al*., 2006), rice (Adebiyi *et al*., 2009) and wheat (Wang *et al*., 2007). Rice (*Oryza sativa* L.) is the most staple food produced in Asian countries.

Rice bran is one of the most abundant byproducts in the rice milling industry. Rice bran protein is of high nutritional value and has nutraceutical properties. Kokkeaw and Thawornchinsombut (2007) prepared rice bran protein hydrolysates using the commercial proteolytic enzyme, Protex 6L. It's maximal radical scavenging activity was only 27.08%. Chanput *et al*. (2009) reported high antioxidant activity from KDML 105 rice bran proteins fractioned through digestion of pepsin followed by trypsin. There is less information on preparation of antioxidants from Thai rice bran protein and its hydrolysates. The antioxidant properties of the hydrolysates largely depend on protease specificity and the nature of released peptides, have been attributed to cooperative or combined effects of a number of properties, including their ability to scavenge free radicals or act as hydrogen donor (Tang *et al*., 2009). In the present study, we investigated antioxidant activity of RBPE, its hydrolysates that hydrolyzed with Alcalase 2.4 L and the effect of RBPE combined with commercial antioxidants on antioxidant activity Antioxidant activity is evaluated by the scavenging effect on DPPH radicals and FRAP.

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MATERIALS AND METHODS

Materials: Khao Dawk Mali (KDML) 105 rice bran was obtained from Patum Rice Mill and Granary Company, Ltd. (Bangkok, Thailand). The samples were packed in aluminum foil bags and kept at -18°C until use. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) and butylated hydroxyanisol (BHA), were purchased from Sigma Co. (St. Louis, MO, USA). Tripyridyltriazine (TPTZ) was purchased from Fluka BioChemika (Buchs, Switzerland). Alcalase 2.4 L FG was acquired from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). All other chemicals used in the experiments were of analytical grade.

Preparation of rice bran protein extracted (RBPE): Full-fat rice bran (KDML 105) was defatted by extracting twice with three volumes of hexane. The defatted rice bran was air-dry over night under fume hood, then ground and sieved through 0.5 mm screen.

RBP was prepared using alkali extraction method followed by isoelectric point precipitation (Adebiyi *et al*., 2008; Gnanasambandam and Hettiarachchy, 1995) with some modification. Briefly, the defatted rice bran (200 g) was stirred in water (rice bran:water 1:4) and control pH at 9.5 with 1 N NaOH for 45 min. The slurry was centrifuged at 10,000 x g, 25°C for 30 min. The sediment was discarded and the supernatant was collected. The pH of the supernatant was adjusted to 4.5 and left to stand for 1 h at 4°C. The precipitate was washed twice with distilled water by centrifuging at 10,000 x g, 25°C for 30 min. The sediment was rice bran protein extract (RBPE). The RBPE was dispersed in distilled water, adjusted pH to 7.0 and lyophilized. The freeze-dried RBPE rice bran protein (FD-RBPE) was stored at -5°C until use. Crude protein and total nitrogen in RBPE and FD-RBPE were determined by the Kjeldahl method (AOAC, 1990).

Preparation of rice bran protein hydrolysates: RBPE was suspended in 50 mM Tris-HCl buffer, pH 8.0 (1 g rice bran protein/100 mL) and hydrolyzed with Alcalase 2.4L (48 mAU/g RBP) at 50°C in a water bath with constant agitation. The degree of hydrolysis (DH) was determined at hydrolysis times of 30, 60, 90 and 120 min. The enzymatic hydrolysis was terminated by heating for 10 min in a boiling water bath. The hydrolysates were centrifuged at 9,800 x g, 15°C for 20 min. The supernatants, as RBP hydrolysates (RBPHs), were stored at -20°C until use.

Degree of hydrolysis: The degree of hydrolysis (DH) was determined by the ratio of the percentage of 10% trichloroacetic acid (TCA)-soluble nitrogen to total nitrogen in the sample (Qi *et al*., 1997). An aliquot of hydrolyzed CRBP was mixed with 20% TCA to create 10% TCA and then centrifuged at 9,800 x g, 25°C for 15 min. The soluble nitrogen in the supernatant was determined by the Kjeldahl method (AOAC, 1990). The percent DH was calculated as follows:

$$DH\ (\%) = \frac{\text{Soluble nitrogen in 10\% TCA solution (mg)}}{\text{Total nitrogen in RBPE (mg)}} \times 100$$

Determination of antioxidant activities

2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity: RBPE and FD-RBPE were dispersed in distilled water at concentration of 2% protein (w/v). DPPH radical-scavenging activity was determined by the method of Bougatef *et al*. (2009) with a some modification. A 0.1 mM DPPH in ethanol (1.50 mL) was mixed with 0.10 mL sample, vortexed thoroughly and left to stand in dark at room temperature for 60 min. Samples were diluted to 50-80% DPPH radical scavenging activity range. The absorbance of the resultant solution was read at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The blank was prepared in the same manner, except that distilled water was used instead of a sample. BHA ranging from 5 to 15 µg was used for the calibration curve. DPPH radical-scavenging activity was expressed as BHA equivalents using the standard curve. The scavenging effect was calculated as follows:

$$\text{Radical-scavenging activity (\%)} = \frac{(B-A) \times 100}{B}$$

where, A and B are an absorbance at 517 nm of sample and blank, respectively.

Ferric reducing ability power (FRAP): The reducing ability of the samples was measured using the ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996). In the assay, 1.80 mL of freshly prepared FRAP reagent (mixture of 2.5 mL of 10 mM tripyridyltriazine (TPTZ), 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 300 mM acetate, pH 3.6) was warmed to 37°C and 50 µL of sample, along with 150 µL of H_2O, was then added. Absorbance at 593 nm of samples as well as blank solutions was taken at 6 min. The absorbance at 6 min was compared with standard aqueous Fe^{2+} and expresses the amount of Fe^{2+} which come from the reduction of Fe^{3+}.

Effect of using FD-RBPE combined with commercial antioxidant: The commercial antioxidant, namely BHA, was combined with FD-RBPE and its antioxidant activity were determined. DPPH radical scavenging activity of FD-RBPE, BHA and the mixture of FD-RBPE and BHA were determined according to the methods described above by preparing an amount of protein for each sample in the range of 20-60% DPPH radical scavenging activity. Those samples were also used to determine antioxidant activity by FRAP.

Statistical analysis: Three sample replications (three replicate preparations of each sample) were performed. Data were analyzed by ANOVA and least significantly different procedures to separate means and differences were reported as significant at $p<0.05$, using standard statistical software package.

RESULTS AND DISCUSSION

Preparation of rice bran protein: Protein content in defatted rice bran KDML 105 was 14.66% (w/w). The value is similar to the range of 13.1-15.4% found in U.S. rice bran (Gnanasambandam and Hettiarachchy, 1995). Total protein was 3.63 g when it was extracted from defatted rice bran with alkali solution and precipitated at pH 4.5. The yield of RBP was 23.21 g/100 g of total protein. The protein might be glutelin because alkali-soluble protein in rice bran consists mainly of glutelin (Hamada, 1997; Adebiyi *et al*., 2007).

Enzymatic hydrolysis of rice bran protein: The hydrolysis of rice bran protein with Alcalase 2.4 L proceeded at a rapid rate during the initial 30 min and then slowed down afterward (Fig. 1). DH values varied from 27.8 to 35.9% during 30-120 min of incubation. This was typical for hydrolysis curves reported by Zheng *et al*. (2006) who evaluated the hydrolysis of corn gluten with Alcalase 2.4 L where the DH value was 24.0-31.2% during the same incubation time. Adebiyi *et al*. (2009) also reported that rice bran protein hydrolyzed with protease showed a high rate of hydrolysis for the first 1 h and DH only slightly increased thereafter.

The DPPH radical scavenging activity of RBPHs are shown in Fig. 2 (a). The DPPH radical scavenging activity of RBPHs was 32.03±4.75-35.49±1.83% and appeared to be constant during hydrolysis times of 0-120 min. FRAP of RBPHs showed a similar trend as DPPH radical scavenging activity over the same hydrolysis times. FRAP of RBPHs were 951-1018 µmol of Fe^{+2}/mL of hydrolysate; they are not significantly different from RBPH at hydrolysis times of 0-120 min. This indicates that antioxidant activity of RBPE is a similar constant to its hydrolysates. Therefore, RBPE was freeze-dried and antioxidant activity was compared to BHA.

Chemical and physical properties of FD-RBPE: When RBPE was freeze-dried (FD-RBPE), the product showed as in Fig. 3. FD-RBPE moisture content was 4.09%, protein was 53.65% and phenolic compounds was 18.78 µg Gallic acid equivalent/g. The colour of FD-RBPE was brown. The brown colour may have resulted from phenolic compounds in the sample. In addition, the formation of brown pigment might result from carbonyl products of carbohydrate reaction with free amino groups in proteins via the Maillard reaction.

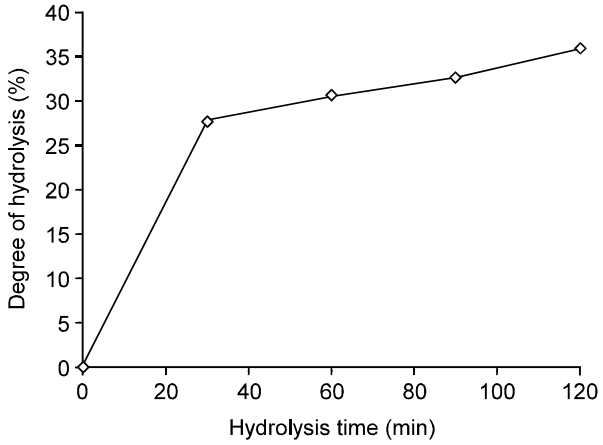

Fig. 1: Degree of hydrolysis of RBPE hydrolyzed with Alcalase 2.4L

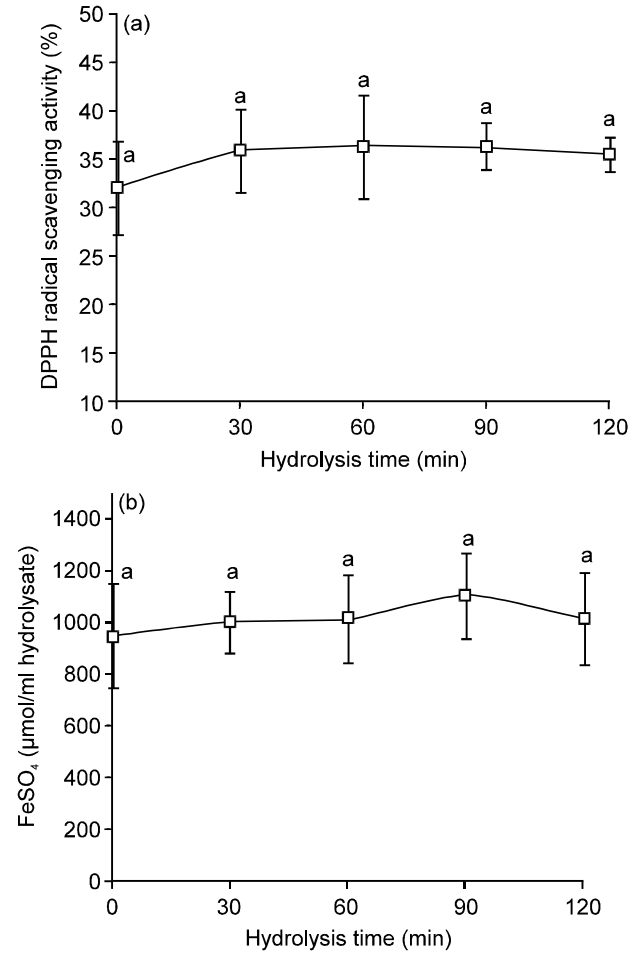

Fig. 2: Antioxidant activity on DPPH radical scavenging activity (a) and FRAP (b) of RBPHs at different hydrolysis time. Means±SD (n = 3) with different letters on the curve are significantly different ($p<0.05$)

Fig. 3: FD-RBPE powder

Antioxidant activities of RBPE and FD-RBPE: DPPH scavenging activity and FRAP of FD-RBPE is shown in Table 2. DPPH scavenging activity was 39.10±4.97 and 41.91±3.44% for RBPE and FD-RBPE, respectively. A reducing power assay is often used to evaluate the ability of natural antioxidants to donate electron. A number of researches have revealed that there was a direct correlation between antioxidant activity and reducing power. FRAP of RBPE and FD-RBPE were 94.8 and 92.6 µmol $FeSO_4$ g/protein, respectively and also not significantly different. This result indicates that the freeze drying process has no effect on antioxidant activity of rice bran protein. We next tested FD-RBPE combined with commercial antioxidant BHA.

Table 1: Comparison of antioxidant activities of RBPE and FD-RBPE

Sample	DPPH scavenging activity (%)	FRAP (µmol $FeSO_4$ g/protein)
RBPE	39.10±4.97[a]	94.8±15.6[a]
FD-RBPE	41.91±3.44[a]	92.6±6.7[a]

Means±SD (n = 3) with different letters in the same column are significantly different ($p<0.05$)

Effect of using FD-RBPE combine with commercial antioxidants: DPPH scavenging activity and FRAP of FD-RBPE and BHA of different amounts are shown in Fig. 4 and 5, respectively. The relationship of DPPH radical scavenging activity and BHA content are linear at 0-15 µg while the relation of DPPH radical scavenging activity and protein content of FD-AE-RBP is linear to 1500 µg (Fig. 4).

The relationship of FRAP value and BHA content are linear at 0-4 µg while the relationship of FRAP value and protein content of FD-AE-RBP is linear at 0-636 µg (Fig. 5).

FRAP of FD-RBPE increased rapidly as the amount of protein increased from 0 to 636 µg. When protein content was higher than 636 µg, FRAP only slightly increased and its relation was no longer linear. The results indicate that protein content should be in the range of 0-636 µg for the FRAP assay. FRAP of BHA increased linearly from 0-4 µg. The results indicate that the amount of FD-RBPE was limited on FRAP and different from BHA. Therefore, the amount of protein must be in the range of 0-636 µg when the effect of RBPE combined with BHA is studied.

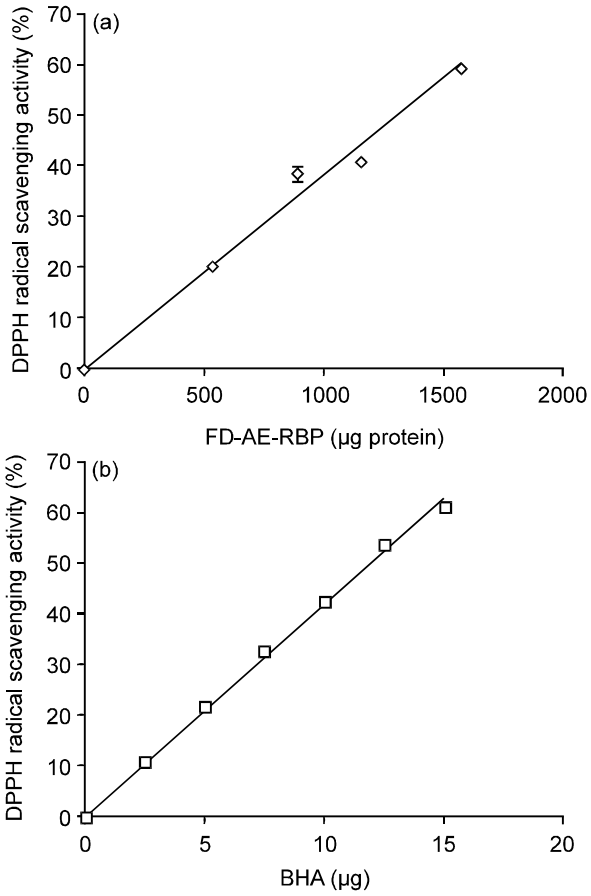

Fig. 4: Effect of the amount of FD-AE-RBP (a) and BHA (b) on DPPH radical scavenging activity. Data represents the average ±SD of two independent experiments

DPPH scavenging activity of FD-RBPE, BHA and mixture of FD-RBPE and BHA are shown in Table 2. DPPH scavenging activity of 530 µg protein of FD-RBPE was not significantly different compared to 5 µg of BHA ($p<0.05$). The antioxidant activity of 530 µg protein of FD-RBPE combined with 5 µg of BHA was close to the sum of FD-RBPE and BHA, but less than FD-RBPE. The results suggest that FD-RBPE can replaced BHA by using of 106 µg protein to replace BHA of 1 µg. Alternatively, FD-RBPE can be combined with BHA as antioxidants in food products.

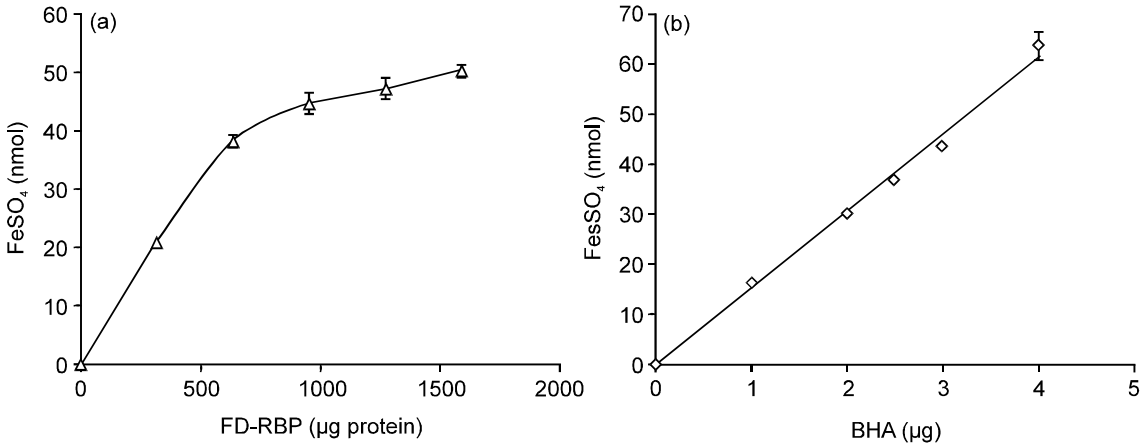

Fig. 5: Effect of the amount of FD-RBPE(a) and BHA (b) on FRAP. Data represents the average ±SD of two independent experiments

Table 2: DPPH scavenging activity of individual FD-RBPE, BHA and its combination

	DPPH scavenging activity (%)
FD-RBPE (530 µg protein)	20.12±0.28[a]
BHA (5 µg)	22.16±0.61[a]
FD-RBPE (530 µg proein)+BHA (5 µg)	41.79±1.46[b]

Means±SD (n = 3) with different letters in the same column are significantly different (p<0.05)

Table 3: FRAP of FD-RBPE, BHA and its combination

Sample	FRAP (nmol $FeSO_4$)
FD-RBPE (640 µg proein)	38.30±0.98[a]
BHA (2 µg)	30.01±0.61[b]
FD-RBPE (640 µg proein)+BHA (2 µg)	62.07±1.46[c]

Mean±SD (n = 3) in raw with same letters are not significantly different (p<0.05)

FRAP of FD-RBPE, BHA and mixture of FD-RBPE and BHA are shown in Table 3. The concentration of FD-RBPE, BHA and combination of them were different for each sample assay from our experiments (Fig. 4). When the concentration of them is increased to the specific level, FRAP was not directly correlated with antioxidant activity and concentration. FRAP of FD-RBPE (640 µg protein) combined with BHA (2 µg) was lower than the sum of FD-AE-RBP and BHA (Table 4). This revealed that FD-RBPE had inhibited antioxidant effects on BHA. These results suggested that FD-AE-RBP should not be used in combination with BHA, but it should be considered to replace BHA in food products. The amount of FD-RBPE used as BHA equivalent will depend on kind of antioxidant activity assay.

Conclusion: RBPE was extracted from defatted rice bran with alkali solution and precipitated at pH 4.5. RBPE was hydrolyzed with Alcalase 2.4L at hydrolysis times of 30, 60, 90, 120 min. Antioxidant activity of the hydrolysates obtained from hydrolysis times of 30, 60, 90 and 120 min were not significantly different from 0 min (p<0.05). RBPE was freeze-dried and it was found that freeze-drying did not affect antioxidant activity of RBPE. FD-RBPE contained protein 53.65% (w/w of FD-RBPE) and its antioxidant activity was not significantly different from RBPE (p<0.05). When FD-RBPE was used together with BHA, DPPH scavenging activity of it was not enhanced or reduced. But FRAP of FD-RBPE combined with BHA was lower than the sum of FD-RBPE and BHA. Therefore, FD-RBPE should not be combined with BHA but it should be considered to replace BHA in food products.

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