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Research Article

Optimization of rice bran protein hydrolysate production using alcalase

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Abstract

The aim of this research was to study the optimum condition for rice bran protein hydrolysate (RBPH) production using a microbial protease, Alcalase. RBPH was prepared from Kao Dok Mali 105 defatted rice bran. The experimentation was conducted using central composite design (CCD) and Response Surface Methodology (RSM) was used to determine the optimum conditions to obtain maximum total RBPH. The three independent variables including temperature (T: 30 – 70°C), enzyme to substrate ratio (E/S: 0 – 2%) and hydrolysis time (t: 30 – 360 minutes) were studied, while the ratio of rice bran to water and pH were fixed at constant rate of 3% and 8.0, respectively. The results showed that temperature and E/S had significant effects ($p \leq 0.05$) on the total rice bran protein but hydrolysis time showed no significant effects. The coefficient of determination (R^2) of total protein and %DH (degree of hydrolysis) were 0.80 and 0.967, respectively. The optimum condition was using a temperature of 63°C and E/S at 1% and 340 minutes incubation time. The RBPH obtained from this condition had 1876.2 mg of total protein and 14.5 %DH.

Keywords: rice bran, protein hydrolysate, alcalase, response surface methodology, RBPH, Thailand

Introduction

Thailand is the world's largest exporter of rice, sending over 8.5 million metric tons of milled rice around the globe in 2008/2009, while also ranking first in global production [1]. Rice bran is the pericarp and germ of *Oryza sativa* seeds and constitutes about 10% of rough rice grain [2]. Rice bran is an inexpensive, underutilized milling co-product of rough rice. Rice bran has a high nutritional value with 12−15% protein content [3]. Rice bran protein is higher in lysine content than rice endosperm protein or any other cereal bran proteins [4]. The protein efficiency ratio (PER) has been widely used as an indicator of protein nutritional quality. The PER values for rice bran concentrates range from 2.0 to 2.5, compared to 2.5 for casein. Protein digestibility of rice bran is greater than 90%. Rice bran is considered a good source of hypoallergenic proteins, and as such, rice bran protein may serve as a suitable ingredient for infant food formulations [5], thus adding variety to the restricted diets of children with food allergies.

However, large portions of rice bran protein cannot be solubilized by ordinary solvents such as salts, alcohol and acids due to extensive disulfide bonding and aggregation. The most common method of isolating rice bran proteins is alkali extraction. Although this treatment with high temperatures and concentration of alkaline solutions solubilizes most of the rice bran proteins, it also leads to the occurrence of denaturation and hydrolysis of proteins, increased Maillard reaction (which causes dark-coloured products) and increased extraction of nonprotein components which co-precipitate with protein and lower the isolate quality [6].

Protease enzyme has been used to enhance solubilization of rice bran protein and obtain a wide range of protein hydrolysates [7]. There are two classes of proteases, exoproteases and endoproteases. Commercial proteases are mainly endoproteases. They attack peptide bonds in the interior of the polypeptide chain producing a range of polypeptides, which differ in molecular weight, depending on the extent of hydrolysis [8]. Wang *et al*. [6] reported a procedure for protein isolate using alkaline protease. The protein extractability increased as they increased the degree of hydrolysis (DH). A higher DH is required to indicate the protein. However, for optimization of the functional and nutritional properties of proteins a lower DH is desired. Hamada *et al.* [7] used alcalase to hydrolyze rice bran protein and found that the enzymatic treatment facilitated the total protein extraction of 83% from rice bran recovered at 7.6% of DH. Hamada [9] reported extraction yields of rice bran protein increased with the use of protease as the DH increased, reaching 92% at a 10% DH value. Hamada [8] used alcalase to produce rice bran protein hydrolysates and found that the hydrolysates consisted of 27.6% protein and 7.5% DH.

Response surface methodology (RSM) is the one of most effective tools for optimizing the process when many factors and interactions affect the desired response [10]. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions. It usually uses an experimental design such as a central composite design (CCD) to fit a first- or second- order polynomial by least significant technique. The contour plots can be usefully employed to study the response surfaces and locate the optimum. Kokkeaw and Thawornchinsombut [11] optimized the conditions for rice bran (Jasmine 105) protein hydrolysates by using Protex 6L and determined radical scavenging activity (RSA) and yield using RSM. The objective of this study was to apply RSM for optimizing the production of rice bran protein hydrolysates by using a microbial protease, alcalase.

Materials and Methods

Jasmine rice bran was obtained from the Royal Chitralada Villa. A commercial protease enzyme; Alcalase® (Novo Nordisk, Denmark) was used in this study.

Sample preparation

Rice bran was defatted twice using hexane in a 1:3 rice bran-to-solvent ratio with continuous stirring at 300 rpm for 30 min and filtered with filter paper no. 4. The defatted rice bran was air-dried overnight in a hood (adapted from Gnanasambandum and Hettiachchy [12]).

Enzymatic Hydrolysis

RBPH was prepared according to the method described by Yuan *et al.* [13] with modification as shown in Fig 1.

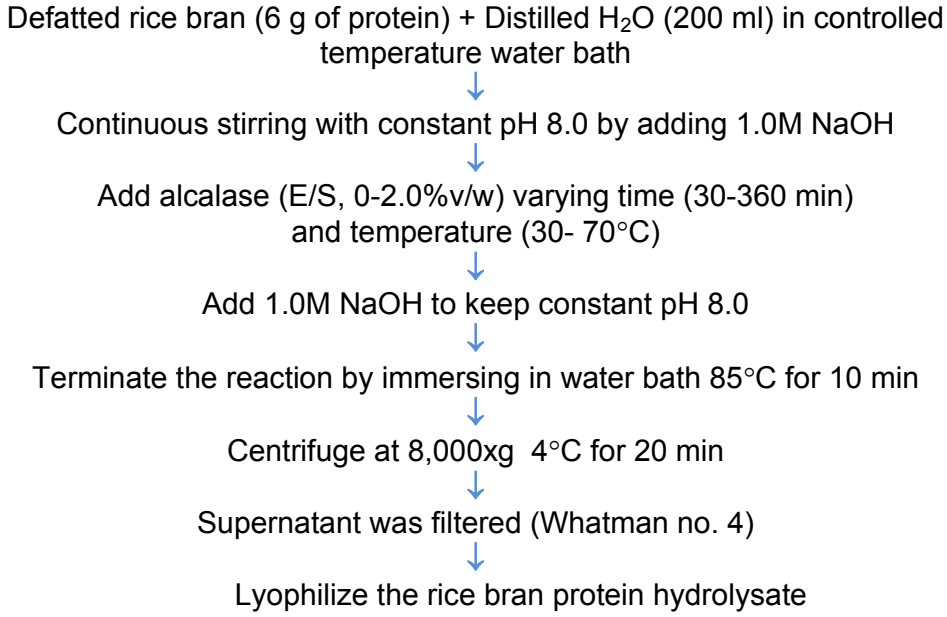

Figure 1. The process of rice bran protein hydrolysate preparation using Alcalase.

Experimental Design

A central composite design (CCD) with three factors and five levels was applied, as shown in Table 1. Five levels were adopted and coded -1.68, -1, 0, +1 and +1.68. The two dependent Y variables evaluate the total protein and %DH.

The regression model between dependent variables (Y) and independent variables (x) was according to the equation (1):

$$Y_1 = \beta_0 + \sum_{i=1}^{3} \beta_i \chi_i + \sum_{i=1}^{3} \beta_{ii} \chi_i^2 + \sum_{i<j=2}^{3} \beta_{ij} \chi_i \chi_j \quad (1)$$

Where Y is the measured response variable, β_0, β_i, β_{ii} and β_{ij} are the constant, linear, quadratic and interaction regression coefficients of the model, respectively. χ_i and χ_j represent the independent variables.

Table 1. Factors and levels of central composite design (CCD) in rice bran protein hydrolysis.

Factor	Levels				
	-1.68	-1	0	+1	+1.68
Temp.(^oC): X_1	30	38	50	62	70
E/S (%) (v/w): X_2	0	0.4	1	1.6	2
Hydrolysis time (mins): X_3	30	97	195	293	360

Statistical analysis

All data were expressed as means for all experiments and a two-tailed student's t-test was used to calculate the significant difference between treatments. A p-value less than 0.05 was considered statistically significant.

Dependent variables determination

The degree of hydrolysis (%DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of bonds per unit weight (h_{tot}), in each case, was calculated from the amount of base consumed [14]:

$$DH\% = \frac{h \times 100}{h_{tot}} = \frac{B \times N_b \times 100}{\alpha \times M_p \times h_{tot}} \quad (2)$$

B is base consumption in ml;
N_b is normality of the base;
α is average degree of dissociation of the α -NH_2 groups;
M_p is mass of protein ($N \times 5.95$) in g;
h is the hydrolysis equivalents in meqv/g protein
h_{tot} is total number of peptide bonds in the protein substrate (8.4 meq/g rice bran protein)

The total protein was analyzed by Lowry method. [15]

Results and Discussion

Statistical analysis

The experimental value and analysis of variance for two response variables; total protein and %DH under different treatment conditions are presented in Table 2. It shows that the response surface model developed for all response variables was adequate. The determination coefficient (R^2) values for all response variables were higher than 0.80, indicating that the regression model explained the reaction well.

Table 2. **Actual levels of independent variables along with the tested values for the response variable.**

Treatment	Factors			Total Protein (mg)	DH (%)
	T (min)	E/S (%)	t (^{o}C)		
1	38	0.4	97	1314.11 f	4.18 l
2	38	0.4	293	1382.20 f	8.12 g
3	38	1.6	97	2171.88 b	5.17 j
4	38	1.6	293	1579.63 e	8.12 g
5	62	0.4	97	2202.15 b	5.85 i
6	62	0.4	293	1882.62 c	11.70 c
7	62	1.6	97	1964.48 c	7.76 h
8	62	1.6	293	1858.74 c	14.04 a
9	30	1.0	195	975.62 g	4.86 k
10	70	1.0	195	1566.86 e	8.46 f
11	50	0	195	1733.29 d	8.30 fg
12	50	2	195	1612.27 e	11.66 c
13	50	1.0	30	1944.51 c	1.79 m
14	50	1.0	360	2369.20 a	12.56 b
15	50	1.0	195	2124.98 b	9.42 e
16	50	1.0	195	2150.44 b	9.53 de
17	50	1.0	195	2144.51 b	9.64 d

a-k Values in the same column with different letters are significantly different ($p \leq 0.05$)

The results of the analysis of variance (ANOVA) for %DH, shown in Table 3, demonstrate that the statistical model is significant at a 95% confidence level ($p \leq 0.05$).

The applications of RSM yield on total protein and on %DH are calculated from the following model equation (3) and model equation (4), respectively:

$$Y_1 = -529.478 + 24.146x_1 + 204.107x_2 - 2.286x_1x_2 - 0.204x_1^2 - 41.265x_2^2 \quad (R^2 = 0.800) \quad (3)$$

$$Y_2 = -17.471 + 0.702x_1 + 0.034x_3 + 0.028x_1x_2 + 0.001x_1x_3 - 0.007x_1^2 - 8.661E\text{-}05x_3^2 \quad (R^2 = 0.967) \quad (4)$$

Where x_1 is temperature, x_2 is E/S ratio and x_3 is hydrolysis time.

The determination coefficient (R^2) of the total protein is 0.800. This implies that the regression models explained the reaction well. As the test of lack of fit hypothesis was not significant ($p > 0.05$) in model equation therefore the model was fitted to the total protein data. A good fit means that the generated models adequately explained the data variation and significantly represented the actual relationships between the reaction parameters.

Table 3. **ANOVA of equation model on lack of fit of the total protein.**

Source of variance	df	SS	MS	F
Residual	11	4515.220		
Pure error	8	3252.459	406.557	
Lack of fit	3	1262.761	420.920	1.035^{ns}

ns means not significant ($p>0.05$)
*means significant ($p \leq 0.05$)

Effects of parameters

The effect of different enzyme treatment conditions on total protein and %DH are reported by the coefficient of secondary order polynomials. To aid visualization, response surface plots and the contour plot of the total protein, are shown in Figures 2 and 3. Figure 3 shows the contour plot for the independent variables on the total protein. As shown in Table 4, total protein was positively related to the linear effect of temperature ($p \leq 0.001$) and E/S ratio ($p \leq 0.01$) and negative quadratic effect of temperature ($p \leq 0.001$) and E/S ratio ($p \leq 0.05$). It can be seen from Table 4 that there is negative effect of an interaction between temperature and E/S ration on the total protein ($p \leq 0.05$). At the lowest temperature, the total protein of RBPH was found to increase rapidly at the beginning but then slowly decreased toward the end with the increase in enzyme concentration (E/S ratio) (Fig. 3). It can also be seen from Table 4, that the positive linear effect and negative quadratic effect of temperature and E/S ratio explain the observed nature of the curve shown in Figure 3. The temperature increases the rate of enzymatic reactions; hence the rate of total protein increases as long as the temperature is below the denaturation temperature of the enzyme.

Table 4. **The regression coefficients, R^2 and *p* of dependent variables; total protein and %DH for RBPH.**

Regression coefficient	Total protein (mg)	DH (%)
b_o	-5294.653***	-17.471**
b_1	241.449***	0.702***
b_2	2041.147**	
b_3		0.034*
b_{12}	-22.860*	0.028**
b_{13}		0.001**
b_{11}	-2.036***	-0.007***
b_{22}	-412.677*	
b_{33}		-0.00008661**
R^2	0.800	0.967
p or probability	0.001	0.000

Subscripts: 1= temperature, 2 = E/S ratio, 3 = hydrolysis time
* Significant at 0.05 level, ** Significant at 0.01 level, *** Significant at 0.001 level

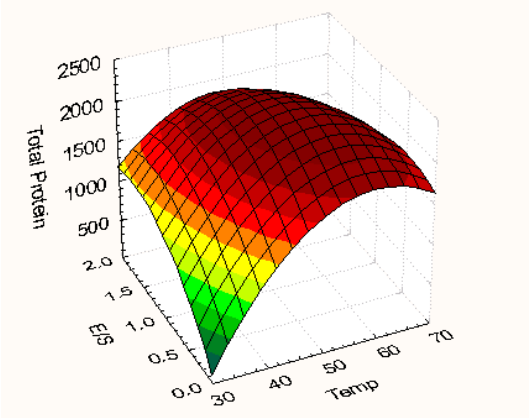

Figure 2. Surface plot to show the combined effect of Temperature and E/S on the total protein of RBPH by alcalase.

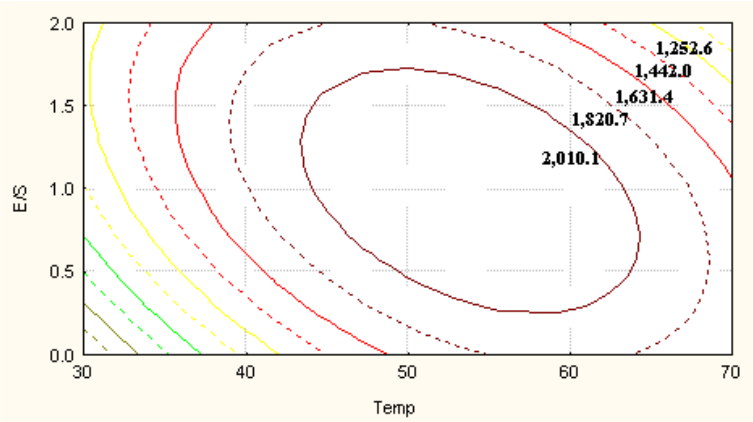

Figure 3. Contour plot showing the combined effect of Temperature and E/S on the total protein of RBPH by alcalase.

As shown in Table 4, DH was positively related to the linear effect of temperature ($p \leq 0.001$) and time ($p \leq 0.05$) and negatively related to the quadratic effect of temperature ($p \leq 0.001$) and time ($p \leq 0.01$). It can be seen that there is an interaction between temperature and E/S ratio and between temperature and time on the DH ($p \leq 0.01$). The DH of RBPH was found to increase rapidly with temperature and time (Fig. 4). The temperature increases the rate of enzymatic reactions; hence the rate of %DH increases as long as the temperature is below the denaturation temperature of the enzyme. Therefore, as time increased, the alcalase enzyme gradually hydrolyzed the extracted protein, then the total protein would decrease at the same time the DH is increasing in reverse.

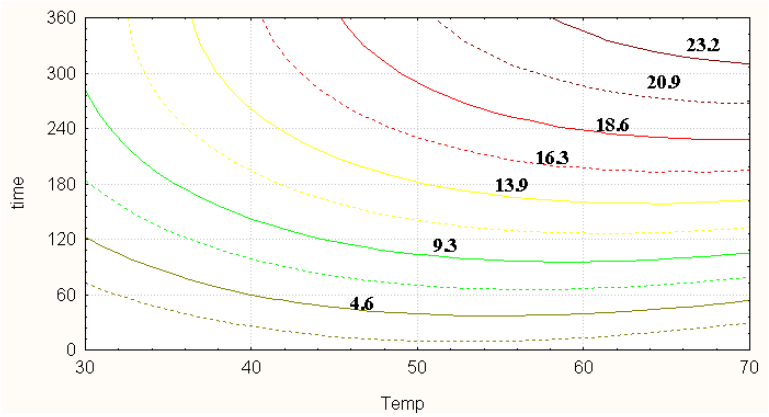

Figure 4. Contour plot showing the combined effect of Temperature and Time on the %DH of RBPH using alcalase enzyme.

The determination coefficient (R^2) of the degree of hydrolysis (%DH) is 0.967. This implies that the regression models explained the reaction well. As the test of lack of fit hypothesis was significant ($p \leq 0.05$) in model equation, the model was not fitted to the %DH data (data not shown). From Figure 4 the incubation time for the highest %DH was at 340 min.

Optimization of enzyme hydrolysis

The optimum extraction condition was determined by the three dimension response surface plots and the contour plot of the total protein, shown in Figures 2 and 3, respectively. They were generated from predicted data to illustrate the effect of independent variables (Temperature, E/S and Time). From equation (3) the total protein was influenced by only temperature and E/S accepted for the time. The optimized temperature and E/S from Figure 3 were 44 to 64^{o}C and 0.3 to 1.7%, respectively. This means that the optimum temperature of alcalase activity is in the range of 55 to 70^{o}C [16]. The optimum conditions of temperature and E/S obtained using superimposed RSM in Figure 5 for maximum total protein were 63^{o}C and 1.0%, respectively. The DH is dependent on time and temperature when it increased, meaning that the percentage of peptide bonds cleaved is more [16]. 340 min was chosen for incubation time in order to get highest DH (Fig. 4).

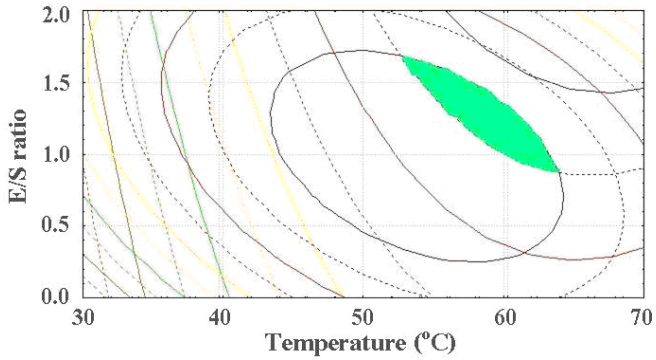

Figure 5. The superimposed contour plot showing the combined effect of temperature and E/S on the total protein and %DH of RBPH using alcalase enzyme.

Verification experiment

The suitability of the model equation for predicting the optimum response values was tested using the selected optimal conditions; Temperature, E/S at 63°C, 1.0 respectively. To save energy, the highest %DH was compared using incubation time 340 min and half time of incubation at 195 min. The experimental total protein was found to be in agreement with the predicted one (Table 5); mean of total protein compared by t-test method, showed that the time was significant for %DH but non-significant for total protein. It was thus confirmed that the equation found no lack of fit for %DH but was good for prediction of total protein. Since %DH at 340 min was higher than at 195 min, an optimum incubation time was selected at 340 min. Kong *et al*. [17] hydrolysed wheat gluten by using several commercially available proteases such as Alcalase at pH 8.5 and 60°C, PTN at pH 8.5 and 47°C, Pepsin at pH 2.0 and 37°C, Pancreatin at pH 8.5 and 37°C, Protamex at pH 6.5 and 50°C and Neutrase at pH 7.0 and 50°C. They found that, after 360 minutes of hydrolysis time, Alcalase gave the highest %DH values (15.8 %DH) and was more efficient for gluten hydrolysis than the others. Cumby *et al.* [18] reported that Canola protein hydrolysates prepared using Alcalase at 50°C and pH 8 for 60 minutes had 20.6 %DH. Yuan *et al.* [13] hydrolyzed *Momordica charantia* L. Var. abbreviate Ser protein using Alcalase. Hydrolysis conditions were optimized by using RSM at 1-3 %E/S, pH 8-10 and 50-60°C and it was found that the %DH ranged from 10.38 – 14.57 %. Hamada *et al.* [7] who generated peptides from defatted rice bran 'Bangal' variety using commercial protease (alcalase) to 7.6% peptide bond hydrolysis (%DH) at 50°C and pH 8.0. Hamada [19] hydrolyzed rice bran protein using alcalase and flavourzyme by controlling temperature at 50°C and pH 8 and found that the %DH was 7.5% and 8.8%, respectively. This indicates that the results of this current research on %DH are comparable to these previously reported results. Meanwhile, particular emphasis must be placed on structural considerations of rice bran protein that are responsible for insolubility and limited protease access to the protein substrate to attain more solubility with very limited proteolysis. Major factors of protein insolubility include the relative occurrences of disulfide bonding and the degree of aggregation of the protein [9].

Table 5. Predicted and experimental total protein of rice bran protein hydrolysis at optimum conditions.

Optimum condition		%DH		Total protein(mg)	
		Predicted	Experimental	Predicted	Experimental
Temperature (°C)	63				
E/S (%)	1.0				
Time1 (min)	195	16.36*	10.38*	200.87 ns	195.73 ns
Time2 (min)	340	23.70*	14.62*	200.87 ns	187.62 ns

ns = non significant ($p > 0.05$), = significant ($p \leq 0.05$)

Conclusion

The different conditions (temperature, enzyme to substrate ratio and time) for RBPH production from alcalase using the critical values of the different conditions were studied to obtain an optimum for total protein and %DH. These related to the enzyme treatment conditions by second order polynomials. Using the contour plots, the optimum set of the operating variables were generated graphically in order to obtain the desired level of the RBPH properties which are suitable for subsequent use. Future studies will be focused on antioxidants of the RBPH that can be used in food and cosmetic products.

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