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OPTIMIZATION OF ENZYMATIC HYDROLYSIS CONDITION OF ANGELWING CLAM (*Pholas orientalis*) MEAT USING ALCALASE® TO OBTAIN MAXIMUM DEGREE OF HYDROLYSIS

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ABSTRACT

Optimization of enzymatic hydrolysis conditions of angelwing clam (*Pholas orientalis*) meat (ACM) catalysed by Alcalase® to obtain the maximum degree of hydrolysis (DH) was carried out using Response Surface Methodology (RSM). A three level face-centered central composite design (CCD) was employed. Four independent variables of enzymatic hydrolysis conditions were applied, which were pH (6.5–8.5), temperature (55–65°C), hydrolysis time (30–120 min), and Alcalase® to substrate concentration (0.5–2.5%). The optimum condition was obtained at pH of 7.34, the temperature of 64.1°C, hydrolysis time of 104.2 min and Alcalase® to substrate concentration of 1.65%. It was found that the quadratic model can be used to describe the relationship between enzymatic hydrolysis conditions of ACM with DH. Verification of the model showed that at optimum condition, the predicted DH value (99.47%) was close to the experimental DH value (98.75%) ($p>0.05$). Lyophilized ACM hydrolysate produced under optimum condition composed of 5.76±0.17% moisture, 65.09±1.09% protein, 1.65±0.06% fat, 19.95±0.49 ash%, and 7.56±0.80% carbohydrate. This study showed that the degree of hydrolysis of the enzymatic hydrolysis of ACM catalysed by Alcalase® could be predicted using RSM.

Key words: Blood cockle, angiotensin converting enzyme, peptide, hydrolysis

INTRODUCTION

Protein hydrolysis is commonly used in the food industry to improve physicochemical properties, nutritional quality, sensory properties as well as to prepare bioactive peptides (Chalamaiah *et al.*, 2012). Because of its tremendous benefits, enzymatic protein hydrolysis has been widely applied in human and animal nutrition, pharmaceutical and cosmetics applications. Enzymatic protein hydrolysis has been applied onto various marine sources such as flying squid muscle (Fang *et al.*, 2012), blood cockle (Amiza & Masitah, 2012), grass carp sarcoplasmic (Ren *et al.*, 2008) and shrimp waste (Sowmya *et al.*, 2014).

Degree of hydrolysis is defined as the ratio of the number of peptide bonds cleaved against the total number of peptide bonds in a substrate (Rahulan *et al.*, 2012). The physicochemical, bioactivity, nutritional and sensory properties of protein hydrolysate are closely related to the extent of DH (Jamdar *et al.*, 2010). Nutrition wise, higher DH will contribute to better absorption in the organisms. As for other applications, the DH can be tailored to achieve the intended physicochemical, bioactivity and sensory properties. DH is mainly affected by pH, temperature, hydrolysis time and enzyme concentration (Salwanee *et al.*, 2013). Optimization of enzymatic hydrolysis conditions can be used to target certain protein properties such as maximum degree of hydrolysis or maximum bioactivity.

Optimization of the hydrolysis conditions of protein hydrolysate can be accomplished by implementing response surface methodology (RSM) with a central composite design (CCD), as it has been found to be effective in optimizing and monitoring various food processes (Roslan *et al.*, 2015). It is a collection of mathematical and statistical approaches capable of determining effects, as well as the interactions of multiple factors (Asadi & Zilouei, 2017). Furthermore, it benefits researchers by minimizing the experiment runs while maximizing the generation of large amounts of information

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(Bezerra *et al.*, 2008). RSM has been used in the optimization of enzymatic protein hydrolysis condition to obtain the maximum degree of hydrolysis from various protein sources including silver catfish frame (Amiza *et al.*, 2011), blood cockle (Amiza & Masitah 2012) and visceral waste proteins of catla (Bhaskar *et al.*, 2008).

Angelwing clam (*Pholas orientalis*) is a marine bivalve belonging to the family Pholadidae. It inhabits the tidal flats in Southeast Asian countries and can range from 6.1cm to 13.0cm in size (Ng *et al.*, 2009). Its flesh is described as sweet, juicy, and tender, making it highly sought in Malaysia, Thailand, Philippines (Normah *et al.*, 2014). It also has high demands from Hong Kong and Taiwan. Angelwing clam has numerous name in Southeast Asia, such as "Siput Mentarang" in Malaysia, "Pim" in Thailand, and "Diwal" in the Philippines. Due to the seasonal nature of angelwing clam, converting it into protein hydrolysate powder is an alternative to ensure its availability all year round and convenience (in terms of transportation and application as a food ingredient) and storage stability. Based on a previous study, angelwing clam hydrolysate contained high nutritive value including all essential amino acids (Normah & Razak, 2015). Several studies have been reported on physicochemical and functional properties of angelwing clam hydrolysate (Normah & Nurfazlika, 2013), effect of degree of hydrolysis on bitterness (Normah *et al.*, 2013) and optimization enzymatic hydrolysis condition to obtain maximum angiotensin-converting enzyme inhibitory activity from angelwing clam (Amiza *et al.*, 2017b). However, little information is available regarding the optimization of enzymatic protein hydrolysis to prepare angelwing clam hydrolysate with a maximum degree of hydrolysis. Thus, the objective of this study was to optimize the enzymatic protein hydrolysis condition of angelwing clam using Alcalase® to obtain a maximum degree of hydrolysis.

MATERIALS AND METHODS

Materials

Twelve kilograms of whole fresh angelwing clams was purchased from Telok Ayer Tawar, Butterworth, Penang. They were transported in the icebox with ice cubes to the laboratory. Next, they were then cleaned and washed with tap water for several times to remove contaminants such as mud and soil. After that, the angelwing clams shells were pried open with a knife. Next, the meat was removed from the clams using a spoon. Finally, the angelwing clam meat (ACM) was homogenized using a food processor and then stored frozen in airtight containers for further use.

Chemicals and reagents

Alcalase® was purchased from Sigma-Aldrich Sdn. Bhd, while all other chemicals used in this study were of analytical grades.

Proximate analysis of angelwing clam meat (ACM) and lyophilized ACM hydrolysate

The proximate analysis of ACM and lyophilized ACM hydrolysate involved the determination of moisture, ash, crude protein and crude fat content according to AOAC methods (AOAC, 2000) in duplicate. The crude protein content of ACM was needed prior to optimization study as the data was required to calculate the mass of ACM, water and enzyme needed for each run of enzymatic protein hydrolysis (Kristinsson & Rasco, 2000).

Experimental design of optimization study for ACM hydrolysis

A face-centered central composite design (CCD) was used in this optimization study combined with response surface methodology (RSM). Design Expert 10.0.7 software (Stat-Ease Inc., 2016) was used to generate the 30 experimental runs as well as to analyze the optimization data. Four independent variables were pH, temperature (°C), hydrolysis time (min) and enzyme concentration (%). Each variable was given three equidistant levels (-1, 0, +1). The ranges used for each independent variables were determined by referring to the previous study on optimization of protein hydrolysis using Alcalase®. The response variable was the degree of hydrolysis (DH). The levels of independent variables used in this study are shown in Table 1. There were six center points in this experimental design, in which all six used the intermediate level of the independent variables. Once the hydrolysis was completed for each run, the resulting supernatant from the hydrolysed ACM was lyophilized. This was followed by determination of DH on the lyophilized ACM hydrolysate samples. The data obtained were then analyzed by Design Expert 10.0.7 software.

For verification of the model, four replications of ACM hydrolysis were prepared close to the predicted optimum condition were carried out. The resulting supernatant from the hydrolysates was then freeze-dried prior to DH measurement. This experimental value of DH was then compared with the predicted DH value obtained from RSM under optimum condition, using one sample t-test.

Table 1. Experimental range and values of three equidistant levels for independent variables

Independent variables	Symbol	Range and levels		
		-1	0	+1
pH	X_1	6.5	7.5	8.5
Temperature (°C)	X_2	55	60	65
Hydrolysis time (min)	X_3	30	75	120
Enzyme to substrate concentration (E/S ratio, % w/w)	X_4	0.5	1.5	2.5

Enzymatic hydrolysis of ACM

Hydrolysis mixture of ACM, enzyme and water was prepared according to Hordur & Barbara (2000). First, the homogenized ACM was boiled at 85°C for 20 min in a water bath (TE-10D Temp., Techne, UK) to inactivate endogenous enzymes. The enzymatic protein hydrolysis for preparing ACM hydrolysate was performed according to the method by Zaliha *et al.* (2017). The pH of hydrolysis mixture was adjusted and constantly maintained throughout the hydrolysis as suggested in the experimental design using 1 N NaOH solution using a dropper (the volume used was less than 5% of the total hydrolysis mixture volume). Once the required temperature of the hydrolysis mixture was achieved, the enzyme solution was added and the hydrolysis time was started. Alcalase® activity was terminated by heating the sample in a water bath at 85°C for 20 min. After the hydrolysate was cooled, it underwent centrifugation at 10,000 rpm at 4°C for 10 min. The resulting supernatant was collected and freeze-dried. The freeze-dried hydrolysate was crushed into powder form and kept in airtight containers for further analysis.

Determination of degree of hydrolysis (DH) of lyophilized ACM hydrolysate

DH of the lyophilized ACM hydrolysate was carried out using trichloroacetic acid (TCA) method as described by Hoyle and Merritt (1994). TCA was selected as it is a well-known protein precipitator to remove non-hydrolyzed proteins and higher molecular weight peptides from the hydrolysate, which would result in the retention of soluble peptide and amino acids in the supernatant.

Statistical analysis

Analysis of variance of the RSM model was carried out using Design Expert software (version 10.0.7) (StatEase Inc.). The experimental data from the experiments were fitted into an empirical second-order polynomial using the steepest ascent method according to the following equation:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

Here, Y is the response variable. β_0 is the offset term. β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction regression coefficient variables, respectively. While X_i and X_j are experimental factors. For the models calculated from the linear regression, analysis of variance (ANOVA) was performed and the R^2 value, residual error, pure error and lack of fit were calculated. Optimization was carried out as described in the Design Expert tutorial (https://www.statease.com/docs/v11/tutorials/multifactor-rsm.html#response-surface-design-and-analysis). Model reduction was carried out by eliminating model terms with $p<0.15$. Mean values were accepted as significantly different at 95% level ($p<0.05$). The mean of experimental DH and predicted DH under optimum condition were compared using one-sample t-test (IBM SPSS version 20).

RESULTS AND DISCUSSION

Proximate analysis of raw angelwing clam meat (ACM)

It was found that based on a wet basis, raw ACM contained 88.88% moisture content, 7.94% protein content, 0.81% fat content, 1.99% ash content and 0.39% carbohydrate content.

The moisture content of ACM in this study (88.88%) is comparable to that of Normah and Nurfazlika (2013) (79.60%). While, the protein

Table 2. Proximate composition of raw angelwing clam meat

Components	Angelwing clam meat	
	Wet basis	Dry basis
Moisture (%)	88.88±0.23	–
Protein (%)	7.94±0.13	71.41±0.30
Fat (%)	0.81±0.05	7.24±0.30
Ash (%)	1.99±0.11	17.84±0.59
Carbohydrate (%)	0.39±0.06	3.51±0.58

Data were presented as mean±standard deviation, duplicate (n=2).

content of angelwing clam in this study (7.94%) was lower than those reported by Normah and Nurfazlika (2013) (13.72±0.56), Normah and Razak (2015) (25.91±0.19) and Amiza *et al.* (2016) (9.04±0.06). The differences in the protein content of ACM samples among these studies could be due to the different methods used for protein content determination. This study and Amiza *et al.* (2016) used the Kjeldahl method while Normah and Nurfazlika (2013) and Normah and Razak (2015) used a modified Lowry method. Furthermore, the difference in protein content may be due to differences in the habitat of angelwing clam, sexual changes in connection with spawning and feed intake (Amiza *et al.*, 2016). The fat content of ACM in this study (0.81%) was lower compared to those of Normah and Nurfazlika (2013) (2.82%) and Normah and Razak (2015) (6.43%).

Generally, marine sources, especially molluscs, consisted of high moisture content, followed by protein content and had low fat and ash content as reported in the proximate composition of mud clam, dog conch, and blood cockle (Bawani, 2015; Amiza *et al*., 2017a; Amiza & Masitah, 2012). Likewise, the proximate composition of ACM in this study followed this trend as well.

Analysis of variance (ANOVA) for optimization data

The range of DH obtained from lyophilized ACM hydrolysates prepared from 30 experimental runs was from 67.47% to 98.54%. The highest DH was observed at pH 7.5. the temperature of 60°C, hydrolysis time of 75 min and enzyme concentration of 1.5%, while the lowest DH was observed at pH 6.5, the temperature of 55°C, hydrolysis time of 30 min and enzyme concentration of 2.5%.

The range of DH in this study was comparable to that of mud clam (68.81%–100%), dog conch (70.59%–97.79%) and blood cockle (68.77%–99.28%) (Bawani, 2015; Amiza *et al*., 2017a; Amiza *et al*., 2017b). On the other hand, the DH range obtained from this study was higher than that of mussel meat (7.02%–28.13%) (Silva *et al.*, 2010).

The differences in DH between the studies could be contributed by the differences in protein sources, type of enzymes used, the different levels of parameters (pH, hydrolysis time, enzyme concentration and temperature) applied during enzymatic protein hydrolysis (Sbroggio *et al.*, 2016). In addition, the methods for determination of DH could result in the differences between the DH found in the previous studies. As demonstrated by Morais *et al.* (2013), the DH of whey protein concentrate obtained using soluble protein content method (TCA) was the highest at 44.7%, followed by OPA method, formaldehyde method and osmometry method at 4.1%.

Design Expert 10.0.7 software was used to analyze the optimization data. Table 3 shows the ANOVA table of the quadratic model after model reduction ($p<0.0001$). The model reduction was carried out by eliminating model terms with $p<0.15$. A non-significant lack-of-fit ($p = 0.0816$) shows a good fit to the suggested model. The coefficient of determination (R^2 of 0.8341) was in good agreement with the adjusted R^2 of 0.7996. The table shows that all enzymatic hydrolysis factors (pH, temperature, enzyme concentration, hydrolysis time) affected the DH significantly and there is also 1 significant quadratic term for A.

Final equation for degree of hydrolysis

The final equation in terms of coded factors is as follows:

$$DH\ (\%) = +92.48 + 1.72A + 1.65B + 8.77C + 3.20D - 9.95A^2$$

Table 3. ANOVA table for quadratic model for degree of hydrolysis after model reduction

Source	Sum of Squares	dF	Mean square	F-value	p-value Prob > F	
Model	2384.44	5	476.89	24.14	< 0.0001	significant
A-pH	53.01	1	53.01	2.68	0.1144	
B-Temperature	48.81	1	48.81	2.47	0.1291	
C-Hydrolysis time	1385.84	1	1385.84	70.15	< 0.0001	
D–Enzyme concentration	183.81	1	183.81	9.30	0.0055	
A^2	712.98	1	712.98	36.09	< 0.0001	
Residual	474.10	24	19.75			
Lack of fit	441.58	19	23.24	3.57	0.0816	not significant
Pure error	32.52	5	6.50			
Cor Total	2858.54	29				

$R^2 = 0.8341$; Adjusted $R^2 = 0.7996$.

The final equation in terms of actual factors is as follows:

$$DH (\%) = -519.31991 + 150.98278A + 0.329333B + 0.194988C + 3.19556D - 9.95111A^2$$

Where A represented pH, B represented temperature, C represented hydrolysis time, and D represented enzyme concentration.

For the final equation in terms of coded factors, the higher the coefficient of estimation for a particular term, the stronger the effect of the term towards DH. The positive sign of the coefficient of estimation for all factors (A, B, C and D) shows that each individual factor gave linear effect which resulted in increased DH. For linear effect, DH was most affected by hydrolysis time > enzyme concentration > pH > temperature. While for A at its quadratic level (A^2), the term gave decreasing effect for DH.

Optimization of the degree of hydrolysis (DH)

Design Expert software suggested 100 optimum conditions with the desirability of 1. The desirability of 1 indicated that it was the best solution. Only selected optimum conditions are listed in Table 4. Solution number 1 was chosen which gave a pH of 7.31, temperature of 64.08°C, hydrolysis time of 104.24 min and an enzyme concentration of 1.65%.

The selected optimum condition in this study was compared with those of previous studies. The optimum pH was similar to those of dog conch and tilapia by-product (Amiza *et al*., 2017b; Roslan *et al.*, 2015). However, it was slightly lower than that of mud clam, which was 7.9 (Bawani, 2015). It was also lower than that of salmon skin, eel and horse mackerel, which had an optimum pH of 8.39 and 9, respectively (See *et al.*, 2011; Jamil *et al.*, 2016, Yang *et al.*, 2015). The optimum temperature in this study was higher than those of previous studies reported for hydrolysis of and mussel meat (51°C), silver catfish (55°C) and tilapia scale gelatin (57.6°C) (Silva *et al.*, 2010; Amiza *et al.*, 2011; Mohammad *et al.*, 2015). However, the optimum temperature of hydrolysis of blood cockle (65°C) and fish protein (61.23°C) were comparable to this study (Amiza & Masitah, 2012; Wisuthiphaet *et al.*, 2016).

In terms of hydrolysis time, the optimum hydrolysis time of ACM (104.2 min) was within similar range with that of salmon skin (120 min), horse mackerel (100 min) and tilapia by-product (120 min). However, it was shorter than the hydrolysis time of mud clam and blood cockle, which required 180 min. Furthermore, it took a longer hydrolysis time compared to the visceral waste of yellowfin tuna, shrimp waste and grass carp, which were 90.25 min, 84.42 min and 83.83 min, respectively (Ovissipour *et al.*, 2010; Dey & Dora, 2014; Wasswa *et al.*, 2007).

Lastly, the optimum enzyme concentration in this study was 1.65%, which was in a similar range to that of grass carp and horse mackerel at 1.70% and 1.60% (Wasswa *et al*., 2007; Yang *et al.*, 2015). However, it was slightly lower than that of eel and shrimp waste, which required an enzyme concentration of 1.80% and 1.84% (Jamil *et al.*, 2016; Dey & Dora, 2014). On the other hand, the optimum enzyme concentration for hydrolysis from fish protein and mussel meat were 6% and 4.5% respectively, which were significantly higher than the required concentration in this study (Wisuthiphaet *et al.*, 2016; Silva *et al.*, 2010).

Verification of the model

The verification of optimum condition as suggested by Design Expert 10.0.7 was carried out with four replicates. The mean of the experimental value was 98.75% while the predicted value was 99.48%. By using one sample t-test, it was found

Table 4. Selected suggested optimum conditions to produce the maximum degree of hydrolysis from angelwing clam meat hydrolysis

Number	pH	Temperature	Hydrolysis time	Enzyme concentration	Degree of hydrolysis (%)	Desirability	
1	7.342	64.084	104.237	1.647	99.474	1.000	Selected
2	7.31	63.132	118.614	0.718	98.826	1.000	
3	7.31	64.491	106.621	1.365	99.007	1.000	
4	7.328	59.301	98.239	2.487	99.342	1.000	
5	7.33	62.494	101.148	1.96	99.289	1.000	
6	7.335	57.872	114.446	1.917	100.248	1.000	
7	7.363	55.315	119.047	1.523	99.176	1.000	
8	7.365	64.184	107.608	1.237	99.063	1.000	
9	7.393	57.107	106.139	2.257	99.721	1.000	
10	7.4	64.203	117.348	0.756	99.471	1.000	

Table 5. Comparison of proximate composition of angelwing clam meat and its hydrolysate produced under optimum condition

Components	Angelwing clam meat		Freeze-dried angelwing clam meat hydrolysate	
	Wet basis	Dry basis	Wet basis	Dry basis
Moisture (%)	88.88±0.23	–	5.76±0.17	–
Protein (%)	7.94±0.13	71.41±0.30	65.09±1.09	69.07±1.28
Fat (%)	0.81±0.05	7.24±0.30	1.65±0.06	1.75±0.06
Ash (%)	1.99±0.11	17.84±0.59	19.95±0.49	21.17±0.49
Carbohydrate (%)	0.39±0.06	3.51±0.58	7.56±0.70	8.02±0.73

Data were presented as mean±standard deviation, duplicate (n=2).

that there was no significant difference ($p>0.05$) between the experimental value and the predicted value. Hence, it was concluded that the predicted model could be used to describe the relationship between the DH and enzymatic protein hydrolysis conditions in ACM catalyzed by Alcalase®.

Proximate composition of angelwing clam meat hydrolysate prepared under optimum condition

Table 5 shows the comparison of the proximate composition of ACM as well as its hydrolysate prepared under optimum condition. The protein content of ACM hydrolysate was in the range of 60% to 90% in wet basis, which was mostly reported by many researchers on fish hydrolysates (Chalamaiah *et al.*, 2012). Since protein content could be used as an indicator of the purity of protein hydrolysate (See *et al.*, 2011), this indicated that the ACM hydrolysate has a high level of purity as its major content was protein. The high protein content was contributed by the solubilisation of proteins during hydrolysis as well as the removal of insoluble solid matters through centrifugation.

Several studies have reported on proximate composition of ACM hydrolysate. Normah and Razak (2015) reported that the ACM hydrolysate contained 74.41±0.35% protein and 3.43±0.11% fat. However, the moisture content, ash content and carbohydrate content were not reported. When compared to this study, the findings by Normah and Razak (2015) revealed higher protein content as well as higher fat content. However, ACM hydrolysate in Normah and Nurfazlika (2013) study was composed of 9.51±1.51% moisture, 43.0±0.04% protein and 0.81±0.56% fat, which gave lower protein and fat content than this study.

The difference in the proximate composition of ACM hydrolysates could be due to differences in proximate composition of the raw materials used (as mentioned previously) and the application of different enzymes for enzymatic protein hydrolysis. For instance, Normah and Razak (2015) applied bromelain while Alcalase® was applied by Normah and Nurfazlika (2013) and the current study.

The proximate components of ACM and its hydrolysate were compared based on a dry basis. The protein content of ACM hydrolysate (69.07%) decreased slightly when compared to that of ACM (71.41%). The decrease of protein content in ACM hydrolysate could be explained by the loss of protein during the preparation of lyophilized protein hydrolysate especially the removal of unhydrolyzed protein precipitation after centrifugation. This phenomenon was also observed in the proximate composition of ACM and its hydrolysate produced by Normah and Razak (2015) as well as Normah and Nurfazlika (2013). Likewise, the protein content in lyophilized cockle hydrolysate was also higher than that of homogenized steamed cockle (Amiza and Masitah, 2012).

Conversely, the fat content of ACM hydrolysate (1.75%) based on dry basis was significantly lower than that of ACM (7.24%). This is due to the removal of fat during the hydrolysis process. This was likely due to the heat treatment during the inactivation of enzyme and hydrolysis (Bhaskar *et al.*, 2007). It was also possible that the fat loss occurred during sample storage and centrifugation. The low fat content of ACM hydrolysate improves its lipid oxidative stability since it is less prone to lipid oxidation (See *et al.*, 2011). This also leads to better keeping quality and overall quality.

Unlike protein content and fat content in terms of a dry basis, the ash content of ACM hydrolysate (21.17%) was slightly higher than that of ACM (17.84%). The increase in ash content was due to the addition of NaOH for adjusting pH to optimum pH prior to enzymatic hydrolysis (Amiza & Masitah, 2012).

CONCLUSION

The quadratic model can be used to describe the relationship between enzymatic hydrolysis conditions of angelwing clam meat hydrolysis with its degree of hydrolysis. The optimum condition was obtained at pH of 7.34, the temperature of 64.1°C,

hydrolysis time of 104.2 min and Alcalase® concentration of 1.65%. This study shows that the Response Surface Methodology can be used to predict the effect of hydrolysis conditions to produce the highest degree of hydrolysis from angelwing clam meat. It was found that lyophilized angelwing clam meat hydrolysate produced under optimum condition composed of 5.76±0.17% moisture, 65.09±1.09% protein, 1.65±0.06% fat, 19.95±0.49 ash%, and 7.56±0.80% carbohydrate. Further study should be carried out on the incorporation of angelwing clam on product development and optimization of other bioactive peptides from angelwing clam such as antimicrobial peptide and antioxidative peptide.

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