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Optimized Nitrogen Recovery and Non-Bitter Hydrolysates from Porcine Hemoglobin

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The combined effects of pH, temperature, buffer/substrate ratio, time and enzyme concentration on nitrogen recovery (NR) from porcine hemoglobin, a by-product of industrial abattoirs, with Pancreatin and Flavourzyme® 500MG enzyme mixture were characterized. The effect of hydrolysis variables on NR was described through response surface analysis (RSA). The results showed that pH and time were the most important parameters and that the buffer/substrate ratio had less of an effect on NR. The mathematical model presented an optimum hydrolysis conditions were as follows: temperature, 50.4 ºC; pH, 7.8; buffer/substrate (containing 33.1% protein) ratio (w/w), 1.4:1; time, 15.4h; and enzyme concentration, 2.0g/kg. The predicted NR value was 98.99%, and the actual value obtained was 97.69%. Molecular mass of recovered hydrolysates ranged from >15 kDa to free amino acid (<1 kDa). The admixture of enzyme had specificity for terminal a variety of hydrophobic amino acids which resulted in recovery of non-bitter hydrolysates from porcine hemoglobin.

Keywords: porcine hemoglobin; enzymatic hydrolysis; response surface methodology; nitrogen recovery; hydrolysates; molecular mass distribution; amino acid composition

Introduction

Porcine blood generated in industrial abattoirs is a by-product that can be used in the food industry, at a low cost, improving the nutritional status of the population, especially in the developing countries. In this way, animal blood could turn into an excellent quality raw source to be used (Dill and Landmann, 1998; Miller and Menichillo, 1991). Currently, animal blood is mainly used for the production of plasma. Plasma could be widely used to increase the nutrients in meat and other foods (Breer, 1978; Perunovic *et al.*, 1991), and its functional properties have been extensively studied (Howell and Lawrie, 1981).

However, hemoglobin, the major fraction of blood, is rarely used in food for dark color and blood flavor, which appear when it is added in trace amounts to food products. The lack of a greater use of animal blood generates a surplus volume that is usually discarded elevating the pollutant level of the residues coming from slaughterhouses, due to its high chemical oxygen demand of about 500,000 mg/L, or increasing the costs of residue treatment as well as generating overload problems in the residue treatment units (Moure *et al.*, 1998). Large-scale utilization of hemoglobin in foods consequently requires decoloration or technological manipulation to mask the hem pigment in processing (Wismer-Perdesen, 1998). Some methods exist for decolourization of blood, such as treatments with acidified acetone (Tybor *et al.*, 1975; Tybor *et al.*, 1973), hydrogen peroxide (Wismer-Perdesen, 1998; Oord *et al.*, 1979), carboxymethyl-cellulose (Autio *et al.*, 1984; Hayakawa *et al.*, 1986), and enzymatic hydrolysis (Adler-Nissen, 1986; Houlier, 1986; Synowiecki *et al.*, 1996; Ockerman and Hansen, 2000). Some of these treatments are inefficient with low protein recovery, while others impart residual solvent or a bitter taste to the recovered protein. So, how to recover effectively a palatable protein from porcine hemoglobin is a challenge faced by food scientists in this area.

Response surface analysis (RSA) is a useful tool applied

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towards the optimization of several food processing operations (Diniz and Martin, 1996). RSA defines the effects of the independent variables themselves, either alone or in combination, during the process. In addition, the methodology may generate a mathematical model that accurately describes the overall process.

The aim of this work was to determine through RSA the combined effects of temperature (T), initial pH, buffer/substrate ratio (B: S, w/w), time (t) and enzyme concentration (E) on the high nitrogen/protein recovery and non-bitter hydrolysates from meat industry byproduct porcine hemoglobin, by using an extensive enzymatic hydrolysis treatment.

Materials and Methods

Materials Hygienically collected porcine blood from an industrial abattoir was used. Sodium citrate solution (0.33%w/v) was added as an anticoagulant (Renata, *et al.*, 1999). At the laboratory, the red cell fraction (RCF) was obtained after blood centrifugation for 15 min at 3000×g and 5–10 ºC (Sigma 3K30, Germany) and plasma decanting (Synowiecki *et al*, 1996; Saguer *et al.*, 2003). Hemoglobin concentrate (HC), the protein content of 33.12±2.57g/100g, was obtained by provoking the hemolysis of RCF using a high pressure homogeniser (APV-1000, Denmark) at 60MPa which can improve the solution property of hemoglobin resolving in water, and then kept frozen at -20 ºC until use. Unlike the previous reports (Gómez-Juárez *et al.*, 1999; Toldrà *et al.*, 2004), the hemolyzed HC did not need further centrifuging to remove erythrocytic stroma and erythrocyte membranes.

Pancreatin was commercially obtained from Hangzhou Sanye Chemical Co., Ltd. (Hangzhou, China) and fulfilled the purity demands for food-grade enzymes, with a declared protease activity of 3000Ug^{-1}. Flavourzyme® 500MG fungal protease complex with a declared activity 50 LAPU (leucine aminopeptidase units) g^{-1} was purchased from Novo Industry A/S (Bagsvaerd, Denmark).

Experimental design and set-up Response surface analysis (RSA) was used to characterize the effect of the hydrolysis variables temperature (T), pH, buffer/substrate ratio (B: S, w/w), enzyme mixture concentration (E) and time (t) on the nitrogen recovery (NR) of HC. It was noted that the enzyme mixture included Pancreatin (P) and Flavourzyme® 500MG (F) and P: F=3: 1, (w/w) in the present study. A Box –Behnken factorial design (Box and Behnken, 1960; Box, *et al.*, 1978) with five independent variables (T, pH, B: S, E and t) was used. Ranges of T, pH, B: S, E and t (Table 1) were coded and selected based on manufacturer's information and preliminary experiments.

Experiments were performed in triplicate in 250 ml conical flask with close-fitting rubber closure placed in a thermostatically controlled water bath oscillator. The pH of reaction system was detected by pH electrode (Sartorius AG PB-20, Germany).

Enzymatic hydrolysis HC was thawed at 5 ºC prior to the assay. The enzymatic hydrolysis was carried out as stated in Table 1 according to the experimental design described in Table 2. The reaction system (50 g of HC and 25, 50, 75, 100 or 125 g of distilled water buffer) was adjusted to the required pH using 2M NaOH or 2M HCl and preheated to the required temperature. Once equilibrium was reached, the enzyme solution was added. After the set reaction time interval (5, 10, 15, 20 and 25 h), the reaction system were taken and heated (100 ºC, 5 min) to end the reaction. Controls were run under identical experimental conditions but without enzyme addition.

Nitrogen recovery Hydrolysed samples were heated at 100 ºC for 5 min and cooled to ambient temperature before centrifugation (3000×*g*, 10min) to separate insoluble from soluble protein (Synowiecki *et al*, 1996). The volume of supernatant (soluble hydrolysate) was measured and freeze-dried. The nitrogen content was determined according to the Kjeldahl method (AOAC, 1995). The nitrogen recovery was defined as follows: NR (%) = (N in hydrolysed supernatant/ N in initial sample) ×100. A control sample without enzyme was also run for each experiment.

Taste determination The bitterness of the hydrolysates, based on Aubes-Dufau *et al.* (1995), was estimated in aqueous solution. The test was performed by a bitterness expert panel of 6 people. The sample (triplicate) concentration for tasting was 10 g/100 mL and the degustation with 200 μL of sample put with a syringe onto the base of the tongue, where bitterness is especially perceptible. The bitterness intensity was scored by comparison with a quinine sulfate concentra-

Table 1. Coding of hydrolysis variables and experimental design levels for response surface analysis.

Code	T(ºC)	pH	B: S	E(g/kg)	t(h)
-2	40	6.6	0.5: 1	1.2	5
-1	45	7.1	1: 1	1.6	10
0	50	7.6	1.5: 1	2.0	15
1	55	8.1	2: 1	2.4	20
2	60	8.6	2.5: 1	2.8	25

T, temperature; B: S, buffer/substrate ratio; E, enzyme concentration (g/kg HC); t, reaction time.

Table 2. Box–Behnken design matrix, Experimental Data, and Predicted Values for Response Surface Analysis.

std	T(ºC)	pH	B: S	E(g/kg)	t(h)	NR (%)
1	-1	-1	-1	-1	1	30.08
2	1	-1	-1	-1	-1	26.14
3	-1	1	-1	-1	-1	28.04
4	1	1	-1	-1	1	75.03
5	-1	-1	1	-1	-1	22.17
6	1	-1	1	-1	1	38.41
7	-1	1	1	-1	1	43.13
8	1	1	1	-1	-1	51.23
9	-1	-1	-1	1	-1	22.68
10	1	-1	-1	1	1	46.05
11	-1	1	-1	1	1	41.84
12	1	1	-1	1	-1	22.63
13	-1	-1	1	1	1	52.90
14	1	-1	1	1	-1	22.76
15	-1	1	1	1	-1	45.73
16	1	1	1	1	1	50.17
17	-2	0	0	0	0	40.28
18	2	0	0	0	0	27.51
19	0	-2	0	0	0	47.64
20	0	2	0	0	0	86.95
21	0	0	-2	0	0	86.61
22	0	0	2	0	0	87.53
23	0	0	0	-2	0	36.49
24	0	0	0	2	0	50.48
25	0	0	0	0	-2	34.76
26	0	0	0	0	2	93.83
27	0	0	0	0	0	97.05
28	0	0	0	0	0	98.53
29	0	0	0	0	0	94.99
30	0	0	0	0	0	92.27
31	0	0	0	0	0	95.01
32	0	0	0	0	0	93.14

tion scale and expressed as an isointensity quinine concentration.

Statistical analysis Data from the Box–Behnken factorial design were analyzed by multiple regression through the least squares method to fit the second-order model given by the following equation:

$$NR = \beta_0 + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{ij} X_i X_j \qquad (1)$$

where NR was the measured response variables, β_0, β_i, β_{ii} and β_{ij} are respectively constant, linear, quadratic and cross-product regression coefficients of the model, X_1, X_2, X_3, X_4 and X_5 represent the five independent variables T, pH, B:S, E and t respectively. A central composite design was used and 32 experiments (see Table 2) for the study of five experimental factors in coded units were required. NR was measured as dependent variable after the set interval of hydrolysis time.

Data were analyzed using the statistical program Design-Expert 7.0.0, trial (Stat-Ease Inc., Minneapolis, USA) and 3D graphic surface were drawn using the same program. Experiments and analytical determinations were conducted in triplicate and the statistical analysis was done on the mean of three determinations.

Optimization Analysis Optimal conditions for the nitrogen recovery, defined the desired limits at maximum, from HC depended on temperature (T), initial pH, buffer/substrate ratio (B: S), time (t) and enzyme concentration (E) were obtained using the predictive equations of RSM. NR was determined after enzymatic hydrolysis under optimal conditions. The experimental and predicted values were compared using SPSS10.0 for Windows in order to determine the validity of the model.

Analytical method for HC hydrolysates The molecular mass distribution of HC hydrolysates was estimated by gel permeation chromatography (Superdex™ Peptide HR 10/300 GL, Amersham Biosciences Corp., NJ, USA) with UV detection at optical density of 214 nm and 280nm. The mobile phase (isocratic elution) was 0.02M phosphate buffer containing 0.25M NaCl, pH 7.2. The flow rate was 0.5 mL/min. UNICORN 5.0 software was used to collect, plot and process the chromatographic data. Peptides of known molecular weight (Amersham Biosciences) were used to calibrate the column. A relationship between the retention volume and the log of the molecular mass of peptides used as standards has been established. Samples were filtered at 0.2 μm before injection.

Amino acid composition was determined by high performance liquid chromatography (Waters, equipment with PICO.TAG column, ϕ3.9mm×150mm), among which, Free amino acids (FAA) were analyzed in triplicates after precolumn derivatization with phenyl isothiocyanate (Bidlingmeyer *et al.*, 1984), total amino acids (TAA) were determined in triplicates after hydrolysis at 110 °C for 24 h with hydrochloric acid (6 mol/L) prior to the derivatization with phenyl isothiocyanate and alkaline hydrolysis for tryptophan. All determinations were performed in triplicate.

Results and Discussion

The effect of the variables on the dependent response for the enzymatic hydrolysis of HC using Pancreatin and Flavourzyme® 500MG mixture was presented in Table 2.

NR varied from 22.17 to 98.53%. Values were obtained at different codes for each variable. The results of the second-order response surface model in the form of analysis of variance (ANOVA) for NR were shown in Table 3. From the analysis, the quadratic regression model demonstrates that the model is highly significant as the Fisher F-test (F model, mean square regression/mean square residual) 10.3246 with a very low probability value [(P model >F) =0.0002]. The fit of the model was checked by the determination coefficient (R^2). In this case the value of the determination coefficient (R^2=0.94942) indicates that only 5.058% of the total variation is not explained by the model. The value of the adjusted determination coefficient (adjusted R^2=0.85747) is also high to advocate a high significance of the model (Myers and Montgomery, 2002). A higher value of the correlation coefficient (R=0.97438) justifies an excellent correlation between the independent variables. Simultaneously, a relatively low value of the coefficient of variation (CV=18.731%) indicates good precision and reliability of the experiments (Ivone *et al*, 2000).

The following regression equation is the empirical models in terms of coded factors for NR:

$$NR=97.26+0.85A+7.30B+1.49C+0.77D+10.60E-17.41A^2-9.06B^2-4.12C^2-15.02D^2-9.81E^2+2.17AB-3.04AC-5.56AD+2.35AE+0.71BC-4.04BD-0.70BE+2.67CD-3.17CE+1.13DE \quad (2)$$

The second-order polynomial model (equation 2) was highly significant and adequate to represent the actual relationship between the response (NR) and the significant

Table 3. ANOVA for Response Surface Quadratic Model.

Source	Sum of Squares	DF	Mean Square	F value	Prob > F
Model	22468.74	20	1123.44	10.32	0.0002
A	17.19	1	17.19	0.16	0.6986
B	1279.40	1	1279.40	11.76	0.0056
C	53.55	1	53.55	0.49	0.4975
D	14.28	1	14.28	0.13	0.7241
E	2696.00	1	2696.00	24.78	0.0004
A^2	8895.47	1	8895.47	81.75	< 0.0001
B^2	2410.02	1	2410.02	22.15	0.0006
C^2	498.03	1	498.03	4.58	0.0557
D^2	6614.71	1	6614.71	60.79	< 0.0001
E^2	2825.35	1	2825.35	25.97	0.0003
AB	75.65	1	75.65	0.70	0.4221
AC	147.44	1	147.44	1.36	0.2690
AD	494.28	1	494.28	4.54	0.0564
AE	88.22	1	88.22	0.81	0.3872
BC	8.17	1	8.17	0.08	0.7892
BD	261.23	1	261.23	2.40	0.1496
BE	7.77	1	7.77	0.07	0.7942
CD	114.01	1	114.01	1.05	0.3280
CE	161.23	1	161.23	1.48	0.2490
DE	20.45	1	20.45	0.19	0.6730
Residual	1196.92	11	108.81		
Lack of Fit	1169.51	6	194.92	35.55	0.0006
Pure Error	27.41	5	5.48		
Cor Total	23665.66	31			
	Std. Dev.	10.431		R-Squared	0.94942
	Mean	55.689		Adj R-Squared	0.85747
	C.V.	18.731		Pred R-Squared	-0.2299
	PRESS	29106		Adeq Precision	8.99286

A, temperature(ºC); B, pH; C, buffer/substrate ratio; D, enzyme concentration (g/kg HC); E, reaction time(h).

variables. Furthermore, the overall effect of the five variables on the NR was further analyzed by a joint test (Table 2). The results revealed that pH and time in their linear form, as well as quadratic T, pH, E and time, were the significant variables. Other model terms were not significant, in which, the buffer/substrate ratio (B: S) in the experimental scale (0.5–2.5:1, w/w) of this study was the least important factor compared to the other reaction parameters from the statistical analysis. This is mainly because RCF was haemolyzed by using homogeniser and easily hydrolyzed. Therefore, the buffer/substrate ratio was constant at zero level (1.5:1) in the following discussion.

The predicted versus actual plot for NR is shown in Fig. 1. The figure proves that the predicted data of the response

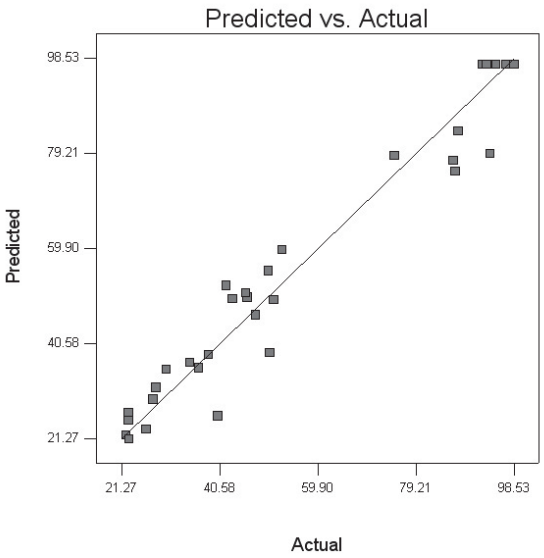

Fig. 1. DESIGN-EXPERT plot. Predicted versus actual data for NR.

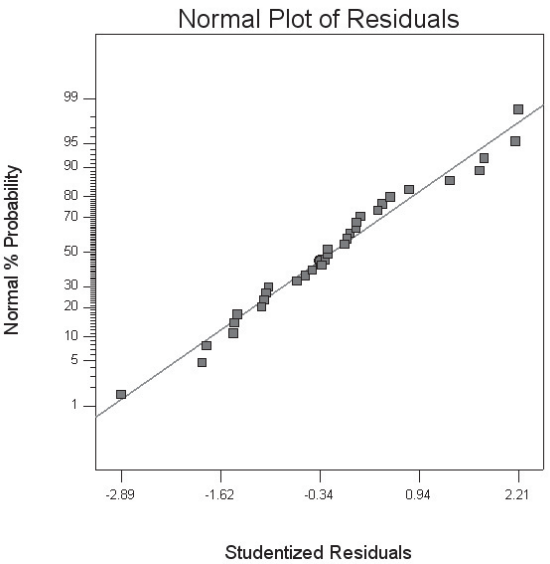

Fig. 2. DESIGN-EXPERT plot. Normal probability plot of studentized residuals.

from the empirical model is in agreement with the actual values in the range of the operating variables. The high value of adjusted R2 (0.85747) indicated that the model well fit the observed data (Nahit, 2005).

The residuals from the least squares fit, which is defined by $e_i = y_i - \hat{y}_i$, i=1, 2, . . ., n, play an important role in judging model adequacy (Myers, *et al.* 2002). By constructing a normal probability plot of the residuals, a check was made for the normality assumption, as given in Fig. 2. The normality assumption was satisfied as the residuals plot approximated along a straight line.

The plot of studentized residuals versus the run order (not given) was tested and the residuals scattered randomly on the display, suggesting that the variance of the original observations was constant for all values of response (Myers *et al*, 2002).

Enzyme concentration and B:S were investigated in the range of 1.6–2.4 g/kg and 1:1–2:1, respectively. Fig. 3 shows the effect of Enzyme concentration, B:S, and their mutual interaction on NR at temperature 50°C, pH 7.6 and reaction time 15h. Comparing with the effect of enzyme concentration on NR, the effect of B:S on NR was not obviously, and at a relative wide range of B:S, from 1.1:1 to 2.0:1, NR maintained high level about 93.6%. One of the reasons why this phenomenon occurred was that homogenization can both provoke the hemolysis of RCF and improve the solution property of hemoglobin leading to easily interact with enzyme. So, it was convenient to select appropriate B:S according to subsequent process such as decoloration and concentration. A reaction with an enzyme amount of 2.0 g/kg and B:S at 1.5:1 led to the maximum NR (>95%). An interesting phenomenon was also found that as enzyme concentration

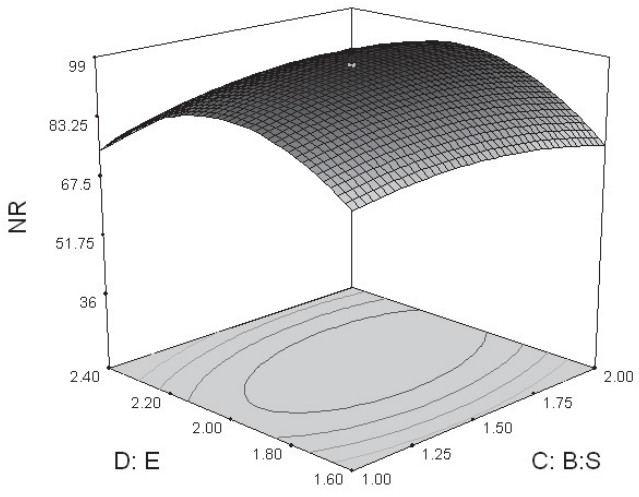

Fig. 3. Response surface plot showing the effect of reaction enzyme concentration, B:S, and their mutual interaction on NR. Other synthesis parameters (temperature, pH, and reaction time) are constant at zero level.

increase from 2.0 g/kg decrease NR. This could be explained by the mixed enzyme system. The chance of Pancreatin and Flavourzyme® 500MG interaction between themselves will be more as both enzymes concentration increasing in solution and thus weak the activity of enzyme system.

The effect of varying enzyme concentration and pH on NR at constant reaction time (15 h), buffer/substrate ratio (1.5:1), and temperature (50 ºC) is show in Fig. 4. At the range of initial reaction pH from 7.1 to 8.1, an increase of pH led to higher recovery until 7.6; after this point, following increase of pH resulted in a little decrease of NR compared to that of 7.6. It indicated that the mixed enzyme system is reliable to weak alkalesence demonstrating activity. Moreover,

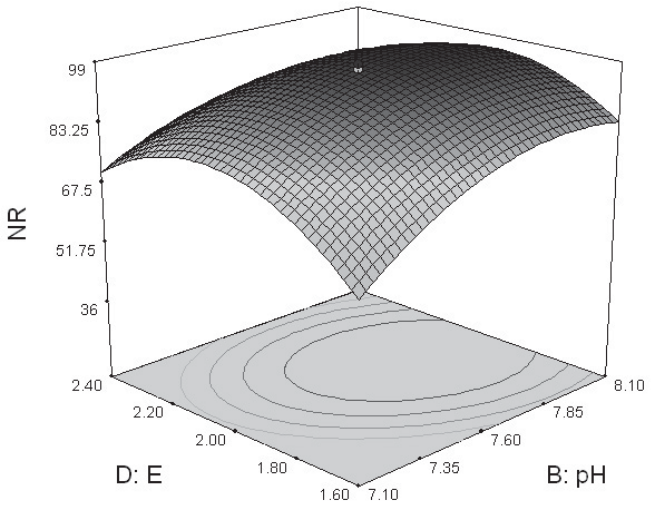

Fig. 4. Response surface plot showing the effect of reaction enzyme concentration, pH, and their mutual interaction on NR. Other synthesis parameters (buffer/substrate ratio, temperature, and reaction time) are constant at zero level.

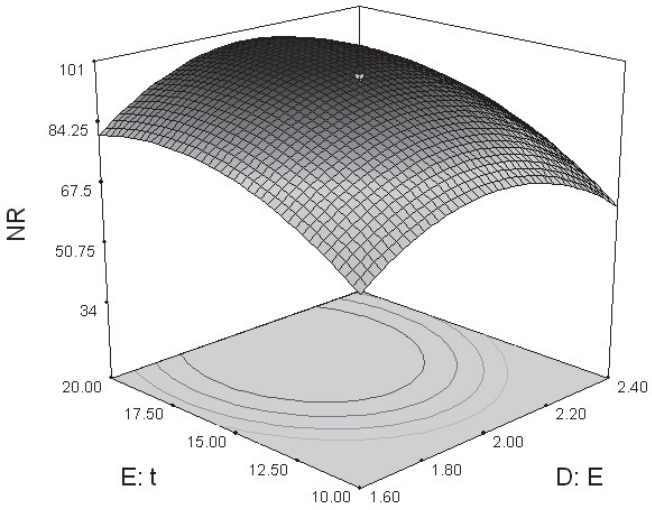

Fig. 5. Response surface plot showing the effect of reaction enzyme concentration, time, and their mutual interaction on NR. Other synthesis parameters (buffer/substrate ratio, temperature, and pH) are constant at zero level.

pH of the solution during the hydrolyzing process decreased mildly and then constant, unlike that of Alcalase. So, in this study, pH did not resort to control by NaOH or HCl, which conduced to economical interest and facility in the hydrolyzing process.

Fig. 5 represents the effect of varying substrate enzyme concentration and reaction time on NR at constant buffer/substrate ratio (1.5:1), pH (7.6) and temperature (50 ºC). At any given enzyme concentration from 1.6 to 2.4 g/kg, an increase of time led to higher yields. Even though only a very small increase in NR in the period 15–20 h of hydrolysis during which NR is higher than 90%, undoubtedly the increase in NR is most profound in the early stages of the hydrolyzing process. Thereby, optimizing the reaction time was critical for economical interest in the hydrolyzing process and easy from this figure.

The optimal point was also obtained by statistical program Design-Expert and cost-effective. The stationary point initial pH (7.8), temperature (50.4 ºC), buffer/substrate ratio (1.4: 1), time (15.4h) and enzyme concentration (2.0g/kg) was located in the experimental region with the predicted value of 98.99%. The adequacy of the predicted model here was examined by additional repeat five independent experiments at the suggested optimal hydrolysis conditions, and the actual value was 97.69±2.11%. One-Sample Test (p value= 0.2400, degrees of freedom=4) indicated that observed values were significantly the same as the predicted NR. In order to further testify this method, 2.5 kg HC was hydrolysis according to the optimal conditions and the experiments were carried out in triplicate. The mean NR was 95.31% and also closed to the predicted value.

Proteolytic Degradation of HC during hydrolysis in optimal conditions at different hydrolysis time (4h, 8h, 12h, 15.4h) was analyzed by gel permeation chromatography. Fig. 6a (214 nm) and 6b (280 nm) show the main peaks of the molecular mass distribution of optimal HC hydrolysates. The recovery hydrolysates showed seven main peaks in the 280 nm chromatogram, however, in the 214 nm chromatogram the hydrolysates showed a complex, containing more peaks, especially at the range of retention volume 12mL–25mL. This was done because monitoring at 280 nm is more specific for protein analysis, but relies on absorption by tyrosine or tryptophan residues which are both amino acids with relatively low abundance, so many peptides generated by the initial hydrolysis will contain neither of them, and hence have no significant absorption at 280 nm. Thus, the less specific wavelength of 214 nm was also used to give a more comprehensive picture. Both the wavelengths were usually applied to determine protein hydrolysates elution from gel permeation (Spellman *et al.*, 2005). So, some of the

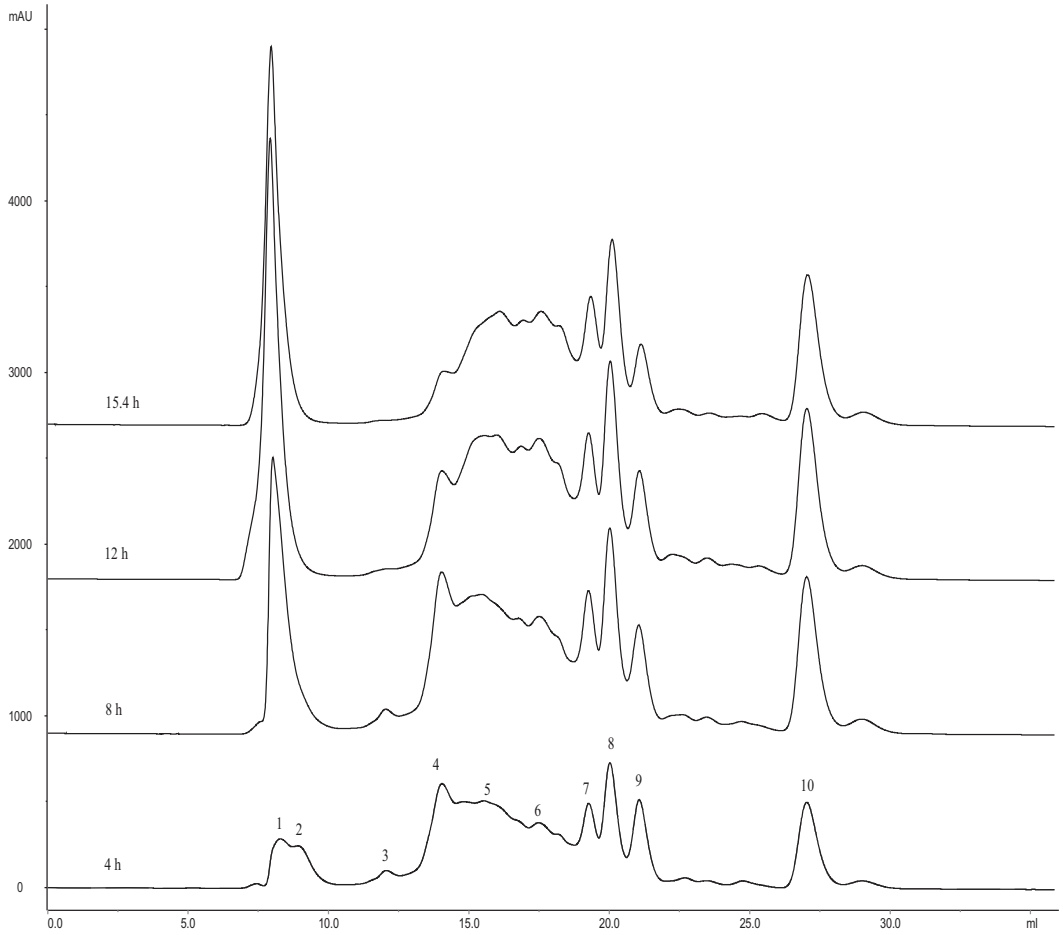

Fig. 6a. Gel permeation chromatograms of different optimal reaction interval (4h, 8h, 12h, 15.4h) extracts of HC (214nm).

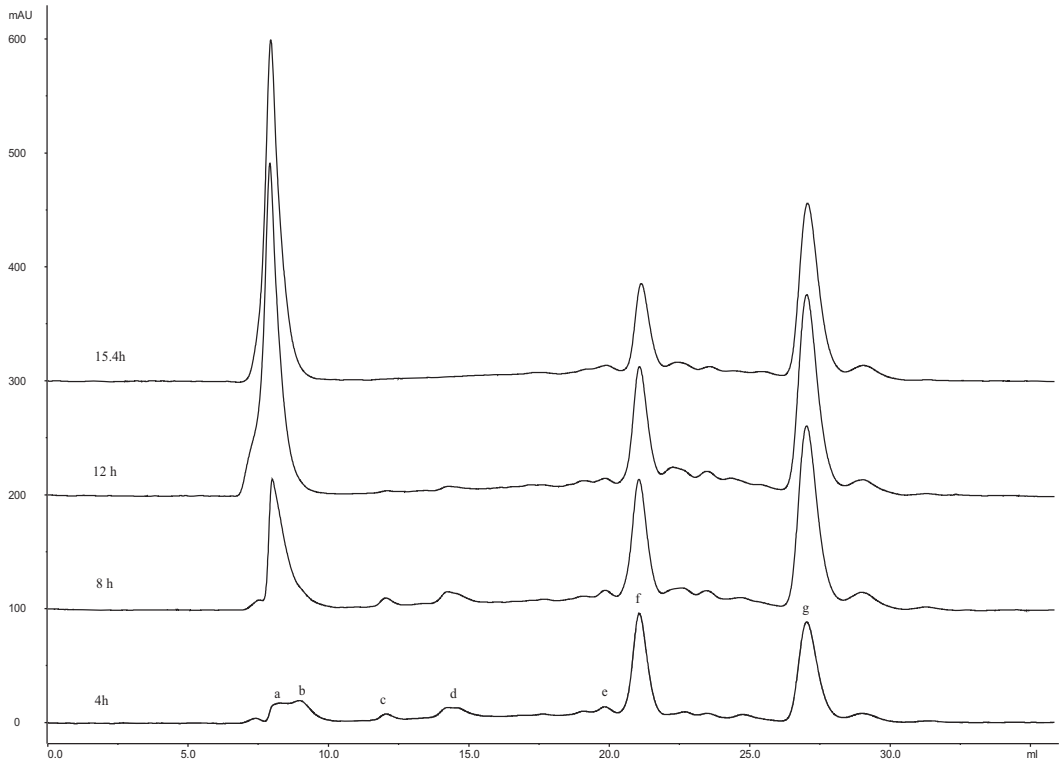

Fig. 6b. Gel permeation chromatograms of different optimal reaction interval (4h, 8h, 12h, 15.4h) extracts of HC (280nm).

peptide peaks seen at 214 nm were missing from the 280 nm profiles. This showed that some recovered fraction peptides, after hydrolysis, did not contain a significant tyrosine and/or tryptophan content. In the chromatogram (214 nm), the molecular weight of peaks of 1, 2 is >15 kDa (<9.0 ml), of 3 is 15–5 kDa (9.0-13.3 ml), of 4, 5 is 5–1 kDa (13.3-16.0 ml), of 6, 7, 8, 9, 10 is < 1kDa (>16.0 mL). Peaks of a, b, c, d, e, f, g in 280nm correspond to peaks of 1, 2, 3, 4, 8, 9, 10 in 214 nm according to retention volume, respectively. Peak 1 increased with increasing hydrolysis time, which indicated the substrate released some amino acids and peptides and became soluble though this fraction had high molecular weight and consisted of more tyrosine and tryptophan (seen from 280nm). Two minor peaks of c and d appear in 4h and 8h of Fig. 6b. This phenomenon indicated that, to some extent, hydrogen bonds (and possibly other non-covalent bonds) were probably affecting the size of the extracted material (aggregation) (Fischer *et al.*, 2002). These bonds were easily destructed as the hydrolysis progress, which could be seen from other curves of Fig. 6b and even from Fig. 6a, the minor peak 3 missing and peak 4 shrinking. The reason why peak b disappear quickly with hydrolyzing might be for that of peak c and d. Other peaks, molecular weight lower 5 kDa, were generally increased with prolonging hydrolysis. The average chain length of HC hydrolysates was 3.3.

Since Carr *et al.* (1966), it has been well-known that bitter peptides occurred during the hydrolysis and resulted from the degradation of the protein substrate itself (Adler-Nissen, 1986). This problem is more acute at high values of hydrolysis(Guigoz and Solms, 1976). Matoba and Hata (1972) proposed that the bitterness was caused by peptides with high content of hydrophobic amino acids, regardless of their primary structure. The higher the content of hydrophobic amino acids in a certain protein, the more pronounced its tendency to form bitter peptides. Belitz *et al.* (1979) confirmed that hydrophobicity was essential for the bitter taste and established a quantitative relationship between the bitterness of amino acids, di- and tripeptides, and their hydrophobicity. Though Adler-Nissen (1986) evaluated Alcalase, its specificity for terminal hydrophobic amino acids, generally leads to the production of non-bitter hydrolysates, Synowiecki, *et al* (1996) reported that protein hydrolysates at high degree of hydrolysis from bovine red blood cells, prepared using the enzyme, had a strong bitter taste. In this study, average quinine detection threshold value for the panelists is 4.3 mg/L in these tasting conditions. Bitterness of the hydrolysates was very weak, as mean isointensity quinine concentration was scored 4.2 in this study. Table 4 shows the mixed enzyme at optimal conditions has specificity for terminal a variety of hydrophobic amino acids. Obviously, the average FAA release rate of hydrophobic amino acids was 41.03%, higer than 27.06% of hydrophilic amino acids (Glu, Asp, Arg, Lys, Ser, Thr, and His). Though release rate of Pro was low, just 8.34%, Lemieux and Simard (1992) reported that Pro does not contribute bitterness. This result led to the recovered peptides with low content of hydrophobic amino acids which reduced the bitter taste of hydrolysates because the bitter-tasting peptides exhibit a high content of hydrophobic amino acids (Matoba and Hata, 1972).

Table 4. Amino acid composition of HC and recovered hydrolysates from optimal conditions as well as FAA release rate.

amino acids	H C		recovered hydrolysates		FAA release rate*
	TAA (mg/100g)	FAA (mg/100g)	TAA (mg/100g)	FAA (mg/100g)	(%)
Asp	3520.70	1.66	1251.33	133.17	8.88
Glu	3161.73	6.11	1087.42	129.29	9.46
Ser	1381.16	0.00	575	139.02	23.75
Gly	1435.56	4.55	572.84	87.56	14.08
His	2431.74	2.57	1052.77	288.91	27.93
Arg	1467.41	2.57	308.76	124.34	19.82
Thr	1168.32	3.25	386.3	159.4	31.93
Ala	2413.44	1.51	883.65	224.71	21.91
Pro	2073.57	6.41	672.34	75.95	8.34
Tyr	790.92	1.60	267.56	141.97	42.16
Val	2545.40	2.33	1186.99	355.2	32.84
Met	325.60	0.00	163.81	118.95	86.22
Cys	99.54	0.10	24.37	16.72	39.54
Ile	179.51	0.74	71.11	25.62	33.28
Leu	3371.39	2.15	1457.41	572.06	39.98
Phe	2040.84	1.56	899.55	511.74	59.10
Try	1505.14	4.48	756.37	473.29	73.91
Lys	2237.47	5.18	1118.79	643.63	67.66
total	32149.44	46.76	12736.37	4221.55	30.85

*: FAA release rate (%) =100 × (FAA in the recovered hydrolysates-control) / TAA in the substrate HC.

Conclusions

High nitrogen recovery and palatable protein hydrolysates can be produced from HC, and enzymatic hydrolysis was successfully optimized by RSM. Optimal conditions for the starting pH, temperature, buffer/substrate ratio, time, and enzyme concentration were 7.8, 50.4 ºC, 1.4: 1, 15.4h, and 2.0g/kg, respectively. Under optimized conditions the experimental values agreed with the values predicted by ridge analysis. Gel permeation chromatography suggested that molecular mass of recovered hydrosates ranged from >15 kDa

to free amino acid (<1 kDa) and that the content of 15 kDa-5kDa was very low. The combination of enzyme at optimal conditions has specificity for terminal a variety of hydrophobic amino acids which might be reduced the bitterness of hydrolysates. This method allowed a fast, quantitative and maximum extraction of non-bitter taste protein from HC.

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