# Preparation of rice bran protein isolates using three-phase partitioning and its properties

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**Abstract**

Rice bran is an economical source of natural plant-based protein. Nowadays, there are many inventions for trying to extract rice bran protein. However, the methods for recovering protein from the extract usually provide low yield. So, the application of three-phase partitioning (TPP) was studied. To recover the proteins from crude rice bran extract, TPP condition including the ratio of *t*-butanol to crude rice bran extract and the concentration of ammonium sulfate was optimized. The optimal condition achieving the highest rice bran protein isolates (RBPI) recovery (98.59±3.52%) was the ratio of *t*-butanol to crude rice bran extract of 2.0:1.0 with 40% of ammonium sulfate. Molecular weight (MW) of rice bran protein hydrolysates (RBPH) obtained by using crude enzyme from papaya latex (activity=8,658.67±44.06 Unit/mL) ranged between <7.51 to 31.83 kDa. The total phenolic content (TPC) of RBPI and RBPH was 244.93±5.01 and 367.40±4.68 µg Gallic acid/mL, respectively. Moreover, these rice bran proteins also showed antioxidants activity in terms of radical scavenging activity (17.84±0.45 to 44.51±0.44%) and ferric reducing activity (584.67±2.65 to 924.33±6.11 µmoL $FeSO_4$/L). According the results, TPP could be used as an alternative method to recover protein with a high yield and bioavailability properties.

**Keywords:** Three-phase partitioning, Rice bran, Protein isolates, Protein hydrolysates, Antioxidant activities

## 1. Introduction

Rice is the main staple food in many Asia countries. The Rice Department of Thailand reported that 2.8 million metric tons rice bran was produced in 2012. During rice milling, rice bran is produced as a by-product approximately 10% weight of the whole rice grain. In general, rice bran is comprised of 11–15% proteins. It has been recognized as nutritionally superior to other proteins especially on its reported hypoallergenicity, anticancer (Jiamyangyuen *et al*., 2004) and also anti-free radical or oxidation reaction especially a small peptides (Chanput *et al*, 2009 and Wang *et al*., 2013). Nowadays, there are many inventions for trying to extract

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such as alkaline extraction, enzymatic extraction, microwave or ultrasound-assisted extraction and also supercritical fluid extraction (Watchararuji *et al.*, 2008; Silpradit *et al.*, 2010 and Fabian *et al.*, 2011). However, several methods to precipitate protein from crude extract e.g. rice bran protein precipitation at isoelectric point (pI) usually provide low protein recovery. Three-phase partitioning (TPP) is a bioseparation technique for protein precipitation, a technique which is based on partitioning of hydrophilic constituents, proteins and hydrophobic constituents in three phase comprising of water, ammonium sulfate and organic solvent (Rawdkuen *et al.*, 2010 ). The TPP has widely been used for the extraction and purification of various proteins with higher recovery than other methods. Ozer *et al*. (2010) reported that TPP recovered invertase from tomato in a level of 190% and its purity reached to 8.6 folds. Moreover, TPP had been used for proteases recovery from fish viscera and plant latex. The results showed that the recovery were higher than 100% (Rawdkuen *et al.*, 2010 and 2012). However, there are no researches works discuss about optimal condition of TPP for rice bran protein recovery. The objective of this study was to optimize three-phase partitioning (ratio of organic solvent to crude rice bran extract and salt concentration) to recover rice bran protein. Chemical properties of rice bran protein and rice bran protein hydrolysates were also determined.

## 2. Materials and Methods

### 2.1 Chemicals and raw materials

Ammonium sulfate ($(NH_4)_2SO_4$) was purchased from Univar (Ajax Finechem, Australia). *t*-Butanol ($C_4H_{10}O$) was purchased from Panreac (Barcelona, Spain). Albumin bovine serum (BSA) was purchased from Sigma-Aldrich (USA). Other analytical grade chemical reagents used in this study were purchased from Merk (Darmstadt, Germany). Stabilized Thai Jasmine 105 organic rice bran (RB) was supported by Urmatt Ltd. (Chiang Rai, Thailand)

### 2.2 Rice bran preparation

Stabilized rice bran was dried with hot air oven at 60°C for 8 hours, grinded and then passed through a 35-mesh sieve. The rice bran was kept in vacuumed-polypropylene bag at -18°C for further experiments.

### 2.3 Preparation of crude rice bran extracts (CE)

Rice bran were mixed with distilled water (1:5) and then homogenized at 20,000 rpm for 10 min at room temperature before placing into microwave machine at 800W for 40 sec.

The solution was centrifuged at 4,000*g* for 10 min, the supernatant (crude rice bran extract: CE) was collected and subjected for further experiments.

**2.4 Three-phase partitioning (TPP)**

TPP was used for rice bran protein recovery as described by Rawdkuen *et al*. (2012) with slightly modification. There are two factors for optimization including CE to *t*-butanol ratio and ammonium sulfate concentration on rice bran protein partitioning.

**2.4.1 Effect of *t*-butanol to CE ratio**

*t*-Butanol was added to CE at the ratios of 0.5:1.0, 1.0:1.0, 1.5:1.0 and 2.0:1.0 (v/v) following 30% (w/v) of ammonium sulfate. The solutions were mixed thoroughly and shake at 90 rpm for 60 min before subjecting to centrifuge at 5,000*g* for 10 min. After phase separation, the lower and upper phases were removed. The interphase was collected, solubilized, and then determined for protein content. The best ratio resulting in the highest protein recovery was selected for the next experiment.

**2.4.2 Effect of ammonium sulfate concentration**

The ammonium sulfate concentration of 30, 40, 50 and 60% (w/v) were added to the selected ratio with the highest protein recovery from previous step. Phase partitioning procedure was performed as mentioned above.

**2.5 Preparation of rice bran protein isolates (RBPI)**

The optimal ratio of CE to *t*-butanol and ammonium sulfate concentration were combined and used for rice bran protein isolates preparation. After the complete phase separation, the interphase was collected (Rice bran protein isolates: RBPI) and determined for protein recovery as equation below;

$$\text{Protein recovery (\%)} = \frac{[\text{Protein content in RBPI (mg/mL)} \times \text{Volume diluted (mL)}] \times 100}{\text{Protein content in CE (mg/mL)} \times \text{Volume of extract used}}$$

**2.6 Production of rice bran protein hydrolysate (RBPH)**

To produce the rice bran protein hydrolysate (RBPH), the RBPI obtained from the optimal condition was used a starting material for enzymatic hydrolysis using crude enzyme extract from papaya latex.

**2.6.1 Crude enzyme preparation**

Latex of local papaya was collected and diluted with distilled water (1:1, v/v). The solution was mixed and centrifuged at 8,000g at 4°C for 10 min. The supernatant (crude

enzyme extract; protein content of 27.47±0.56 mg/mL and enzyme activity of 8,658.67±44.06 Unit/mL) was kept in a clean tube at -18°C before using in hydrolysis reaction.

**2.6.2 Hydrolysis of RBPI**

RBPI was suspended in distilled water, mixed with crude enzyme extract in the ratio of 1.0:0.5 (based on protein content), and then incubated at 37°C for 60 min. Enzyme reaction was inactivated by heating at 95°C for 5 min. The hydrolysates were subjected to determine the antioxidant activities and protein pattern compared with original crude rice bran extract, RBPI and commercial soy protein isolates (SPI).

**2.7 Analytical method**

**2.7.1 Protein contents determination**

Protein contents were measured by Bradford method (Bradford *et al*., 1976) using BSA as a protein standard.

**2.7.2 Electrophoresis by SDS-PAGE**

SDS-PAGE was used to investigate for protein pattern of all protein fractions obtained according to the method of Laemmli *et al*. (1970). The protein solutions were mixed at the ratio of 1:1 (v/v) with sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS and 20% glycerol). The CE, RBPI RBPH (5 and 10 µg) and crude enzyme extracts were loaded onto 15% of separation gel, respectively and 4% of stacking was used. The samples were subjected to electrophoresis at a constant current of 15 mA/gel. After that, the gels were stained overnight with a solution of 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) of methanol and 7.5% (v/v) of acetic acid. Moreover, the gels were destained with 50% (v/v) of methanol and 7.5% of acetic acid for 40 min, followed by 5% (v/v) of methanol and 7.5% (v/v) of acetic acid for 20 min before being washed and dried.

**2.7.3 Bioavailability properties**

**TPC determination**

Folin-Ciocalteu reagent was diluted with distilled water 1:9 (v/v) followed by 1.25 mL of this reagent and 50 µL of sample were mixed. One milliliter of $Na_2CO_3$ (7.5%) was added. The mixture was incubated for 15 min at 50°C. The absorbance at 760 nm was measured by using a UV-Vis spectrophotometer within 15 min. Gallic acid was used as a standard, and results were calculated as gallic acid equivalents (µg GAE/mL).

**DPPH radical scavenging activity**

The free radical scavenging activity was measured using the method of Mao *et al*. (2006). The 0.1 mM DPPH radical in ethanol was prepared and 2 mL of this solution was added to 3 mL of the extracts. The absorbance was measured at 517 nm after 30 min. BHT

was used as positive standard. The scavenging of DPPH radical in percent was calculated by the equation:

$$\text{Scavenging activity (\%)} = (1 - A_1 / A_0) \times 100$$

where $A_0$ was the absorbance of the control reaction and $A_1$ was the absorbance in the presence of sample and reported as percentage of inhibition activity.

**Ferric Reducing (FRAP)**

Ferric Reducing Activity Power (FRAP) assay was performed according to the method of Benzie and Strain (1996). The FRAP reagent was prepared by mixing of 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ (10:1:1), respectively. The 1800 µL of FRAP reagent was mixed with 180 µL of distilled water and 60 mL of samples. The absorbance was measured at 593 nm after 10 min. $FeSO_4$ was used as a standard (0–1000 µM), and absorbance of samples were calculated as FRAP value (µmoL $FeSO_4$/L).

**2.8 Statistical analysis**

The results were statistically analyzed by analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Statistical significance was accepted at a level of $p<0.05$.

**3. Results and Discussion**

**3.1 Effect of the ratio of *t*-butanol to CE on protein partitioning**

The ratio of *t*-butanol to crude extract (CE) of 0.5:1.0, 1.0:1.0, 1.5:1.0 and 2.0:1.0 were studied at the constant concentration of ammonium sulfate (30%). The result showed that protein recovery increased with increasing of the ratio of *t*-butanol to CE (Figure 1). The highest protein recovery (81.78±2.62%) was obtained from the ratio of 2.0:1.0 and followed by the ratio of 1.5:1.0 (70.06±1.54%). At the ratio of 0.5:1.0 and 1.0:1.0, the protein recovery was not significantly different (59.29±3.09 and 64.07±1.10%, $p<0.05$). There are many studies also mentioned that the lower content of *t*-butanol may not adequately synergized with ammonium sulfate (Ozer *et al*., 2010 and Vidhate and Singkal, 2013); whereas, the increasing of *t*-butanol to slurry ratio from 1.0:1.0 to 3.0:1.0 increased the protein precipitate (Vidhate and Singkal, 2013). This result may be attributed to protein is easier to precipitate out at the higher amount of *t*-butanol used. Furthermore, the higher volume of *t*-butanol affected to the extractability of hydrophobic impurity molecules such as fat or lipids resulting in the high protein recovery. Therefore, the ratio of 2.0:1.0 was used to study for the optimal concentration of ammonium sulfate.

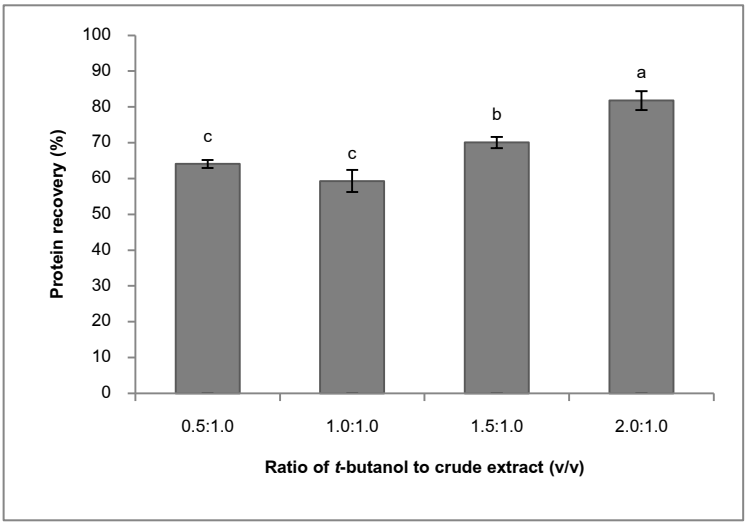


**Figure 1** Effect of the ratio of *t*-butanol to CE on rice bran protein recovery using three-phase partitioning

### 3.2 Effect of ammonium sulfate concentration on protein partitioning

The ratio of *t*-butanol: CE of 2.0:1.0 was used in this experiment; whereas, the concentrations of ammonium sulfate varied from 30 to 60% (w/v). It was observed that the protein recovery increased with the increasing of salt concentration (Figure 2). The concentration of 50% showed the highest protein recovery (99.27±2.61%) but insignificantly different with the using of 40% salt (98.59±23.25%) ($p$>0.05). Furthermore, the lowest protein recovery (80.84±1.57%) was occurred when 30% salt was used. However, the using of 60% ammonium sulfate is not recommended because there is some salt crystal cannot dissolve in the system. The effect of salting out plays a major role in the system contained high salt concentration. The principle of sulfate ions for salting out protein has been viewed in five different ways including ionic strength effects, kosmotropy, cavity surface tension and enhancement osmotic stressor (dehydration), exclusion crowding agent, and binding of sulfate ion to cationic sites of protein (Rawdkuen *et al*., 2010). Since, the using of 40 and 50% ammonium sulfate could not make the different percentage of protein recovery; thus, 40% ammonium sulfate was selected to set the condition of RBPI preparation.

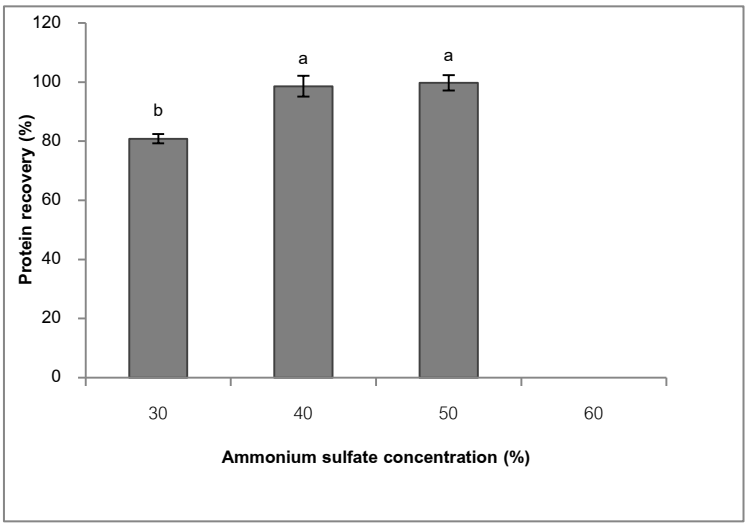


**Figure 2** Effect of ammonium sulfate concentration on rice bran protein recovery using three-phase partitioning

### 3.3 Rice bran protein isolates and hydrolysates

The condition of 40% ammonium sulfate and *t*-butanol to crude extract ratio of 2.0:1.0 was selected to use for RBPI preparation. The RBPI was hydrolyzed with the crude enzyme (activity of 8,658.67±44.06 Unit/mL) to obtain the RBPH. Protein patterns, TPC and antioxidant activity of CE, RBPI, SPI, RBPH and crude enzyme extract were monitored and presented in Figures 3–6.

### 3.4 SDS-PAGE

Protein patterns of CE, RBPI and RBPH are shown in Figure 3. Five and ten microgram of each samples were loaded in 15% separating gel. It was observed that the molecular weight (MW) of protein component in CE and RBPI ranged between 7.51 to 31.83 kDa. Moreover, there are no protein bands with MW upper 11 kDa after hydrolyzing by crude enzyme (RBPH) was observed. It is hypothesized that by using this condition high MW protein in RBPI were hydrolyzed into peptide subunits or amino acid (MW<7.51 kDa). This result supported by the study of Chanput *et al*., (2009) who found that the MW of albumin, globulin, and glutelin fraction from rice bran were between 10 and 60 kDa. Furthermore, Tang *et al*., (2003) also reported that the MW of proteins from freeze-dried and spray dried rice bran protein concentrate ranged between 6.5 and 66.2 kDa. Hamada *et al*., (1998) determined that the MW of rice bran protein hydrolysates ranged between 1 and 150 kDa as well. In the other hand, it was found that the commercial papain plays a role to reduce MW of soy protein isolate from ~66 kDa to <42 kDa (Tsumura *et al*., 2005).

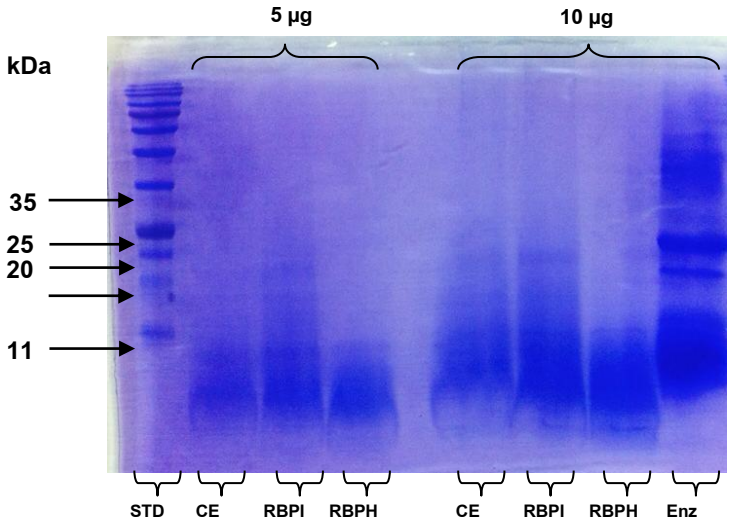


**Figure 3** Protein patterns of crude rice bran extract (CE), rice bran protein isolates (RBPI), rice bran protein hydrolysates (RBPH), crude enzyme (Enz) and standard protein marker (STD)

### 3.5 Total phenolic contents (TPC) and antioxidant activities

The total phenolic content of CE, RBPI, SPI, RBPH and crude enzyme (1 mg/mL based on protein content) were determined using Folin-Ciocatule reagent. The result showed that the crude extract from rice bran showed the highest amount of TPC (518.59±3.16 µg/mL) (Figure 4), followed by RBPH (367.40±4.68 µg/mL), RBPI (244.93±5.01 µg/mL) and SPI (83.28±2.51 µg/mL), respectively ($p$<0.05). The decreasing of TPC in RBPI may cause by TPP step. In general, TPP system consisted of top phase (non-polar), interphase (protein fraction) and bottom phase (polar) resulting in dispersion of some polar and non-polar TPC to optimal phase. In addition, it was observed that TPC of RPBH slightly increased when compared with the original RBPI ($p$<0.05). This result may cause by the function of enzyme to release some phenolic amino acid from RBPI such as tyrosine, which is believed to be associated with the special ability of phenolic groups (Bernardini *et al*., 2012). However, in crude enzyme also may contain phenolic compound that play a role to this value obtained.

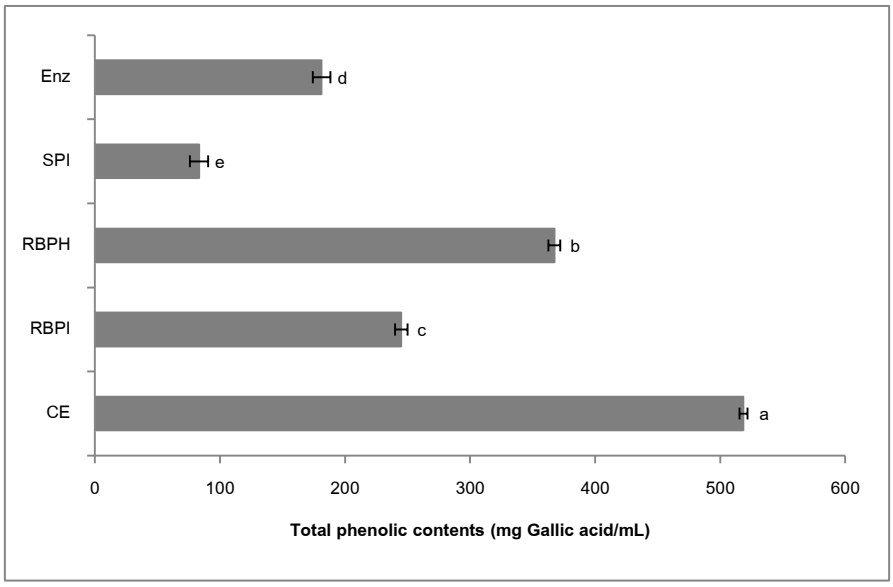


**Figure 4** Total phenolic contents of crude extract (CE), rice bran protein isolate (RBPI), rice bran protein hydrolysate (RBPH), soy protein isolates (SPI) and crude enzyme (Enz).

DPPH radical scavenging assay has been widely used to evaluate antioxidant properties of compounds as free radical scavengers or hydrogen donors (Intarasirisawat *et al*., 2012). At the same concentration of each protein samples (1 mg/mL based on protein content), CE showed the highest scavenging effect on DPPH radical (70.82±1.38%) (Figure 5) followed by RBPH (44.51±0.44%) and RBPI (19.76±0.61%), respectively ($p$<0.05). Commercial SPI showed the lowest scavenging effect on DPPH radical (13.92±0.33%) ($p$<0.05). However, all protein fraction obtained from this investigation showed lower DPPH radical scavenging when compared with synthetic antioxidant (BHT) ($p$<0.05). This might be governed by a difference amount of TPC, amino acid composition, amino acid side chain and hydrophobicity. In addition, the enzymatic cleavage affected to the formation of shorter chain peptides possessing more hydrophilicity resulted in it could not interact properly with an oil-soluble free radicals (hydrophobic) (Intarasirisawat *et al*., 2012). When compared between RPBI and RBPH, it was found that the later showed higher radical scavenging activity than the former about 2.25 folds. During proteolysis, a newly created antioxidative function of the resultant peptides and/or amino acids released may be occurred (Chanput *et al*., 2009). Fang *et al*. (2012) and Bernardini *et al*. (2012) also indicated that amino acid composition especially aromatic amino acids including tyrosine, histidine, methionine and phenylalanine and hydrophobic amino acid containing valine, alanine, proline and luecine showed ability to scavenge free radicals. However, this result indicated that CE, RPBI and RBPH acting as electron donors and free radical scavengers.

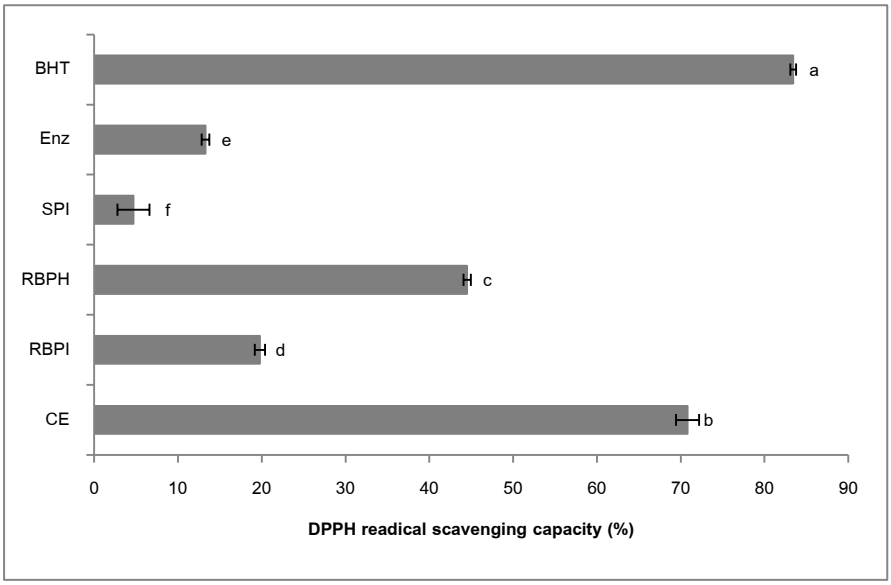


**Figure 5** Inhibition activity of DPPH radical of crude extract (CE), rice bran protein isolates (RBPI), rice bran protein hydrolysates (RBPH), soy protein isolates (SPI), crude enzyme (Enz) and BHT.

FRAP values was measured by monitoring the amounts of $Fe^{2+}$ (micromolar) yielded by reducing the $Fe^{3+}$ complex. It was clearly seen that the crude extract had the highest reducing capacity with FRAP values of 1,662.50±6.61 μmoL $FeSO_4$/L, followed by RBPH (1,110.50±7.09 μmoL/L) and RBPI (924.33±6.11 μmoL/L), respectively (Figure 6). However, this result may be diminished because CE is likely to be contaminated with other soluble components such as sugars from the starting materials which can be acted as reducing agent. Whereas, the reducing capacity of the RBPH was stronger than RBPI, might be due to a newly created antioxidative function of the resultant peptides and/or amino acids released. This result conformed to the study of Chanput *et al*. (2009) who reported that rice bran protein hydrolysates can be act as stronger reducing agent than rice bran protein isolate, which had FRAP value at about 1,808.8–8,744.0 μmoL $FeSO_4$/L. Furthermore, RBPI and RBPH from this study showed higher reducing capacity than commercial SPI at about 5.8–11.0 folds.

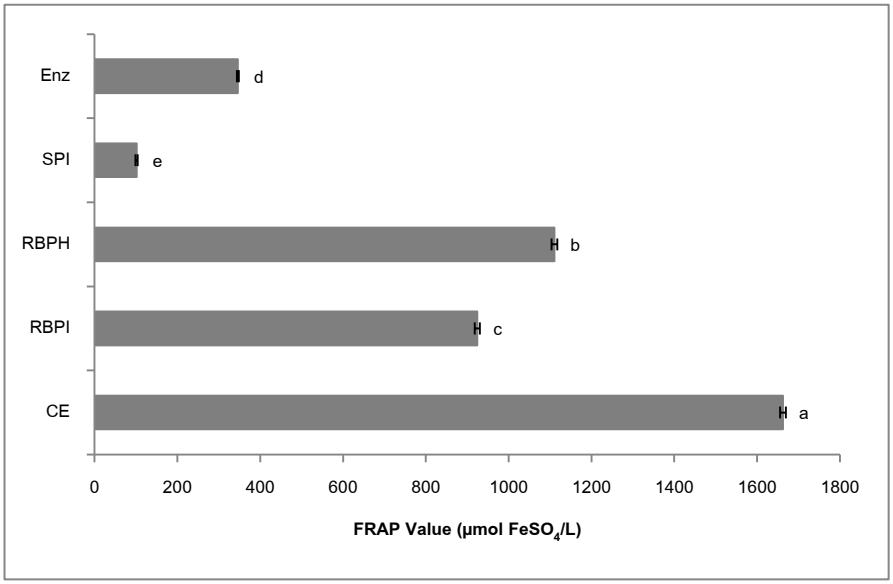


**Figure 6** FRAP values of crude extract (CE), rice bran protein isolates (RBPI), rice bran protein hydrolysates (RBPH), soy protein isolates (SPI) and crude enzyme (Enz).

## 4. Conclusion

Three-phase partitioning is an effective method to recover protein from rice bran extract. The best condition to achieve the highest protein recovery was the ratio of *t*-butanol to crude rice bran extract of 2.0:1.0 with 40% of ammonium sulfate. For bioavailability properties, RBPI and RBPH showed antioxidants activity both radical scavenging activity and ferric reducing activity.

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