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Enzymatic Optimization of Riceberry Bran Protein Hydrolysate Extraction and Characterization

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Abstract

Defatted riceberry bran (DRBB) by screw press contains high nutrition including protein and phytochemicals. This research is aimed to optimize the protease enzymatic extracting condition for riceberry bran protein hydrolysate (RBBPH) and characterize protein hydrolysate compositions. The experimentation was conducted using central composite design (CCD). The Response surface methodology (RSM) was used to determine the optimum condition in order to obtain maximum protein yield, total phenolic content (TPC) and anthocyanin. The three independent variables including enzyme to substrate ratio (E/S; 0.1-2%), temperature (T; 30-70°C), and hydrolysis time (t; 30–360 min) were studies while the ratio of rice bran to water by weight and pH were fixed constant at 1:5 and 8.0, respectively. The results showed that the model of protein yield and anthocyanin content were significant ($p< 0.05$). The coefficients of determination (R^2) of protein yield, TPC and anthocyanin were 0.87, 0.55 and 0.80, respectively. The optimal condition was 0.82% enzyme/substrate at temperature of 43.75ºC for 140.38 min. Under this condition, the values for protein yield, TPC and anthocyanin content were 14.98 g, 226.84 mg GAE and 3.25 mg Cyn-3-Glu per 80g DRBB, respectively with degree of hydrolysis (%DH) 21.26%. The RBBPH after freeze-dried contained 21.62% of total protein, 33.85 mg GAE/g of TPC and 0.47 mg Cyn-3Glu/g of anthocyanin content. The extract exhibited high antioxidant activities (DPPH; IC_{50} = 0.052 mg/mL and FRAP; IC_{50} = 2.27 mg/mL). The RBBPH was high in glutamic acid and molecular weight was between 5-50 kDa.

Keywords: proteinhydrolysate, riceberry bran, enzymatic extraction, anthocyanin, antioxidant

1. Introduction

Rice is feeds about one half of the world's population, mainly in Asia, Africa, and SouthAmerica. It has a long cultivation history and like religion or tradition. Its use is deeply ingrained in the daily lives of Asian people [1]. There are many special cultivars of rices. Black rice, an economically important special rice species, derives its name from the rich natural anthocyanidin

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compounds in pericarp and seed capsule of brown rice (caryopsis) [2]. However, in the recent years pigmented rice varieties have received increased attention from the researchers and consumer preferences have shifted towards pigmented types having high nutritional properties [3]. Black rice has a number of nutritional advantages over common rice, such as a higher content of protein, vitamins and minerals, although the latter varies with cultivar and production location [4].

Riceberry, a Thai black rice, had been recently developed with aim to provide optimum nutritional benefit to general consumers as well as supplementation to anaemic and diabetes mellitus patients. Riceberry bran contains a high amount of major bioactive components such as phenol compounds and anthocyanins. The most common anthocyanin is the Cy-3-glucoside in vegetable and fruits and the stability of anthocyanin depends on pH, temperature, enzyme and other [5]. The anthocyanin content was purple-red pigment of plant. Anthocyanin was an antioxidant which gave health benefits such as modulating cardiovascular disease biomarkers, reducing tumor development in rodents, and preventing oxidative damage to DNA [6]. These compounds have shown anti-cancer [7] and antioxidant activities [8]. Protein hydrolysate from enzymatic hydrolysis of Riceberry bran has great potential to become an important value-added product of rice. The characterization and antioxidant properties of rice bran protein hydrolysate from Riceberry bran has been reported by Thamnarathip *et al.* [9]. Riceberry bran has a hight anthocyanin, especially cyanidin and peonidin-3-glucoside. It has a high protection of endothelial cells from oxidative stress events and a considerable protection against angiogenesis induced by vascular endothelial growth factor [10].

Rice bran, the residue of brown rice during the production of white rice, this agricultural by-product contains a number of carbohydrate and other nutrients such as proteins, lipid, fiber, Ca^{2+}, Mg^{2+}, phosphate, silica, Zn^{2+}, thiamin, and niacin [11]. Generally, Rice bran is extracted to rice bran oil. The residue after extraction of rice bran oil from rice bran is called defatted rice bran (DRBB). Thus, defatted rice bran still contains many other nutrition. Chanput *et al.* [12] studied antioxidant properties of rice bran and barley protein hydrolysate and it was found that protein hydrolysate has potential anti lipid peroxidation of polyunsaturated. Antioxidant and peptide protein in rice bran have many functions such as antioxidant, antihypertensive, reduce cholesterol, immunosti-mulating [13]. Dark rice bran has more benefits than other rice bran. For example, riceberry bran, homnil bran and leumpua glutinous rice etc.

Rice bran excellent source of protein and bioactive materials. Plant protein hydrolysates can contribute as alternative source of protein and health-promoting ingredients in specific nutritional products [9]. Enzymatic proteolysis is a valuable bioprocess to enhance the functional properties of the original protein. The functionality of hydrolysate is tied to the nature and the composition of peptides generated during hydrolysis. Many potential applications of hydrolysates require well defined and reproducible peptides. Thus, the feasibility of a hydrolysis process of a specific enzyme/ substrate system depends on the qualitative control of hydrolysis products [14]. This research has been focused on bioactive protein and peptide from Riceberry bran protein. The aim of this research to optimum RBBPH extraction and characterize protein hydrolysate compositions.

2. Materials and Methods

2.1 Materials

DRBB obtained from Rice Science Center & Rice Gene Discovery Kasetsart University Kamphaengsaen Campus, NakhonPathom Thailand. DRBB was ground using electric blender and was kept at 4ºC in laminated aluminum foil container. Alcalase was purchased from the East Asiatic Company (Thailand) Ltd. (Bangkok, Thailand). All other chemicals and reagents were analytical grade and commercially available.

2.2 Preparation of Riceberry bran protein hydrolysate (RBBPH)

Eighty gram of DRBB was dissolved in 400 ml distilled water (1:5) weight by volume. The suspension was adjusted to pH 8.0 for hydrolysis Alcalase according to the method of Silpradit [15] using 0.1 M NaOH, the content of alcalase was followed by Table 1. The pH was maintained at an optimum according to the pH-stat method. The mixture was stirred at 500 rpm. After extraction, the enzyme was inactivated by heating at 85ºC for 10 min. Then cooled down in cold water for 15 min. The protein supernatant was recovered using centrifugation at 8,000 g for 15 min at 4ºC and filtered by four layer of cheesecloth. The remaining protein in the riceberry bran precipitate was washed with 250 ml of distilled water and centrifuged at 8,000 G, 15 mins twice by centrifugation (Tommy, Suprema 25, Japan). The protein solution was adjusted to pH 7.0 with 0.1 M citric acid. Then, RBBPH solution was evaporated and freeze dried (Super Modulyo 220, USA). The dry RBBPH obtained was kept in laminated aluminum foil bag at 4ºC.

Proximate analysis

The proximate of defatted DRBB and RBBPH were determined by AOAC method [16].

2.3 Experimental design and optimization

The Response Surface Methodolo(RSM) and a five-level three-factor central composite design (CCD) were used to evaluate the effect of enzyme/substrate (0.1-2%), Temperature (30-70ºC) and hydrolysis time (30-360 min). The complete design consisted of 20 combinations including five replicates of the center showed in Table 1. The response value of hydrolysis were protein yield, TPC and anthocyanin content. The experimental design and statistical analysis were performed using Stat-Ease software (Design expert version 7.0.0 Trial).

2.4 Determination of the degree of hydrolysis

The degree of hydrolysis (DH) was calculated according to Adler-Nissen [17] as shown in equation 1:

$$DH (\%) = B \times N_b \times 1/\alpha \times 1/M_P \times 1/h_{tot} \times 100 \quad (1)$$

Where B is volume on base (mL) used to control the pH during enzymatic hydrolysis, N_b is the normality of the base (0.1 N NaOH), α is the degree of dissociation of the a $-NH_2$ groups ($1/\alpha$ = 1.13 for hydrolysis of Alcalase), M_P is the mass of protein yield in g and h_{tot} is the total number of peptide bonds in the protein substrate (8.4 meqv/g rice bran protein).

Response of hydrolysis were protein yield, TPC and total anthocyanin content. This experiment was replicated twice. RBBPH yield was calculated according to Adler-Nissen [17] as shown in equation 2:

$$\text{Protein yield (\%)} = \frac{\text{weight (g) of RBBPH x protein content (\%) of RBBPH x 100}}{\text{weight of DRBB x protein content (\%) of DRBB}} \quad (2)$$

Table 1. The hydrolysis conditions of RBBPH using central composite design (CCD)

Factor	X1; E/S (%)	X2;Temp (˚C)	X3;Time (min)
1	1.05 (0)	50 (0)	195 (0)
2	0.11 (-α)	50 (0)	195 (0)
3	1.05 (0)	50 (0)	30.03 (-α)
4	0.49 (-1)	38.11 (-1)	96.91 (-1)
5	1.99 (+ α)	50 (0)	195 (0)
6	1.05 (0)	50 (0)	195 (0)
7	1.05 (0)	50 (0)	195 (0)
8	1.61 (+1)	38.11 (-1)	96.91 (-1)
9	1.61 (+1)	61.89 (+1)	96.91 (-1)
10	1.05 (0)	70 (+ α)	195 (0)
11	1.05 (0)	50 (0)	359.97 (+ α)
12	0.49 (-1)	38.11 (-1)	293.09 (+1)
13	1.05 (0)	30 (-α)	195 (0)
14	1.05 (0)	50 (0)	195 (0)
15	0.49 (-1)	61.89 (+1)	96.91 (-1)
16	0.49 (-1)	61.89 (+1)	293.09 (+1)
17	1.61 (+1)	61.89 (+1)	293.09 (+1)
18	1.05 (0)	50 (0)	195 (0)
19	1.61 (+1)	38.11 (-1)	293.09 (+1)
20	1.05 (0)	50 (0)	195 (0)

2.5 Measurement of color

A spectrophotometer (Minota CM-3500d, UK) with 25 mm petri dish was used to determine the color of the RBBPH. The instrument was calibrated with white and black calibration tiles. Color was measured in the CIE L^* a^* b^* color space, with L^* was the luminance of lightness component range of L^* from 0-100; 0 was darkness and 100 was lightness. Parameter a^* showed green and red color, parameter b^* showed blue and yellow color were two chromatic components range from -120 to 120 [18]. Each color value reported was calculated by averaging ten measurements, repacked for each measurement.

2.6 Chemical properties determinations

2.6.1 Determination of total anthocyanin content

Total anthocyanin content in RBBPH was measured by pH differential method [19]. The RBBPH was dissolved in a potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5), respectively. The mixture waskept in the dark at room temperature for 15 min. Then, the mixture was centrifuged using centrifugation at 3,000 rpm for 30 seconds at 4ºC. The mixture spectral absorbance measurements were read at both 510 and 700 nm. Distilled water was used as the blank. The absorbance value (A) of the diluted sample was then calculated as shown in equation 3:

$$A = (A_{510}-A_{700})_{pH\ 1.0} - (A_{510}-A_{700})_{pH4.5} \quad (3)$$

Anthocyanin content (mg Cyn-3Glu/L) (equation 4)

$$\text{Anthocyanin content} = (A \times Mw \times DF \times V)/(E \times W) \quad (4)$$

When, Mw = 449.2 (Cyanidin 3 glucoside)
DF = Dilution factor
E = molar absorbtivity = 26,900 (Cyd-3-glu)
W = dry weight of sample
V = the volume of the extraction liquid.

2.6.2 Determination of total phenolic content (TPC)

The TPC of RBBPH was determined using Folin-Ciocalteu method according to Chan *et al*. [20] method. The reaction mixture contained 300 µl of hydrolysate solution, 1.5 mL of Folin-Ciocalteu reagent (1:10 v/v) and 1.2 mL of 7.5% Na_2CO_3. The mixture was incubated in the dark at room temperature for 30 min. The absolute was measured at 765 nm. Distilled water was used as the control and gallic acid at 10-100 µg/ mL was used as a standard.

2.6.3 Determination of total protein content by Lowry method

The total protein in soluble was determined using Folin-Ciocalteu method according to Lowry *et al*. [21]. The values were expressed as BSA. The reaction mixture contained 100 µl of sample, 3 mL of Reagent A (1:1:100; 1% $CuSO_4$: 2% $NaKC_4H_4O$: 2% Na_2CO_3*NaOH). The mixture was kept at room temperature for 10 min. The mixture was mixed with 300 µl of Reagent B (1:1 Folin-Ciocalteu: distilled water) and was kept at room temperature for 30 min. The absorbance was measured at 660 nm using a spectrophotometer. After freeze dried, the protein powder of RBBPH was determined by the Kjeldahl method [17] and crude protein was calculated using the 5.95 conversion factor [22].

2.6.4 Determination of DPPH radical scavenging activity

The antioxidant activity of sample was evaluated by DPPH radical scavenging capacity assay [23]. Firstly, 1 mL of diluted sample (0.02-0.10 mg/ mL) was mixed 1 mL of 0.1 mM DPPH solution in 95% ethanol. The mixture was kept at room temperature for 30 min. The absorbance was measured at 517 nm. Ascorbic, α-tocopherol and BHT were used as reference standard. The results of antioxidant activity were expressed as % radical scavenging activity. DPPH radical scavenging activity was calculated as shown in equation 5:

$$\text{DPPH radical scavenging (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (5)$$

A_0 = The absorbance of blank
A_1 = The absorbance of sample

A calibration curve was acquired by plotting the DPPH-scavenging rate against sample/ standard concentration.

2.6.5 Determination of ABTS radical scavenging activity

ABTS radical scavenging activity was determined according to Re *et al*. [24]. Diluted sample with ethanol gave light absorbtion 0.70±0.02 at 734 nm. The ABTS radical scavenging activity of RBBPH was compared with those of ascorbic, α-tocopherol and BHT as standard. The results of antioxidant activity were expressed as % radical scavenging activity. ABTS radical scavenging activity was calculated as shown in equation 6:

$$\text{ABTS radical scavenging (\%)} = [(A_0 - A_1)/A_0] \times 100 \quad (6)$$

A_0 = The absorbance of blank

A_1 = The absorbance of sample

The percentage of scavenging ABTS$^{•+}$ was plotted against the sample/ standard concentration.

2.6.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to investigate for protein patterns. The protein solution of RBBPH was mixed in sample buffer (0.5 TrisHCl, pH 6.8, 10% SDS, 2-mercaptoethanol and glycerol) and heated to 100ºC for 2 min. The mixture (20, 30, 40 and 50 µg) were loaded onto 12.5% of the separation gels and 4 % of the stacking gels were used. The mixture were subjected to electrophoresis at a constant current of 100. After electrophoresis, the gel were stained for 2 hour with comassie brilliant blue R 250 and destained with destaining solution (50 mL methanol: 70 mL acetic acid: 1,000 mL deionized water).

2.6.7 Determination of total amino acid

The total amino acid was analyzed with In-house method according to HPLC-AccQ*Tag [25].

2.7 Statistical analysis

All experimental results from the optimization and measurements expressed as the mean ± SD of three replications using Design expert 7.0.0 Trial. Data of other results were analyzed using the SPSS 11.0 software by one-way analysis of variance (ANOVA) and differences were reported as significant at $p < 0.05$.

3. Results and Discussion

3.1 Riceberry bran photein hydrolysate

3.1.1 Proximate analyze of RBBPH

The proximate of DRBB at moisture of 7.94% contains protein, fat, ash, fiber and carbohydrate 13.35, 5.10, 5.19, 7.47 and 60.95%, respectively.

3.1.2 Model fitting and optimization of RBBPH

The effect of extraction for RBBPH from 20 conditions showed in Table 2. The response surface methodology (RSM) was used to determine the optimum conditions to obtain maximum protein, TPC and anthocyanin.

Table 2. The Response surface methodology (RSM) of Ricerberry bran protein hydrolysate.

Treatment	Factor			Protein (g/80gDW)	TPC (mg80gDW)	Anthocyanin (mg Cyn-3Glu/80gDW)
	E/S (%)	Temperature (˚C)	Time (min)			
1	0.49(-1)	38.11(-1)	96.91(-1)	14.82	220	2.85
2	1.61(+1)	38.11(-1)	96.91(-1)	15.13	222	3.52
3	0.49(-1)	61.89(+1)	96.91(-1)	13.19	237	3.26
4	1.61(+1)	61.89(+1)	96.91(-1)	12.67	187	3.69
5	0.49(-1)	38.11(-1)	293.09(+1)	10.96	204	2.43
6	1.61(+1)	38.11(-1)	293.09(+1)	11.93	181	1.41
7	0.49(-1)	61.89(+1)	293.09(+1)	11.61	157	0.83
8	1.61(+1)	61.89(+1)	293.09(+1)	10.45	144	1.10

Table 2. The Response surface methodology (RSM) of Ricerberry bran protein hydrolysate. (Con.)

9	0.11(-α)	50(0)	195(0)	11.03	209	2.32
10	1.99(+α)	50(0)	195(0)	15.64	252	2.59
11	1.05(0)	30(-α)	195(0)	15.91	206	3.26
12	1.05(0)	70(+α)	195(0)	14.18	236	2.53
13	1.05(0)	50(0)	30.03(-α)	14.12	208	3.60
14	1.05(0)	50(0)	359.97(+α)	9.82	210	2.32
15	1.05(0)	50(0)	195(0)	15.74	255	3.70
16	1.05(0)	50(0)	195(0)	15.67	229	3.05
17	1.05(0)	50(0)	195(0)	14.21	249	3.33
18	1.05(0)	50(0)	195(0)	15.19	236	3.08
19	1.05(0)	50(0)	195(0)	15.41	260	3.62

The result of RBBPH by enzymatic extraction showed that the protein content (Y1) were between 9.82-15.91 g/80 g DW (Table 2). The statistical analysis of the equation model and the significance of each coefficient were determined using the F-test and *p*- value (protein = 0.0024, anthocyanin = 0.0094) (Table 3). Hydrolysis time (X_3), %E/S (X_1^2) and hydrolysis time^2 (X_3^2) were shown an effect on the response value of protein. The hydrolysis time (X_3) and %E/S (X_1^2) of anthocyanin were significant ($p<0.05$). Regression analysis of confidence level 95% showed that coefficient of determination (R^2) of protein, TPC and anthocyanin equation model were 0.86, 0.55 and 0.82, respectively.

Table 3. Final equation in terms of coded factors and coefficient of independent variables and the dependent variable by central composite design (CCD).

No.	Factor	Final Equation in Terms of Coded Factors	R^2	Model Significant
1	Y1	$15.26+0.54*X_1-0.57*X_2-1.32*X_3-0.37*X_1*X_2+2.500E-003*X_1*X_3+0.41*X_2*X_3-0.83*X_1^2-0.23*X_2^2-1.31*X_3^2$	0.8668	0.0024*
2	Y3	$3.48+0.16*X_1-0.19*X_2-0.71*X_3+0.13*X_1*X_2-0.23*X_1*X_3-0.31*X_2*X_3-0.31*X_1^2-0.30*X_2^2-0.28*X_3^2$	0.8190	0.0094*

*Significant difference ($p<0.05$) using response surface methodology. 1 equation of protein, 2 equation of anthocyanin.

Optimization of protein extracted from RSM showed that high protein when %E/S was the highest, low temperature and hydrolysis time in range of 2-3 hour (Figure 1). The response surface for protein yield plot indicated that the hydrolysis time had higher effect on protein yield than %E/S, and temperature (Table 4). The response surface for protein yield with temperature and %E/S show in Figure 1A that a high protein yield was obtain 1.00 - 1.99%E/S and 30-50ºC. Figure 1B showed that a high protein yield was at 0.7-1.7% E/S and 100-195 min of hydrolysis time. However, temperature at 30-50 ºC and hydrolysis time 30-195 min caused high protein yield (Figure 1C).The response surface of TPC plot had no significant effect of RBBPH ($p\geq0.05$). Anthocyanin content in the response surface plot (Figure 2) showed that hydrolysis time had a high effect on anthocyanin content same as protein yield (Table 4). The anthocyanin content was high at %E/S between 0.7-1.5% and temperature 40 to 55ºC (Figure 2A). In Figure 2B showed that a high anthocyanin content was 0.7-1.99% E/S and hydrolysis for 30-195 min. The high anthocyanin content also at 30-195 min at 40-70ºC (Figure 2C).

Table 4. ANOVA of model response surface of RBBPH

Source	p-value Prob > F protein yield	p-value Prob> F Anthocyanin
Model	0.0024*	0.0094*
A-% E/S	0.0779	0.2764
B-temp	0.0630	0.2136
C-time	0.0007*	0.0005*
AB	0.3259	0.4925
AC	0.9946	0.2379
BC	0.2817	0.1220
A^2	0.0111*	0.0473*
B^2	0.4182	0.0516
C^2	0.0006*	0.0684

*Significant difference ($p<0.05$) using response surface methodology

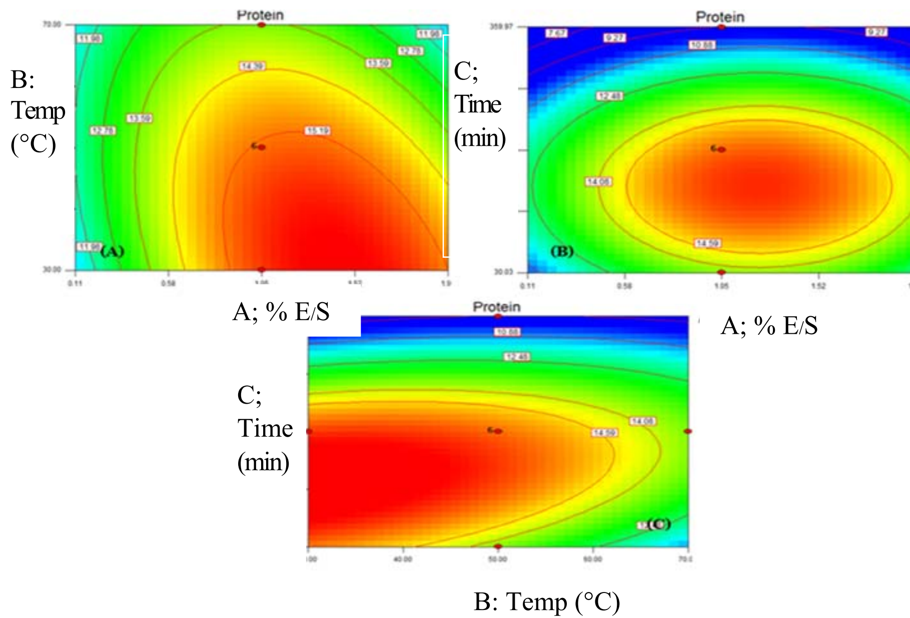

Figure 1. Response surface plots of independent variables on protein yield (g/80gDW); (A): %E/S and Temperature (ºC), (B): %E/S and hydrolysis time (min), (C): Temperature (ºC) and hydrolysis time (min)

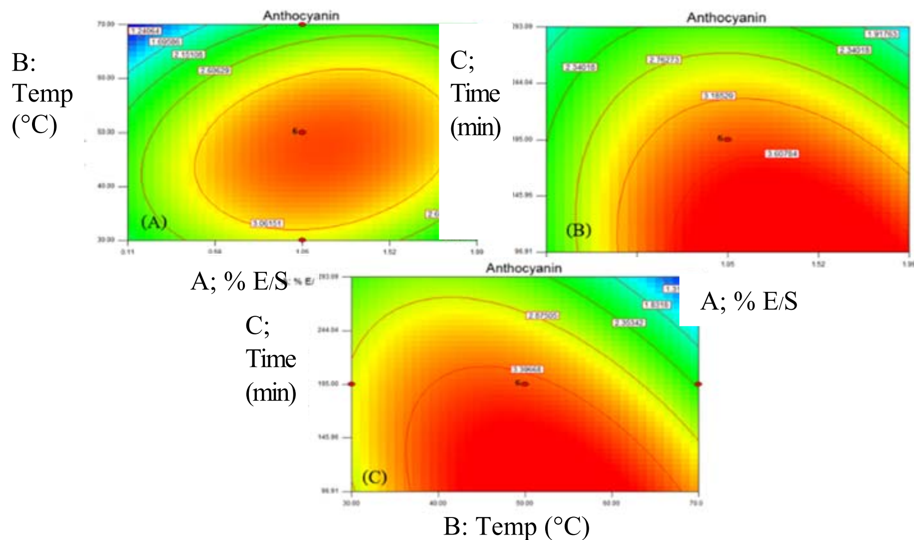

Figure 2. Response surface plots of independent variables on anthocyanin (mg Cyn-3Glu/80gDW); (A): % E/S and Temperature (ºC), (B): % E/S and hydrolysis time (min), (C): Temperature (ºC) and hydrolysis time (min)

The overlay plot and desirability of RBBPH extraction in Figure 3 showed that the shaded area was optimum conditions for maximum protein, TPC and anthocyanin yield by selection of the two most desirability conditions for determining the most reasonable effective condition. The selected conditions were (Figure 4) E/S 0.82%, 43.75ºC, and 140.38 min (condition 1) and E/S 1%**,** 46.05ºC and150.45min (condition 2) (Figure 5). The optimum conditions to obtain maximum protein, TPC and anthocyanin yield showed in condition 1 obtained protein 14.98 g/80gDW, TPC 226.84 mgGAE/80 gDW and anthocyanin 3.25 mg Cyn-3Glu/80gDW. The predicted value and observed values of protein was not significant difference ($p \geq 0.05$) but predicted value and observed values of TPC and anthocyanin content were significant difference ($p<0.05$). While condition 2 obtained protein 15.63 g/80gDW, TPC 236.12 mgGAE/80gDW and anthocyanin 3.58 mg Cyn-3Glu/80gDW. The predicted value and observed values of protein and TPC were not significant difference ($p \geq 0.05$), but anthocyanin contents were significant difference ($p<0.05$) (Table 5).

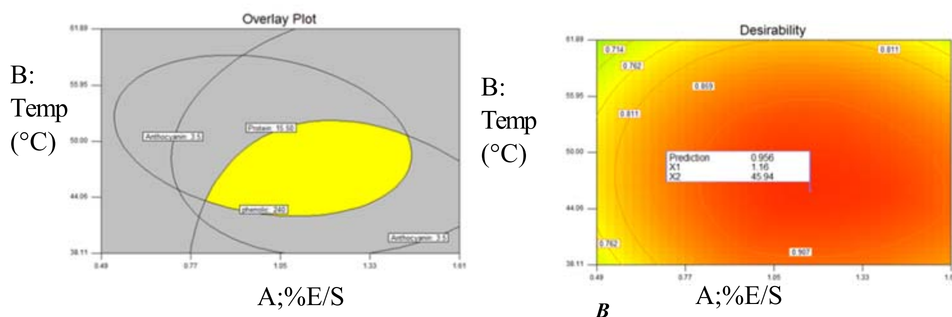

Figure 3. (A) The overlay plot response surface of protein yield, TPC and anthocyanin hydrolysis. (B) Desirability of protein yield, TPC and anthocyanin hydrolysis.

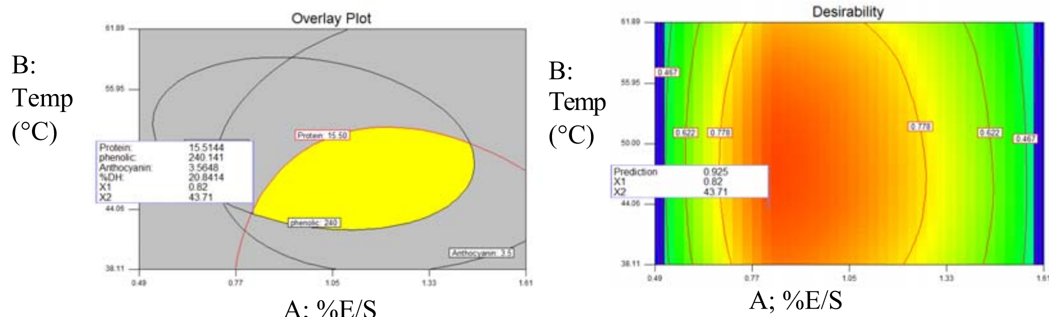

Figure 4. Optimization condition of E/S 0.82% temperature 43.75º C, hydrolysis time140.38 min

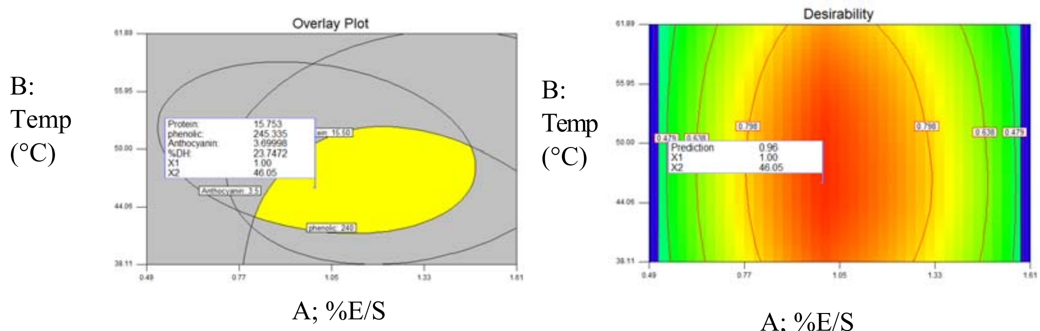

Figure 5. Optimization condition of E/S 1% temperature 46.05 ºC, hydrolysis time 150.45 min.

Table 5. Optimization condition of total protein content, TPC and anthocyanin

Response	Condition1		Condition2	
	Predicted values	Observed values	Predicted value	Observed values
Total protein content (g/80gDW)[ns]	15.51	14.98±0.38	15.75	15.63±0.09
TPC (mgGAE/80gDW)	240.14[a]	226.84±2.00[b]	245	236.12±11.21
Anthocyanin (mgCyn-3Glu/80gDW)	3.56[a]	3.25±0.03[b]	3.70[a]	3.58±0.03[b]

Condition 1 is E/S 0.82%, temperature 43.7 ºC, hydrolysis time 140.38 min.
Condition 2 is E/S 1%, temperature 46.05 ºC, hydrolysis time 150.45 min.
[a-b] average amount and root mean square are significantly different ($p < 0.05$) in row.

Protein yield of RBBPH and Degree of hydrolysis

The protein content of RBBPH after freeze dried was determined by the Kjeldahl method [16] and crude protein was calculated using the 5.95 conversion factor [22] which was 19.18%. The protein yield was observed in RBBPH 52.36% calculated follow by Adler-Nissen [18]. For the protein hydrolysis with enzyme by using pH-stat assay found that, the degree of hydrolysis (DH) of RBBPH in 20 conditions were between 17.87-29.21%. The DH increased with increasing temperature of hydrolysis. The DH of optimal condition was 21.26%. The increase in DH indicated that peptide bonds were cleaved providing amino acids and small peptides during hydrolysis [14]. The results of this research correspond to Thamnarathip *et al*. [9]. The protein yield 53.6-75.1% was observed in hydrolysates using Alcalase.

Color properties

The color parameter of RBBPH were *L**= 18.86, *a**= 5.55, *b**=2.00. The RBBPH with purple to black had lower *L** with dark Maroon color. The pigment distributions found in the RBBPH were similar to those of riceberry bran. Chen *et al.* [26] evaluated the genotypic variation in red and purple bran of rice of diverse geographic origins for concentrations of oligomers and polymers of PAs, and their relationships to the total phenolic and flavonoid concentrations and antiradical capacity and identified purple rice bran had *L**, *a** and *b**value of 28.90, 11.65 and 12.45, respectively.

3.2 Chemical properties

Proximately analysis of RBBPH

RBBPH powder (E/S 0.82%, temperature 43.75ºC, hydrolysis time 140.38 min.) with moisture content at 4.41% contained protein, fat, ash, fiber and carbohydrate 19.18, 4.95, 6.59, 0.10 and 64.77% respectively. The protein content of RBBPH was higher than that of DRBB ash and carbohydrate content were also increased.

Total Proteins

The protein content of RBBPH hydrolysis at optimum condition (0.82%E/S, 43.75ºC and 140.38 min) was analyzed by Lowry *et al*. [21] method. The results showed that the protein content 21.62% (Table 6). After hydrolysis with enzyme, total protein content was increased comparing with before hydrolysis.

Anthocyanin and TPC

The RBBPH after freeze dried has a purple-brown color. The anthocyanin content of RBBPH was 46.51 mgCyn-3Glu/100g (Table 6).The same as Das *et al*. [5] studied the extracted anthocyanin from black and purple rice bran and found that anthocyanin content were 31.95 and 34.86 mg Cyn-3Glu/L, respectively.

The RBBPH was extracted by protease (Alcalase) enzyme hydrolysis. The TPC of RBBPH after freeze dried was 33.85 mgGAE/g (Table 6). Thamnarathip *et al*. [9] also extracted RBBPH with different enzymes (Alcalase, Flavourzyme and Neutrase) and hydrolysis time (2, 4 and 6 h) and found that RBBPH at time 0, 2, 4 and 6 1hr with Alcalase contained TPC 21.3, 22.1, 22.4, 22.2 mg GAE/g sample.

Table 6. Chemical properties of RBBPH after freeze dry

Chemical properties	Amount
Total protein content (%)	21.62
TPC (mgGAE/g)	33.85
Anthocyanin content (mg Cyn-3Glu /g)	0.47

Antioxidant properties

The RBBPH was evaluated for their antioxidant activities with DPPH and ABTS assay. The IC_{50} value of RBBPH (Figure 6) was compared with ascorbic acid, α tocopherol and BHT. The result showed that DPPH radical scavenging activity of RBBPH (IC_{50} of 0.0517 mg/ mL) can the oxidation better inhibitthan BHT (IC_{50} of 0.1299 mg/ mL) (Figure 6A). While ABTS assay had lower antioxidant properties than all standard (ascorbic acid, α to copherol and BHT) (Figure 6B). Wang *et al*. [27] found that rice bran protein hydrolysate had IC_{50} of 1.57 mg/mL.

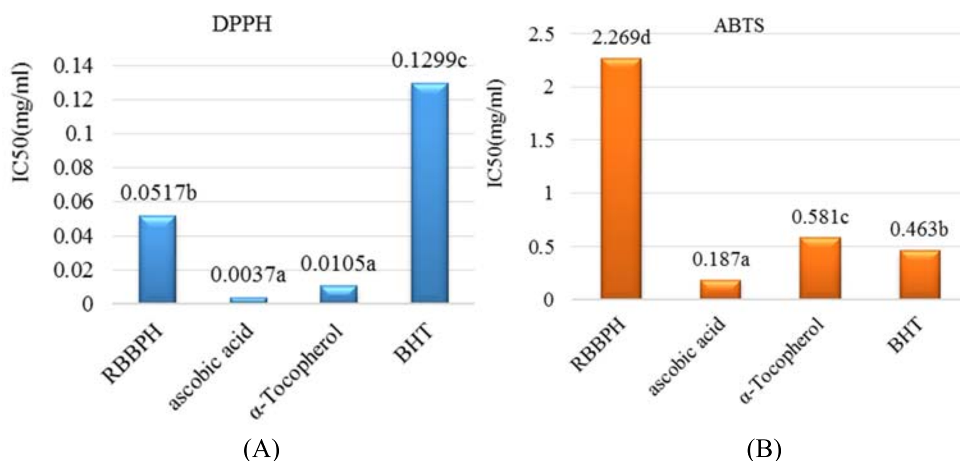

Figure 6. Antioxidant properties of RBBPH (1) DPPH assay (IC_{50}) and (2) ABTS assay (IC_{50})
[a-b]is amount average are significantly different (P < 0.05)

Alcalase hydrolysate an endo-peptidase has ability to produce various peptides, such as. antioxidant due to its endo-peptidase properties [28]. The evaluation of the antioxidant capacity of RBBPH was getting more importance, since it has been found that anthocyanin and TPC compounds were one of the effective antioxidants more than other rice bran.

Amino acid profiles

The total amino acid compositions of RBBPH wasshow in Table 7. The RBBPH contained high amount of the non essential amino acid, glutamic acid, aspartic acid, alanine and essential amino acid, arginine, leucine. glutamic acid was the most amount 18.04 mg/100 g. It was a glutamate receptor agonist enhances retention of memory and Glutamic acid is also useful in lowering blood pressure. Furthermore, glutamic acid is used as a conjugate because it increases the efficacy of anticancer drug and decreases its toxicity and nontoxic toward humans [29].

Table 7. The total amino acid analysis of RBBPH

Total amino	Amount (mg/100 g)
Aspartic acid	12.10
Serine	6.73
Glutamic acid	18.04
Glycine	7.35
Histidine	3.65
Arginine	11.84
Threonine	5.89
Alanine	8.71
Proline	5.74
Tyrosine	3.65
Valine	6.78
Lysine	6.78
Isoleucine	4.17
Leucine	9.12
Phenylalanine	5.47

Protein patterns by SDS page

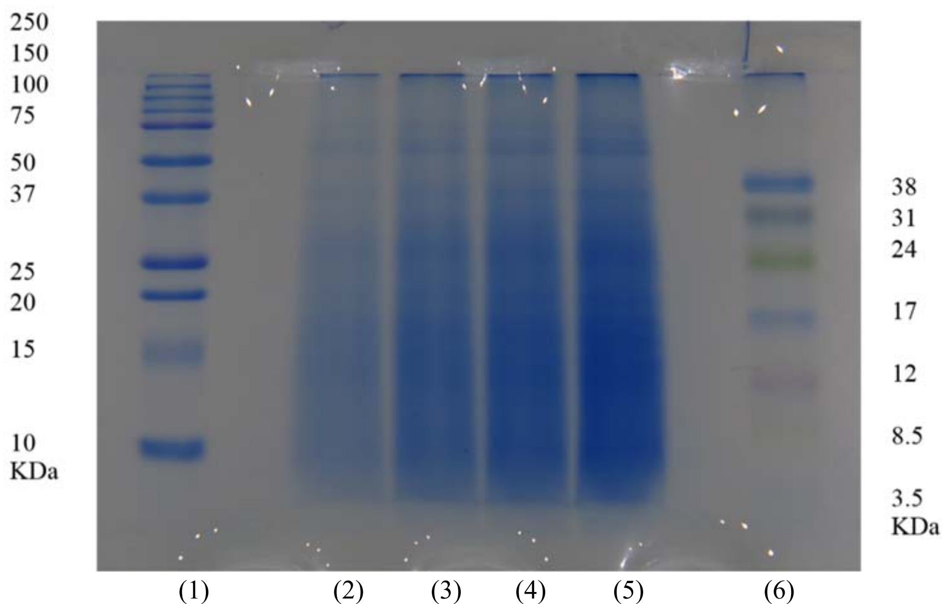

Figure 7. Molecular weight profile of RBBPH using SDS-PAGE (1) Standard protein marker1 (2) RBBPH at 20 µg (3) RBBPH at 30 µg (4) RBBPH at 40 µg (5) RBBPH at 50µg (6) Standard protein marker2

The protein patterns of RBBPH showed in Figure 7 found that the RBBPH with DH 21.26% still had protein bands ranged between 5 kDa to 50 kDa. It was clearly observed RBBPH at 30 µg seen the most obvious band. RBBPH show the four major protein band because hydrolysis protein with enzymatic for bond dissociation change to many amino acid or peptide shortened . The result is consistent with the study of Phongthai *et al*. [30], who suggested that the desirable properties of rice bran protein can be controlled with enzymatic modification. It was found that the hydrolysate between DH 5.04 to 15.04% have molecular weight 18.7, 29.9 and 52.5 kDa [30].

4. Conclusions

RBBPH was prepared by enzymatic extraction using response surface methodology (RSM). The RBBPH optimum extracting conditions was 0.82% enzyme/substrate at temperature of 43.75 ºC for 140.38 min. In optimum condition, total protein, TPC and anthocyanin contents of RBBPH were 14.98 g, 226.84 mg GAE and 3.25 mg Cyn-3-Glu/g, per 80 g riceberry bran, respectively, with the degree of hydrolysis (DH) of 21.26%. Chemical properties of RBBPH were as the following; total protein content of 21.62 %, TPC of 33.85 mg GAE/g and anthocyanin content of 0.47 mg Cyn-3Glu/g. The RBBPH possessed high antioxidant activities (DPPH; IC_{50} = 0.052 mg/mL and FRAP; IC_{50} = 2.27 mg/mL). RBBPH exhibited the strongest antioxidative properties, which includes DPPH radical scavenging and ABTS radical scavenging. The amino acid profile showed high amounted glutamic acid followed by aspartic acid, arginine, leucine and alanine, respectively. The molecular weight (sodium dodecyl sulfate polyacrylamide gel electrophoresis; SDS-page) ranged between 5 to

50 kDa. Protein hydrolysis with enzyme for bond dissociation obtained amino acid or shortened peptide.

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