OPTIMIZATION OF ENZYMATIC HYDROLYSIS OF BLOOD COCKLE (*Anadara granosa*) USING ALCALASE®

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ABSTRACT. *The objective of this study is to establish the optimum hydrolysis conditions for blood cockle (Anadara granosa) using Alcalase® employing response surface methodology (RSM). A three-level face-centered central composite design (CCD) was adapted in the study. The effects of temperature (45-65^{o}C), pH (7.5-9.5), ratio of enzyme to substrate level (1-2%) and hydrolysis time (60-180 minutes) were studied in order to obtain the optimum degree of hydrolysis. The optimal conditions for enzymatic hydrolysis of blood cockle were found to be at 65^{o}C, pH of 9.5, enzyme concentration at 2% and hydrolysis time of 180 minutes. The enzymatic hydrolysis of cockles gave a quadratic fit with the experimental data. Under these optimum conditions, the predicted value for degree of hydrolysis was 34.05%, while the actual experimental data was 37.27%. The lyophilized blood cockle hydrolysate was composed of 8.59% water, 74% protein, 5.80% fat, 10.22% ash and 1.39% carbohydrate.*

KEYWORDS. Alcalase®, blood cockle, degree of hydrolysis, optimization

INTRODUCTION

Shellfish such as blood cockle (*Anadara granosa*) have been recommended in several dietary regimes for their high protein content, low calorific values, low fat/cholesterol profile, the presence of good lipids, significant amounts of omega-3-fatty acids, dietary essential amino acids, vitamin B12 and several important minerals such as iron, zinc and copper and lower proportions of saturated fat (Dong, 2001). It is one of the bivalves that have great potential and economic value to be developed into a source of protein and minerals. Malaysia produced 64,958.51 metric tons of blood cockles in 2009 (Department of Fisheries Malaysia, 2009). Malaysia exported the cockles to Thailand, Singapore and Brunei (Izura & Hooi, 2008).

Since cockle is a filter feeder, it is often associated with safety issues due to accumulation of microbes, heavy metal and polycyclic aromatic hydrocarbon (Koh *et al.*, 2011; Ibrahim, 1995: Mirsadeghi *et al.*, 2011). Thus, this call for more study in developing value added products from cockle as well as ensuring safe cockle for public consumption. Until now, only one study has been reported on the optimization of enzymatic hydrolysis from cockle meat (Haslaniza *et al.*, 2010). No study has been reported on the optimization of enzymatic protein hydrolysis of blood cockles. Besides, cockle hydrolysate offers a more convenient way to use cockle in food preparation. Furthermore, optimization of cockle protein enzymatic hydrolysis is important in order to have an effective process control for later up-scaling.

Soluble protein hydrolysate is produced from protein-based raw materials that undergo protein enzymatic hydrolysis. A common enzyme used to hydrolyse protein for producing fish protein hydrolysate is Alcalase® as it shows better proteolytic activity for most sources of protein. Several studies have reported on the optimization of fish protein hydrolysate such as in Catla viscera (Bhaskar & Mahendrakar, 2008), Pacific whiting solid waste (Nilsang *et al.*, 2005), threadfin bream (Normah *et al.*, 2005) and grass carp skin

(Wasswa *et al.*, 2008). However, no study has been reported on optimization of protein enzymatic hydrolysis of cockles.

Response Surface Methodology (RSM) is a statistical model frequently used for the optimization of complex systems and uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate problems (Madamba, 2002). Based on the experimental data, RSM could tell us the optimum conditions to obtain the desired responses, as well as the mathematical model in explaining the relationship between the experimental variable and its response.

The aim of this study is to optimize the enzymatic protein hydrolysis from blood cockle in terms of hydrolysis time, hydrolysis temperature, hydrolysis pH and concentration of enzyme (ratio of Alcalase® to substrate) to achieve the maximum degree of hydrolysis (DH). The proximate analysis of steamed cockle and its lyophilized hydrolysate prepared under the optimum conditions was also determined.

MATERIALS AND METHODS

Materials

About ten kilograms of whole blood cockle (with shell) were purchased from local market. Commercial enzyme, Alcalase® 2.4L in liquid form (2.4 AU/g) was purchased from Novo Nordisk (Bagsvaerd, Denmark). All other chemicals used were of analytical grades.

Methods

The whole blood cockles were rinsed 3 to 4 times to remove the mud and other contaminants. Then the cockles were de-shelled after steaming for 30 minutes. The cockles were then homogenized using Waring blender (model HGB2WTS3). Distilled water added to the cockle with the ratio of 1:10 (w/w) was employed to facilitate the homogenization process. The sample was then sealed in a polyethylene bag and stored at - 40°C until further use.

Prior to protein hydrolysis, the proximate analysis of homogenized cockle was carried out (AOAC, 2002). The mass of homogenized cockle and Alcalase® enzyme used in the cockle hydrolysis depended on the protein content of cockle. The calculation of raw materials to be used in the hydrolysis process was based on a modified calculation from Hordur and Barbara (2000). Preparation of cockle hydrolysate was carried out according to Amiza *et al.* (2011) with some modification.

Optimization of the hydrolysis conditions were accomplished by employing the response surface methodology (RSM) with a central composite design (CCD). Four different independent variables which were temperature (A,°C), time (B, minutes), enzyme to substrate concentration (C, %v/w) and pH (D) were employed at three equidistant levels (−1, 0 and +1). The hydrolysis processes were carried out based on the parameters shown in Table 1. A total of 30 runs were performed.

Table 1. Independent factors and levels used in the optimization of enzymatic blood cockle hydrolysis using RSM.

Independent factors	**Symbol**	**Range and level**		
		-1	**0**	**+1**
Temperature (°C)	X_1	45	55	65
Enzyme to protein concentration (E/S,% v/w)	X_2	1.0	1.5	2.0
Hydrolysis time (h)	X_3	60	120	180
pH	X_4	7.5	8.5	9.5

For each run, 55 g of homogenized cockle was added to30 g of distilled water (including the volume of 1 N NaOH used to adjust to required pH). Then the mixture was heated at 85°C for 20 minutes to inactivate endogenous enzyme in the homogenized cockles, prior to hydrolysis. After cooling down, 20 g of Alcalase enzyme solution (enzyme was diluted to the final volume of 20 g with distilled water) was added to the slurry and the hydrolysis process was carried out using an autotitrator (Metrohm model 799 GPT Titrino). The volume of 1 N NaOH used resulted from the calculation of the degree of hydrolysis in each run. After the specified hydrolysis time each run was terminated by heating the mixture at 85^{o}C for 20 minutes.

Degree of hydrolysis (DH; %) was determined as the response variable (Y) and determined using pH-stat method according to Adler-Nissen (1986). Calculation of degree of hydrolysis was carried out according to Adler-Nissen (1986) as follows:

$$DH = B \times N_b \times 1/\alpha \times 1/MP \times 1/h_{tot} \times 100\%$$

where B is base consumption (in ml), N_b is normality of the base, α is average degree of dissociation of the α-NH groups, MP is mass of protein (in g) and h_{tot} is total number of peptide bonds in the protein substrate.

The hydrolysate that gave the highest degree of hydrolysis was centrifuged at 4000 g for 30 min (High Speed Centrifuge, Sorvall HS23, USA). The supernatant (without removing the oil layer) was frozen at - 80°C and lyophilized in freeze dryer (Labconco Freeze Dryer Freezone 6 liter, USA) and then analyzed for its proximate analyses using AOAC methods (AOAC, 2002).

Statistical analysis

Experimental data obtained in the optimization of protein enzymatic hydrolysis of blood cockle using Response Surface Methodology were analyzed using Design Expert Version 8.0.6 (Stat Ease Inc.). The software generated the experimental runs and automatically analyzed the experimental data and gave the important analysis including sequential model sum of squares, ANOVA table, final equation, diagnostics, suggested solutions for optimization, response surface plots and point prediction table. The model was considered valid if the "lack of fit" given by ANOVA table after model adequacy checking was not significant ($p>0.05$). Optimization criteria were set as stated in Table 5 in results and discussion section.

RESULTS AND DISCUSSION

Optimization of enzymatic hydrolysis

Experimental data obtained in the study is shown in Table 2. Randomized experiments were run and 30 sets of hydrolysis experiments were conducted according to Central Composite Design (CCD) (face-centred). The range of degree of hydrolysis (DH) obtained was 10.7% - 37.27%. The highest DH (37.27%) was given at temperature of 65°C, enzyme concentration 2%, pH 9.5 and hydrolysis time 180 minutes. Design Expert software (version 8.0.6) was used in the study in order to analyze the experimental data. In order to determine whether the fitted model provided an adequate approximation to the true system, model adequacy checking was carried out. Later, the data was analyzed for variance (ANOVA), coefficient variation, diagnostic case statistics and response surface plots and the effects of factors. After the response had been analyzed, the optimization module in Design-Expert was used to search for a combination of factors that simultaneously satisfied the requirements of the response and factors.

Model adequacy checking

The prediction equation for response, degree of hydrolysis was generated automatically by the Design Expert version 8.0.6 software based on the experimental data. The software compared the formats and automatically underlined at least one "Suggested" model as most likely for the response. Model summary statistics actually list other statistics used to compare models. Table 2 shows the model summary for degree of hydrolysis.

Table 3 shows that the suggested model to describe degree of hydrolysis was a quadratic model, which is similar to a previous study on optimization of DH from fish soluble concentrate (Nilsang *et al.*, 2005), visceral waste of Catla (Bhaskar & Mahendrakar, 2008) and silver catfish skeleton (Amiza *et al.*, 2011).

Table 2. Effect of enzymatic hydrolysis conditions on the degree of hydrolysis of cockle using Alcalase®.

		Factor 1	Factor 2	Factor 3	Factor 4	Response
Std	**Run**	**Temperature (°C)**	**Enzyme concentration (E/S %)**	**pH**	**Hydrolysis time (min)**	**Degree of hydrolysis (DH %)**
18	1	65	1.5	8.5	120	23.9535
13	2	45	1	9.5	180	22.1858
19	3	55	1	8.5	120	10.6981
1	4	45	1	7.5	60	11.537
8	5	65	2	9.5	60	24.1462
7	6	45	2	9.5	60	17.119
30	7	55	1.5	8.5	120	15.2232
24	8	55	1.5	8.5	180	22.2612
17	9	45	1.5	8.5	120	15.2095
12	10	65	2	7.5	180	25.915
20	11	55	2	8.5	120	20.825
28	12	55	1.5	8.5	120	16.0267
26	13	55	1.5	8.5	120	15.2844
3	14	45	2	7.5	60	14.7644
10	15	65	1	7.5	180	28.1039
25	16	55	1.5	8.5	120	16.3138
2	17	65	1	7.5	60	18.2009
11	18	45	2	7.5	180	16.019
4	19	65	2	7.5	60	17.6265
29	20	55	1.5	8.5	120	14.8869
27	21	55	1.5	8.5	120	18.3725
9	22	45	1	7.5	180	20.2085
15	23	45	2	9.5	180	23.5238
21	24	55	1.5	7.5	120	11.7342
23	25	55	1.5	8.5	60	13.9
22	26	55	1.5	9.5	120	21.5497
5	27	45	1	9.5	60	14.2919
16	28	65	2	9.5	180	37.2717
14	29	65	1	9.5	180	29.2692
6	30	65	1	9.5	60	19.0174

Table 3. Model Summary Statistics for degree of hydrolysis.

Source	Std. Dev	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS
Linear	< 0.0001	0.0114	0.6549	0.5604	
2FI	0.5782	0.0091	0.6380	0.1704	
Quadratic	0.0010	0.0657	0.8565	0.6176	Suggested
Cubic	0.0097	0.9089	0.9655	0.9365	Aliased

Variance Analysis (ANOVA)

Table 4 shows the ANOVA table of response surface reduced Quadratic model for DH, which was obtained after model reduction was performed. Model reduction was carried out by eliminating the model terms with p-value above 0.05. It is necessary to examine the fitted model to ensure adequate approximation is provided to the true system (Myers & Montgomery, 2002).

Table 4. ANOVA table for response surface quadratic model for DH after model reduction.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	887.13	6	147.85	28.40	< 0.0001	significant
A-Temperature	261.79	1	261.79	50.29	<0.0001	
B- Enzyme concentration	31.20	1	31.20	5.99	0.0224	
C- pH	108.86	1	108.86	20.91	0.0001	
D- Hydrolysis time	305.50	1	305.50	58.68	<0.0001	
BC	27.62	1	27.62	5.31	0.0306	
A^2	152.17	1	152.17	29.23	<0.0001	
Residual	119.74	23	5.21			
Lack of Fit	111.66	18	6.20	3.84	0.0712	not significant
Pure Error	8.08	5	1.62			
Cor Total	1006.87	29				

The "F-value" for the quadratic model was 28.40 indicating that the model was significant and there was only 0.01% chance that the model would occur due to noise. Meanwhile the "p-value" less than 0.05 implied that the model term was significant. In this case A, B, C, D, BC, A^2 are significant model terms for DH. "p- value" for lack of fit was insignificant. In an optimization study, the insignificant result is preferable because it shows that the model fits well with the experimental data. The final equation in terms of coded factors, given by the software was DH = 16.42 + 3.81 *A + 1.32 *B + 2.46 *C + 4.12 *D + 1.31 *B *C + 4.60 *A^2. Meanwhile, the final equation in terms of actual factors was DH =

134.92 – 4.68 *A – 19.70 *B – 1.48 *C + 0.07 *D + 2.63 *BC + 0.05 *A^2. These results show that the most influential factor for cockle hydrolysis is enzyme concentration, followed by temperature, pH and hydrolysis time. This equation could be used to predict and control cockle hydrolysis using Alcalase™.

Optimization of DH

Table 5 shows the optimization parameters for independent variables and dependent response. The goal for all experiment independent variables had been set to be "in range" and their "importance" had been set as 3. There sponse variable, degree of hydrolysis, was considered the most important and the goal had been set as "maximize" and the "importance" had been set as 5.

Table 5. Optimization parameters for independent variables and response.

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
A: Temperature	is in range	45	65	1	1	3
B: Enzyme concentration	is in range	1	2	1	1	3
C: pH	is in range	7.5	9.5	1	1	3
D: Time	is in range	60	180	1	1	3
Degree hydrolysis	Maximize	10.6981	37.2717	1	1	5

After these limitations were set, the optimization tests were evaluated by Design Expert 8.0.6 software and the 5 best solutions of optimization conditions for further evaluation were listed as shown in Table 6. The desirability close to of 1.0 is the best solution. Table 6 shows that the optimum conditions were temperature 65°C, enzyme to substrate level 2%, pH 9.5 and hydrolysis time 180 minutes. The optimum enzymatic hydrolysis conditions for silver catfish skeleton were quite similar to this study with temperature 55^{o}C, hydrolysis time 163 min, pH 9.45 and Alcalase® concentration 2.0%. The optimum conditions were also quite similar to those reported by Bhaskar & Mahendrakar (2008) for Catla visceral waste protein hydrolysis using neutral protease with hydrolysis temperature 55°C, hydrolysis time 165 min and enzyme concentration 1.25%.

Table 6. Solutions of optimization conditions and maximum DH values.

No	Temperature	Enzyme concentration	pH	Time	Degree hydrolysis	Desira-bility	
1	65	2	9.5	180	34.0431	0.879	Selected
2	65	2	8.5	179	33.983	0.876	
3	65	2	8.5	178	33.8856	0.873	
4	65	2	8.5	180	33.8055	0.870	
5	65	2	8.5	180	33.7673	0.868	

RSM has been used successfully to optimize the parameters affecting the protein hydrolysis (Nilsang *et al.*, 2005; Cao *et al.*, 2008). The response surface was used to study the relationship of DH under hydrolysis conditions (temperature, pH, time, enzyme to substrate level). Based on Table 6, the optimum conditions gave a predicted DH of 34.04%. A validation experiment was carried out to determine the actual DH at the optimum conditions. It was found that the actual experimental DH obtained using the optimal condition was 35.97%. The experimental DH was quite close to the predicted DH. DH is higher than that from silver cat fish skeleton (22.73%) (Amiza *et al.*, 2011) but lower than that from fish soluble concentrate (50%) (Nilsang *et al.*, 2005). The difference in optimum degree of hydrolysis of protein could be due to the type of protein and enzyme used.

Proximate composition

The proximate composition of raw materials (steamed homogenized cockle with 10% water) and the lyophilized cockle hydrolysate powder (prepared under optimum conditions) was determined. Table 7 shows the proximate composition of raw cockle and its hydrolysate powder. The yield for raw cockle is the weight of raw materials used, while the yield for cockle hydrolysate powder is the total weight of the freeze dried powder recovered from the soluble fraction of hydrolysate. The yield of lyophilized cockle hydrolysate powder was 15.5% of the original homogenized cockle.

Table 7. Proximate composition of homogenized steamed cockle and lyophilized cockle hydrolysate powder.

Material	Water (%)	Protein (%)	Carbohydrate (%)	Fat (%)	Ash (%)	Yield (g)
Homogenized steamed cockle	83.08 ± 0.74^{a}	14.17 ± 0.17^{b}	0.87 ± 0.59^{a}	1.17 ± 0.08^{b}	0.71 ± 0.08^{b}	55.00
Lyophilized cockle hydrolysate	8.59 ± 0.08^{b}	74.00 ± 0.57^{a}	1.39 ± 0.57^{a}	5.80 ± 0.91^{a}	10.22 ± 0.68^{a}	8.53

a, b – different letters indicate signify difference in the mean of samples within the same column ($p<0.05$)

Based on Table 7, proximate composition of homogenized steamed cockle (with 10% water (w/w) added during homogenization) shows the major composition was water which accounted for 83.08% of raw cockle. Moisture content of steamed cockle would be higher than raw cockle because during steaming, more water is absorbed by the cockle. Besides, during homogenization 10% water was added to aid homogenization. This explains the high moisture content in steamed minced cockle. A previous study on raw cockle reported lower moisture content in the range of 74.37-81.4% (Nur Nadia *et al.*, 2011; Nurjanah & Kustiyariyah, 2005; Basu & Gupta, 1984). Protein was the second major ingredient in raw cockle (14.17%). Previous studies of raw cockle also reported a similar protein content ranging from 11.7 to 19.48% (Nur Nadia *et al.* 2011; Nurjanah & Kustiyariyah, 2005; Basu & Gupta, 1984). The fat, carbohydrate and ash content accounted for 1.17%, 0.87%, and 0.71%, respectively. These values were in a similar range with previous studies where the fat, carbohydrate and ash content were reported in the range of 1.1-2.5%, 1.41-3.5%, and 0.51-2.4% (Nur Nadia *et al.*, 2011; Nurjanah & Kustiyariyah, 2005; Basu & Gupta, 1984).

The freeze dried cockle hydrolysate powder contained 8.59% moisture, 74% protein, 5.80% fat, 10.22% ash, and 1.39% carbohydrate. Protein content of the lyophilized cockle hydrolysate (74%) was higher than tilapia muscle hydrolysate (37.7%– 49.6%) (Azizah *et al.*,

2001) and Catla viscera hydrolysate (63.13%) (Bhaskar & Mahendrakar, 2008), but lower than those of sardine, mackerel and white croacker (82.7% - 85.1%) (Arvanitoyannis & Kassaveti, 2008). The enzymatic hydrolysis and freeze-drying process had increased the protein, ash and fat content. The drastic increase in ash content was due to addition of NaOH during enzymatic hydrolysis to maintain the specified pH throughout the hydrolysis. Other investigators have reported fish protein hydrolysates with the ash content of 6.9% to 22% (Onodenalore & Shahidi, 1996; Amiza *et al.*, 2011; Benjakul & Morrissey, 1997; Liceaga-Gesualdo & Li-Chan, 1999).

CONCLUSION

The optimum conditions for cockle hydrolysis using Alcalase® were temperature 65°C, enzyme concentration 2%, pH of 9.5 and hydrolysis time 180 minutes. The enzymatic hydrolysis of cockles gave a quadratic fit with the experimental data. Under these optimum conditions, the predicted values for degree of hydrolysis was 34.05%, while the actual experimental data was 37.27%, The lyophilized blood cockle hydrolysate was composed of 8.59% water, 74% protein, 5.80% fat, 10.22% ash and 1.39% carbohydrate.

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