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Production of rice bran protein hydrolysates from traditional Thai rice bran (Plai-Ngahm-Prachinburi)

[1*]Charoen, R., [1]Tipkanon, S. and [2]Savedboworn, W.

[1]*Department of Innovation and Product Development Technology, Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok (Prachinburi campus), Prachinburi, 25230, Thailand*
[2]*Department of Agro-Industry Technology and Management, Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok (Prachinburi campus), Prachinburi, 25230, Thailand*

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Abstract

Enzymatic processes for protein extraction of defatted rice bran from Plai-Ngahm-Prachinburi rice were optimized using response surface methodology (RSM). The extraction yield could be influenced by various factors including enzyme concentrations (0.025-0.075 gram / 5 gram of protein), extraction temperatures (40-60°C) and time (2-4 h). The obtained protein solution was further dried using a rotary evaporator and a freeze dryer, respectively. Results showed that the optimal conditions for extracting proteins were 0.075 gram enzyme concentration at 50°C for 3 h. Then, the hydrolyzed protein powder was analyzed for physicochemical and functional properties. The hydrolyzed time comparison between 2 h and 3 h was reported. There was no difference found in moisture content and solubility (pH 4) while, a_w, color and bulk density were significant. The hydrolyzed protein of 2 h affected the greatest enhancement of 2,2 – diphenyl-1-picrylhydrazyl (DPPH) and emulsifying activity index (EAI). For the degree of hydrolysis (DH), solubility (pH 5 and pH 6) and foaming capacity, 3 h was found to have higher capacity than hydrolyzed protein for 2 h. This study shows that hydrolyzed rice bran protein has potential as value-added ingredient food industry.

Introduction

Rice bran has been considered a good source, since it contains 10 to 16% protein (Juliano, 1985). In addition, rice bran proteins consisted of four main proteins such as albumins, globulins, prolamins and glutens (Saunders, 1990; Hamada, 1997). Rice bran contains several beneficial compounds, for example, essential amino acids, vitamins and minerals. Besides amino acids, rice bran also contains several vitamins and minerals such as Vitamin E, thiamine, niacin, aluminum, calcium, chlorine, iron, manganese (Saunders, 1990; Helm and Burks, 1996). It also contains health beneficial compounds, for example, ferulic acid, γ-oryzanol, β-Inositol, campesterol, β-sitosterol, and coumaric acid. (Sunder, 1990; Fabian and Ju, 2011; Patsanguan *et al.*, 2015). Therefore, rice bran and rice bran proteins could be potentially used in several food applications, for example, functional protein beverages, meat and sausage products and infant foods with low allergenic properties.

To recover rice bran proteins, several methods have been reported using a variety of extracting techniques including chemical (alkaline treatment), physical (ultrasonic and colloid mill treatments), and enzymatic (protease, xylanase and phytase) extraction processes. (Hamada, 2000; Anderson and Guraya, 2001; Tang *et al*., 2002; Gupta *et al*., 2008) Enzymatic extraction using some proteases can hydrolyze peptide bonds in protein molecules into small peptides and free amino acids. Several benefits of using a protease for protein extraction have been reported such as having high substrate specificity, low level of usage and performing under mild conditions. It was well known that controlling the degree of hydrolysis during enzymatic treatments is one of the important parameters to extract proteins. Depending on the size of the protein and peptide molecules, obtained protein extracts may have a variety of protein functionalities. It was reported that enzymatic hydrolysis could improve solubility, emulsion and foam properties of proteins and increase digestion and absorption of proteins. Moreover, hydrolyzed proteins and peptides exhibited higher antioxidant activities in comparison with non-hydrolyzed protein sources and their antioxidant activities were dependent on the size and amino acid arrangement of peptides (Ansharullah *et al*., 1997; Wang *et al.*, 1999;

*Corresponding author.
Email: *ch_ratchanee@yahoo.com*
Tel: +(66) 3721-7300 to 9 ext. 7929; Fax: +(66) 3721-7312

Hamada, 1999, 2000; Tang *et al.*, 2003).

Plai-Ngahm-Prachinburi is one of the recommended traditional rice cultivars obtained from Prachinburi, Thailand. It has been usually used as a source of proteins for animal food. To increase the value of rice bran and extend its application, this research aimed to study an enzymatic process using a protease. The obtained hydrolyzed rice bran protein extracts were also investigated on some physico-chemical and functional properties of hydrolyzed rice bran protein extracts for potential use in food industries.

Materials and Methods

Raw material and chemicals

Full-fat rice bran (Plai-Ngahm-Prachinburi cultivar) was obtained from Suan Dusit Rajabhat, Rice mill factory, Co Ltd. (Prachinburi, Thailand) and passed through a sieve (60 mesh). The defatted rice bran was prepared by solvent extraction in a soxhlet apparatus using hexane. After completed extraction and removal of hexane, the defatted rice bran was stored in a vacuum low density polyethylene (LDPE) plastic bag under -18°C for further experiment. Chemical composition of this defatted rice bran was 11% moisture, 16% protein, 5% ash, 8% fiber, 11% fat and 49% carbohydrate, respectively. Chemicals and reagents of analytical grade were obtained from Merck (Darmstadt, Germany) and Univar (USA Inc., USA). The Hexane for oil removal was a commercial grade. Flavourzyme® 500 MG and alcalase 2.4 L was donated by novozymes (Novozymes A/S, Bagsvaerd, Denmark).

Rice bran protein extraction and the recovery

The method of preparing protein extraction from rice bran was described by Hamada (2000). Rice bran suspension (5.0 g proteins in 250 ml water), heated to 50°C, the pH adjusted to 8 then 0.025 g of alcalase 2.4 L and flavourzyme were added for proteolysis. The protease was inactivated by heating at 85°C for 10 min. After proteolysis, the suspensions of rice bran were centrifuged under 20°C at 5,000 rpm for 15 min by refrigerated centrifuge (Rotana A35R, Hettich, Germany). Solubilized protein was recovered after three runs of centrifugation. Protein in the bran was washed with 100 ml water during the second and third centrifugation. Combined supernatant solutions were evaporated under 70°C, 120 mbar using a rotary evaporator (R-205, Buchi, Switzerland) for 45 min and followed by freeze drying under -50°C and 0.11 mbar for 10-12 h. using freeze dryer (Alpha 1-4 LSC, Christ, Germany). The obtained rice bran protein was packed in laminated aluminium bags and stored under 4°C prior to use for analysis.

Effect of temperature, time and enzyme concentrations for rice bran protein hydrolysates

The method of protein extraction is described above. The extraction conditions consisted of enzyme concentrations (0.025-0.075 gram / 5 gram of protein), times (2-4 h) and temperatures (40-60°C). The enzyme was added to rice bran suspension, then the mixture was stirred for 30 min and the pH was controlled during that extraction. The solution was then heated to inactivation the enzyme followed by water evaporated and freeze dried, respectively. The optimization of enzyme-assisted extraction for rice bran proteins using response surface methodology (RSM) was expressed.

Physical properties of rice bran protein extracted

Rice bran protein was measured for color using Hunter LAB (Color Flex 45/0, Color global, USA). Color parameters were L^* a^* and b^* value.

Bulk density: 5 grams of rice bran protein were added into graduated measuring cylinders. The cylinders were gently tapped and the volumes occupied by the samples determined. The bulk densities were calculated as weight per unit volume (g/ml).

Moisture content and a_w: the moisture content of rice bran protein was determined according to the method of AOAC (2000), a_w was measured by Aqua lab (CX3TE, England) under 25°C.

Rice bran protein yield (%) was determined as the equation below:

$$\text{Yield (\%)} = \frac{\text{weight of rice bran protein powder (g)}}{\text{weight of defatted rice bran (g)}} \times 100$$

Chemical and function properties of rice bran protein extracted

Solubility of protein: 0.5 grams of extracted rice bran protein was dispersed in 50 ml of distilled de-ionized water, then shaken for 30 min at room temperature and centrifuged at 5000xg for 15 min. The nitrogen content of the supernatant was determined by the Kjeldahl method (AOAC, 2000) and solubility (%) was calculated.

Degree of hydrolysis: Reactions were observed by measuring the amount of α-amino acid using a modified method described of Adler-Nissen (1979) and Benjakul and Morrissey (1997). Rice bran protein hydrolysates 100 µl was transferred in screw-cap test tube. 2 ml of phosphate buffer 0.2125 M pH 8.2 and 1 ml of 0.02% of 2,4,6-trinitrobenzenesulfonic acid

(TNBS) were added into the protein hydrolysates. The mixture was vortexed for 5 second then heated at 50°C for 30 minutes under dark condition. 2 ml of 0.1 M sodium sulfite was add into the mixture and vortexed for 5 second to stop a reaction and kept in dark condition for 15 minutes. Absorbance of the mixture was read at 420 mm using a UV-visible spectrometer (Helios Alpha, Themo Electron Corporation, Waltham, MA), and α-amino acid was expressed in terms of L-leucine. The DH(%) defined as follows:

DH (%) = (Number of peptide bonds cleaved/ Total number of peptide bonds in sample) x 100

Where DH is the percent ratio between the number of peptide bonds cleaved after hydrolysis and the total number of peptide bonds in the sample before hydrolysis.

DPPH: The DPPH radical-scavenging activity of rice bran protein extracted was determined described by Brand-Williams *et al.* (1999). An aliquot of 0.5 ml of sample solution in methanol (1:10) was mixed with 2.5 ml of a 0.5 mM methanolic solution of DPPH. The mixture was shaken and incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm.

Foaming: Foaming capacity was determined using the method described by Bandyopadhyay *et al.* (2012). The 20 ml of 2.0% (w/v) of rice bran protein was homogenized in a mechanical homogenizer (Ultra turrax® T25 basic, IKA® WERKE, Germany) at 13,500 rpm for 3 min. The foaming was calculated by using the volume of foam after whipping compared to the volume of that before whipping.

Emulsifying properties: Emulsifying properties of protein samples were determined as described by Pearce and Kinsella (1978). Ten grams of soybean oil and 30 g of protein solutions were mixed. The mixtures were homogenized using homogenizer (Ultra turrax® T25 basic, IKA® WERKE, Germany) at a speed of 13,500 rpm for 1 min. After that, 50 µl aliquot of the emulsion was collected from the bottom of the beaker at 0 and 10 min and mixed with 5 ml of 0.1% SDS solution using a vortex mixer. The absorbance was measured at 500 nm using a spectrophotometer (SP300, Optima, Japan). The absorbance at 0 (A0) and 10 (A10) after emulsion was used to calculate the emulsifying activity index (EAI) and emulsion stability index (ESI) as follows;

EAI (m^2/ g protein) = (2 x 2.203 x A_0 x dilution factor)/(L x φ x C x 10,000)

Where as; L = the patch length of cuvette (cm), φ = oil volume fraction of emulsion, C = weight of protein/unit volume (g/ml) of aqueous phase before emulsion formation

ESI (min) = (A_0 x 10)/(A_0-A_{10})

Statistical analysis

This research was conducted using 3^3 factorial in box-behnken design. The study of the interaction of protein hydrolysis variables and the optimization extraction conditions for rice bran protein extracted by using response surface methodology (RSM) (Myers 1971; Wangdee, 2012) were investigated. There were three variables; enzyme concentrations (0.025-0.075 gram / 5 gram of protein), time (2-4 h) and temperature (40-60°C). The variables codes were -1 (low level), 0 (middle level) and 1 (high level) and the correspondences between the codes are presented in Table 1. Rice bran protein extracted in powder form (Yield%) was taken as the dependent variable (Y) of the experiment. The mathematical model used was the following equation (1).

$$Yi = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \quad (1)$$

Where Yi represents the percentage of yield; X_1 represents enzyme concentrations, X_2 represents temperature and X_3 represents time; β_0, β_i represents regression coefficients and β_{ii} represents regression coefficients for quadratic effects. Minitab statistical software (Version 17.0, Minitab Inc., State College, PA, USA) was used in the design of the experiment and to fit the equation of the experimental data. The optimal extraction conditions were obtained by a functional approach using validation at different points. The STATISTICA (Statsoft, Inc. Tulsa, OK) was used for response surface graphs.

All qualities were performed in triplicate and presented as mean±standard deviation. The differences between means were measured by independent t-test by using SPSS statistic program version 18.0. The probability value of less than 0.05 was considered significant.

Results and Discussion

Optimization of enzymatic process for rice bran protein extraction

Optimization of enzyme-assisted extraction for rice bran proteins were performed using response surface methodology (RSM). Flavourzyme, a

Table 1. Coded levels and actual protein extraction yields for testing in response surface methodology (RSM)

Treatment	Code levels			% Yield
	Enzyme	Temperature	Time	
1	0.025	40	3	21.3±1.5
2	0.075	40	3	32.6±2.3
3	0.025	60	3	25.3±2.0
4	0.075	60	3	31.8±2.3
5	0.025	50	2	26.2±1.7
6	0.075	50	2	29.4±2.4
7	0.025	50	4	27.7±1.8
8	0.075	50	4	29.5±2.2
9	0.05	40	2	26.0±1.6
10	0.05	60	2	25.3±1.4
11	0.05	40	4	28.2±2.4
12	0.05	60	4	27.4±2.7
13	0.05	50	3	28.0±2.3
14	0.05	50	3	27.6±1.8
15	0.05	50	3	29.2±1.9

commercial protease enzyme was used and its processing conditions were optimized including enzyme concentration (0.025-0.075 gram / 5 gram of protein), extracting temperature (40-60°C) and time (2-4 h).

As indicated in Table 1, results suggested that enzyme concentration, temperature and time were a strong influence on extraction yields. Increasing enzyme concentrations and extraction times resulted in a higher degree of hydrolysis (%DH) and extraction yield. The obtained results were consistent with the results reported by Sari *et al.* (2012) that the concentration of primary amine in the extract solution increased in relationship to the concentration of enzyme used. Therefore, extraction yields were increased, since protein solubility was increased in the solution due to formation of small peptides.

Table 1 showed the treatments with the coded levels and their experimental results of yield (%), the rice bran protein extracted in powder from the bran. The full quadratics model was established using mathematical equation in Eq.1 and expressed in equation (2)

$$\text{Yield (\%)} = -23.1+319X_1+1.26X_2+4.84X_3-4.80X_1X_2-14.0X_1X_3-0.003X_2X_3-767.0X_1^2-0.01X_2^2-0.55X_3^2 \quad (2)$$

The coefficients of determination (R^2) for protein yield was 0.761 which indicated that the model explains approximately 76.19% for total variance.

Table 2. Physicochemical properties of the obtained rice bran protein extracted

Physicochemical properties	Rice bran protein extracted for 2 h	Rice bran protein extracted for 3 h
Moisture content (%)^{ns}	11.16±0.83	11.58±0.56
Water activity	0.27±0.00^b	0.29±0.01^a
Protein content (%)^{ns}	37.05±3.03	35.87±1.43
Color		
L* (lightness)	81.71±1.02^b	83.06±0.58^a
a* (redness)	0.95±0.64^a	0.72±0.28^b
b* (yellowness)	13.67±0.73^a	11.81±0.42^b
Bulk density(g/ml)	0.43±0.03^b	0.45±0.01^a
Degree of hydrolysis (%)	17.89±0.84^b	20.05±0.53^a
Solubility (%)		
pH 4^{ns}	27.28±1.34	27.60±0.56
pH 5	30.19±2.36^b	41.00±2.16^a
pH 6	38.57±0.84^b	43.00±1.43^a

Means (±SD) with different superscript letters in the same row (a-b) indicate significant differences ($P < 0.05$). The superscript "ns" indicates no significant differences among the means in the same row.

Moreover, results showed the protein yield in relationship to the response surface graph at various concentrations of the enzyme temperature and time (Figure 1), and various concentrations of the enzyme and extraction time at a fixed temperature variable at 50°C (Figure 2). The validation of the reduced equation when considering only one temperature (50°C) suggested that the optimal conditions for rice bran protein extraction were 0.075 gram of enzyme concentration per 5 gram of protein, 50°C of extraction temperature, and 3 h of extraction time.

Physicochemical properties of the rice bran protein extracted

The optimized condition using 0.075 gram of enzyme concentration at 50°C for 3 h was used to prepare the rice bran protein extract for further studies on physicochemical and functional properties in comparison with the rice bran protein extracted for 2 h. As showed in Table 2, the rice bran protein extracted was investigated for some physicochemical properties such as moisture content, water activity, color, bulk density, degree of hydrolysis and solubility. Overall, characteristics of rice bran protein extracted were light weight and a slightly yellowish powder as indicated by bulk density (g/ml) 0.43±0.03

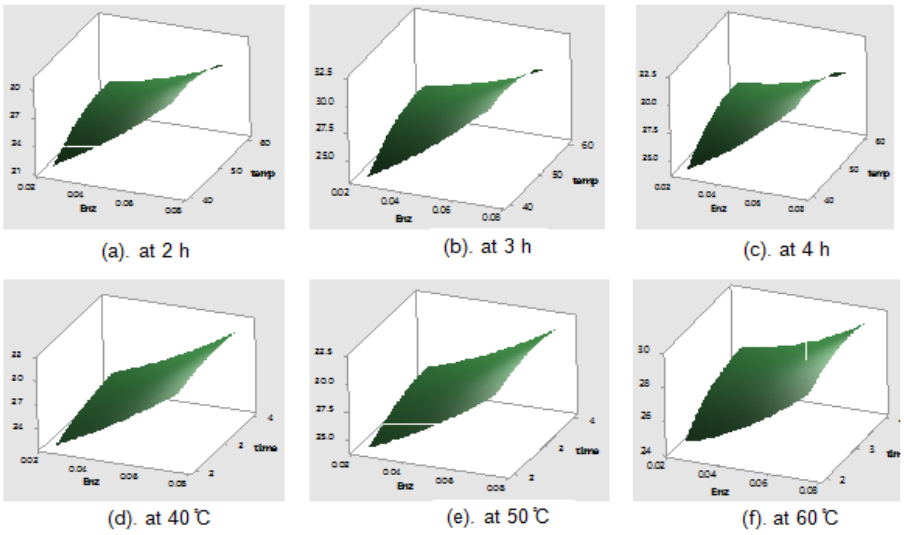

Figure 1. 3D response surface curve of enzyme concentrations temperatures and time on yields (%) a-c) under different extraction times d-f) under different extraction temperatures.

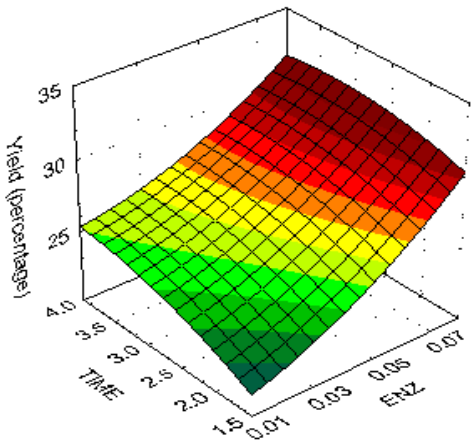

Figure 2. 3D response surface curve of enzyme concentrations and time on yields (%) under 50°C

and 0.45±0.01 for 2 and 3 h, respectively. Lightness and yellowness were ranges between 81.71-83.06 and 11.81-13.67. Water activity and moisture of the protein powder were ranged 0.27-0.29 and 11.16-11.58%, respectively. The lowest in a_w and MC was found in protein extracted for 2 h, while highest lightness was found in protein extracted for 3 h.

In terms of solubility, hydrolyzed rice bran protein extracted for 3 h had the highest protein solubility (43.00±1.43) at pH 6. However, when the pH values of the solution were close to its isoelectric point pH 4.5 which reported by Chandi and Sogi (2007), the protein solubility of rice bran protein extracted was decreased to 27.60±0.56. Proteins are polymers of amino acids covalently linked via peptide bonds. All amino acids sharing a similar basic structure contain carboxylic (-COOH) and amine ($-NH_2$) groups. Therefore, the solubility of amino acids, peptides and proteins is strongly dependent upon their net charge due to the zwitter ion form of amino acids. Additionally, the influence of net charges, the solubility of proteins and peptides could be impacted by not only the length of peptide chains, but also solubility of each amino acid (Mao and Hua, 2012; He *et al.*, 2013). In general, short chain peptides have higher solubility than long chain peptides. Hydrolysis of proteins using an enzyme could increase extraction yield due to increasing protein solubility in water (Chobert *et al.*, 1988; Gbogouri *et al.*, 2004). According to He *et al.* (2013), rapeseed protein extracts contain hydrophobic amino acids such as alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionineand cysteine, which are an influence on their water solubility. In addition, Chandi and Sogi reported that rice bran protein from Basmati 370 had solubility index (%) 47.69% and 72.67% for Basmati 386.

Antioxidant activity

As illustrated in Table 3, antioxidant activity of the rice bran protein was evaluated using DPPH radical scavenging activity. Hydrolyzed rice bran protein obtained from the treatment with 0.06% enzyme concentration for 2 h time extracted had the highest antioxidant activity due to exhibiting the DPPH 4.04% when compared with protein extraction for 3 h (3.90%). Patsanguan *et al.* (2014) reported that DPPH of rice bran extraction by microwave assisted with homogenization (800W for 4 sec, 14,000 rpm for 10 min) was 8.83%. It was noted that formation of short chain peptides may contribute to increasing antioxidant activity of the sample. (He *et al.*, 2013).

Table 3. Functional properties of the rice bran protein extracted.

Functional properties	Rice bran protein extracted for 2 h	Rice bran protein extracted for 3 h
DPPH scavenging activity (%)	4.04±1.03[a]	3.90±0.05[b]
Emulsifying properties		
EAI (m^2/g protein)	7.47±0.36[a]	5.50±0.31[b]
ESI (min)	16.39±0.14[b]	53.27±5.59[a]
Foam capacity (%)		
0 min	100	100
5 min	36.88±5.36[b]	47.33±2.65[a]
10 min	26.39±4.22[b]	28.46±1.84[a]
15 min	22.29±2.85[b]	24.04±0.59[a]
30 min[ns]	16.64±4.42	17.57±0.41

Means (±SD) with different superscript letters in the same row (a-b) indicate significant differences ($P < 0.05$). The superscript "ns" indicates no significant differences among the means in the same row.

Not only does the higher antioxidant activity of short chain peptides contributes to the ability to donate electrons to radicals, but it contributes to the ability to chelate metal ions. It was well-known that metal ions can act as a pro-oxidant that accelerates lipid oxidation, which can be inhibited using metal chelators (Gordon, 2001).

Emulsifying properties

One of the important functional properties evaluated the ability to form oil-in-water emulsions. As shown in Table 3, emulsifying properties of the rice bran protein were expressed in terms of the emulsifying activity index (EAI) and the emulsion stability index (ESI). Results showed that the rice bran protein obtained from the treatment with 0.06% for 3 h exhibited the highest ESI at 53.27 min in comparison with the rice bran obtained from the treatment with 0.06% for 2 h exhibiting 16.39 min. In this study, the results were consistent with previous reports that protease-assisted protein extraction could improve emulsifying properties (Hamada, 2000).

It was postulated that medium- and short-chain peptides and/or proteins after treated with a hydrolytic enzyme may have changed in their structures, resulting in a more surface active property due to altering the balance between hydrophobic and hydrophilic parts (Kaewumporn, 2006). Moreover, it was suggested that peptides and protein with high emulsifying properties could be due to their ability to arrange their structures on the oil/water interfacial region resulting in decreases in surface tensions between oil and water phases (Kinsella, 1976; McClement, 1999). Oil-in-water emulsions emulsifying with proteins had high emulsion stability and low flocculation among emulsion droplets (Zayas, 1997). There were several reports suggesting that emulsifying properties of proteins may be influenced by degree of hydrolysis, protein conformation, molecular weight or size, and net charge of proteins (Chobert *et al*., 1988; Singthong *et al*., 2008).

Foaming property

The foam capacity of the rice bran protein was investigated. As shown in Table 3, results indicated that the rice bran protein treated with 0.075 gram enzyme concentration for 3 h exhibited foam capacity of 47.33% at 5 min while protein extracted under 2 h exhibited 36.88%. Our result was in agreement with the result of previous reports by Chabanon *et al*., (2007) that foam capacity of rapeseed proteins treated with alcalase at 1 min was 44%. However, foam capacity of the rice bran protein decreased over time. Results showed that foam of the rice bran protein at 30 min was 16.64 and 17.57% for 2 and 3 h, respectively.

It was suggested that stability of air/liquid foam structures was strongly influenced by several factors such as ability to form thick layers between air and liquid interfaces, ability to reduce surface tension, and the viscosity of the liquid solution at the air/liquid interfaces (Phillips and Beuchat, 1981; Damodaran, 1996). Phillips and Beuchat (1981) reported that in terms of characteristics of proteins that stabilize foam structures, the net charges of the proteins were too high which may reduce foam capacity due to the prevention of protein-protein interactions.

Moreover, Kinsella (1976) suggested that foaming activity and stability of proteins were strongly influenced by degree of protein denaturation, molecular weight, amino acid sequence and polarity of proteins. Usually, proteins with high foaming activity have low foaming stability. Generally, soluble proteins could associate at the air/liquid interface, and rearrange themselves to form a stable foam structure. However, in the case of peptides, small peptides have a higher ability to associate with the air/liquid interface. The degree of hydrolysis of proteins was also important to foaming property. It was reported that peptides obtained from fish protein hydrolysates had high foaming activity; however, short chain peptides also exhibited low foaming stability due to the lack of a rigid foam structure (Thiansilakul *et al*.,

2007; Singthong *et al.*, 2008).

Conclusion

The use of the enzymatic method under optimal extraction condition 0.025 g of alcalase 2.4 L followed by flavourzyme (0.075 gram protease concentration at 50°C for 3 h) gave the feasible region under the surface plot (RSM). Extraction conditions effected the physicochemical and functional properties of hydrolyzed protein that occurred from protein fractions and differed among rice varieties. Further studies of protein hydrolysis investigated the stability of rice bran protein in food systems under a wide range of environmental stress during processing in the food industry.

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