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Optimization of enzymatic hydrolysis of cockle (*Anadara Granosa*) meat wash water precipitate for the development of seafood flavor

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Abstract

Cockle (Anadara granosa) meat wash water precipitate was hydrolyzed using bromelain. Experiments were carried out to determine optimum conditions for temperature, enzyme concentration and hydrolysis time using response surface methodology (RSM) based on a central composite rotatable design (CCRD) to obtain the highest value of nitrogen content (NC) and degree of hydrolysis (DH). Results revealed that the optimum conditions for temperature, enzyme concentration and hydrolysis time were 33.7°C, 1.45% (E/S) and 28.42 hrs, respectively. At the optimum condition, hydrolysis of cockle meat wash water precipitate using bromelain resulted in a NC of 0.6% and DH of 48%. The NC and DH were significantly influenced by temperature, enzyme concentration and hydrolysis time. When the bromelain concentration, hydrolysis time and temperature were increased, the values of NC and DH also increased. The hydrolysate produced contained flavor compounds found in clam and oyster which were 3-methylbutanol and 1-pentanol. The compound 3-MCPD was not found in the hydrolysate.

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Introduction

Cockle is the common name for a group of small, edible, saltwater clams, marine bivalve mollusks in the family Cardiidae. Various species of cockles live in sandy sheltered beaches throughout the world. The distinctive rounded shells of cockles are symmetrical, and are heart-shaped when viewed from the end. In most but not all genera there are numerous radial ribs. The mantle has three apertures (inhalant, exhalant, and pedal) for siphoning water and for the foot to protrude. Cockles typically burrow using the foot, and feed by filtering plankton from the surrounding water. Like many bivalves, cockles are hermaphroditic and some species reach maturity quickly. Recycling of wash water from cockles to produce protein hydrolysates may contribute to reduced pollution and give benefit to the food industry. During processing, the cockle were de-shelled and the meat washed prior to use. Wash water from this washing step is usually discarded without further processing.

Currently, there are no published information concerning the production of hydrolysate by utilizing the proteins in cockle meat wash water. Enzymes can be used to hydrolyze proteins, thereby allowing the nitrogen to be more soluble, thus making the hydrolyzed mass the most available amino acid source (Espe *et al*., 1989; Vidotti *et al.*, 2003). Also, such hydrolysates can be used as ingredients in aquaculture feeds (Vidotti *et al.*, 2003; Nilsang *et al.*, 2005) and as an effective nitrogen source in microbial growth media (Guerard *et al*., 2001). Several factors, like pH, time, enzyme to substrate ratio and temperature, influence enzymatic activity and thus, offer possibilities to control the process (Viera *et al*., 1995; Liaset *et al.*, 2000). Compared to acidic or alkaline hydrolysis, enzymatic hydrolysis of proteins, using selective peptidases, provides more moderate conditions of the process and few or no undesirable side reactions or products. Enzymatic proteolysis and solubilisation of proteins from various sources has been studied extensively and described by several researchers (Kristinsson and Rasco, 2000a,b; Liaset *et al*., 2000; Nilsang *et al.*, 2005; Bhaskar *et al*., 2007b). Protein hydrolysates with different percentage of degree of hydrolysis were made from minced salmon muscle treated with one of four alkaline proteases (Alcalase 2.4L, Flavourzyme 1000L, Corolase PN-L, and Corolase 7089) or endogenous digestive proteases. Reaction conditions were controlled by pH, temperature, and protein content, and enzymes were added on the basis of standardized activity units (Azocoll units). The preferred commercial enzymes for most researchers are protease preparations of

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bacterial origin like Alcalase, Neutrase, Protease, and also plant proteases like Papain, Bromelain and Ficin. Bromelain and Ficin have yielded a good result in the study of enzymatic fish protein hydrolysate (Lalasidis *et al*., 1978).

The aim of the present study was to elucidate the optimum conditions for the enzymatic hydrolysis of protein hydrolysate from cockle (*Anadara granosa*) meat wash water using bromelain.

Materials and Methods

Optimization of protein hydrolysate production using CCRD

Protein hydrolysate was produced through enzymatic hydrolysis using bromelain. Bromelain was selected based on the study by Stein *et al.* (2005) which showed that it gave the highest total yield of free α-amino groups compared to other proteases such as alcalase, neutrase and papain. The hydrolysis process was carried out based on Montecalvo *et al.* (1984) method. Optimization of the hydrolysis conditions was carried out by employing the response surface methodology (RSM) using a central composite rotatable design (CCRD).

Cockles (*Anadara granosa*) were purchased from a local supplier in Kuala Selangor, Selangor, Malaysia. Samples of cockles were de-shelled after steaming at boiling water temperature for 10 minutes. The cockles meat were then minced using a bowl chopper (A-FW 88100, Beem-Gigant, W. Germany). The minced meat was then subjected to a washing step. Washing was carried out by placing 500 g of minced cockle meat in a beaker (2000 ml). Distilled water was added at a ratio of 1:3 (minced cockle meat: distilled water) and stirred for 30 min at 600 rpm using an overhead stirrer (RW20, Ika Labortechnik, Germany). Subsequently, the cockle meat wash water which had a pH of 6.85 was filtered using a siever and kept at -20°C until further analysis.

Precipitation of the protein in the cockle meat wash water was carried out by adjusting the pH to pH 4 using 4N HCl and 0.1N NaOH. Samples were stirred using a magnetic stirrer for 30 min and left to stand for 1 hr. Samples were then centrifuged at 7800 x g for 30 min. The supernatant was then removed and the precipitate was kept frozen at -20°C before freeze drying (Alpha 1-4 LD Plus, Christ, Germany) for 25 hr. Total nitrogen content of the precipitate was analyzed using Kjeldahl method (AOAC, 1990).

Cockles meat wash water precipitate (2.0 g) from the previous step was defatted using a fat extractor (Soxtex System, Tecator, Sweden) according to the Soxhlet method (AOAC 1990). About 2.0 g of defatted cockle meat wash water precipitate from the previous step was added with distilled water (50 ml) in a conical flask (100 ml). The mixture was then hydrolyzed by heating in an oven at 95°C and cooled down to room temperature before pH was adjusted to pH 6 using 4N NaOH. Subsequently, bromelain was added at different enzyme concentrations (Table 1) along with 2 ml of 0.1M L-Cystein to activate the enzyme. The mixture was hydrolyzed in a shaker incubator (Environ-Shaker) at 200 rpm for different temperature and time period (Table 1). Samples were then heated at 95°C for 15 min to deactivate the enzyme. Each mixture was then divided into two portions which were analyzed for nitrogen content (NC) and degree of hydrolysis (DH). Subsequently, both samples were centrifuged separately at 7800 *g* for 30 min. The supernatant was frozen at -20°C and freeze dried using a freeze-dryer (Alpha 1-4 LD Plus, Christ, Germany) for 24 h. The freeze-dried samples were analyzed for nitrogen content (NC) and degree of hydrolysis (DH).

Experimental data was fitted with statistical models, to produce the response surface. Models were deemed suitable when it is significant based on ANOVA, insignificant lack-of-fit test and R^2 of more than 0.75. The chosen models were subsequently optimized based on the optimization criteria of minimum temperature, enzyme concentration and hydrolysis time while NC and DH were set for maximum.

Nitrogen content (NC) and degree of hydrolysis (DH)

NC was determined using the Kjehdahl method (AOAC, 1990). Degree of hydrolysis (DH) was calculated as described by Hoyle & Merritt (1994) and Fonkwe & Singh (1996). Samples of 20 ml of protein hydrolysate was added to 20 ml 20% trichloroacetic acid (TCA) to get 10% of TCA concentration. The mixture was left to stand for 30 min to precipitate and then followed by centrifugation (7800 x *g* for 15 min) (High Speed Centrifuge, Sorvall HS23, USA). The supernatant was analyzed for nitrogen content by Kjeldahl method (AOAC, 1990) using a protein analyzer (KjeltecTM 2000, Foss-Tecator, Sweden). The hydrolysate was also analyzed for nitrogen content using the same method. DH was determined using the formula below:

$$DH = \frac{\text{Solubility of N in 10\% TCA}}{\text{Total N in the precipitate}} \times 100$$

where DH: degree of hydrolysis; TCA: trichloroacetic acid.

Table 1. Actual and coded (in parentheses) levels of temperature (X_1/x_1), enzyme concentration (X_2/x_2) and hydrolysis time (X3/x3) used for optimization of *Anadara granosa* meat wash water precipitate hydrolysis using bromelain

Run #	$X_1(x_1)$	$X_2(x_2)$	$X_3(x_3)$
1	40.00 (0.000)	3.11 (1.682)	32.00 (0.000)
2	50.00 (1.000)	0.70 (-1.000)	48.00 (1.000)
3*	40.00 (0.000)	1.60 (0.000)	32.00 (0.000)
4*	40.00 (0.000)	1.60 (0.000)	32.00 (0.000)
5*	40.00 (0.000)	1.60 (0.000)	32.00 (0.000)
6	40.00 (0.000)	1.60 (0.000)	5.09 (-1.682)
7	30.00 (-1.000)	0.70 (-1.000)	16.00 (-1.000)
8	50.00 (1.000)	0.70 (-1.000)	16.00 (-1.000)
9	30.00 (-1.000)	2.50 (1.000)	48.00 (1.000)
10	23.18 (-1.682)	1.60 (0.000)	32.00 (0.000)
11	30.00 (-1.000)	0.70 (-1.000)	48.00 (1.000)
12	40.00 (0.000)	1.60 (0.000)	58.91 (1.682)
13	30.00 (-1.000)	2.50 (1.000)	16.00 (-1.000)
14	50.00 (1.000)	2.50 (1.000)	16.00 (-1.000)
15	40.00 (0.000)	0.09 (-1.682)	32.00 (0.000)
16	56.82 (1.682)	1.60 (0.000)	32.00 (0.000)
17*	40.00 (0.000)	1.60 (0.000)	32.00 (0.000)
18	50.00 (1.000)	2.50 (1.000)	48.00 (1.000)
19*	40.00 (0.000)	1.60 (0.000)	32.00 (0.000)
20*	40.00 (0.000)	1.60 (0.000)	32.00 (0.000)

*replication of the centre point

Volatile compounds analysis

Volatile compounds were extracted using Head Space Solid Phase Microextraction (HS-SPME) as described by Martinez-Urunuela *et al.* (2004). About 5 ml of sample was added into a headspace vial (12 ml) and sealed with PTFE/ silicone septum. Then, samples were heated in waterbath at 50°C for 10 min. After that, SPME needle which contained a divinylbenzene-carboxen-polydimethylsiloxane (DVB-CARBOXEN-PDMS) fibre (StableFlex, Supelco, USA) was injected through the septum into the vial and exposed for 10 min. After the extraction, the SPME device was removed from the vial and inserted immediately into a Gas Chromatography Mass Spectrometry (GCMS-QP5050A, Shimadzu, Japan) injection port for thermal desorption. Extraction was done in triplicate using the samples being prepared.

Desorption of volatile compounds from the SPME fibre was carried out with a splitless injector using an inlet SPME 0.75 mm. A non-polar capillary column (HP-5MS, J&W Scientific, USA) (60 m x 0.25 i.d., 0.25 µm film) was used. Helium at a flow of 2 ml/min was used as carrier gas. Oven temperature was programmed as follows: 80°C for 10 min, heated at 20°C/min to 100°C, heated to 150°C at 7.5°C/min and finally raised to 250°C at 30°C/min and held for 2 min.

Amino acid composition

Amino acid content was determined by HPLC according to AOAC (2000) method. Amino acids content were analyzed with three different analysis. For all types of amino acids, the analysis involved hydrolysis using 6N HCl. For cysteine and methionine detection, performic acid analysis were carried out to form the acid derivatives (cysteic acid and sulphonated methionine, respectively). As tryptophan is almost entirely destroyed during acid hydrolysis, the analysis was performed in basic conditions using 4.3N LiOH.

3-MCPD

For 3-MCPD detection, samples protein of hydrolysate from cockle meat wash water precipitate were sent to Doping Control Centre, Universiti Sains Malaysia, Pulau Pinang, Malaysia. This procedure used solid phase extraction followed by derivation to convert the 3-MCPD to 2,2-dimethyldioxolane derivative. Dilution of isotope using deuterated 3-MCPD was used to quantify the analyte through GC-MS analysis. Validation limit for detection of 3-MCPD is 2 ng/ ml.

Data analysis

The experimental design and statistical analysis were performed using response surface methodology (RSM) with Design Expert Version 6.0.10, (Stat Ease, 2003) software. Data was also analyzed using Statistical Analytical System (SAS) version 6.12 for ANOVA and Duncan tests. All experiments were done using three replications. Validation of the optimum point was done using Root Mean Squared Deviation (RMSD) as described by Pineiro *et al.* (2008) using the formula below:

$$RMSD = \sqrt{\frac{1}{n-1}\sum_{i=1}(\hat{y}_i - y_i)^2}$$

where $\hat{y}_i$ = observed value
y_i = predicted value

Results and Discussion

Results of nitrogen content (NC) and degree of hydrolysis (DH) for enzymatic hydrolysis of the cockle wash water precipitate are shown in Table 2. NC and DH experimental data was fitted using linear, quadratic and cubic models. Statistical results suggest that the quadratic model is the most suitable model for both NC and DH. Protein hydrolysate that was produced using different parameters will have different nitrogent content (NC) and degree of hydrolysis (DH). The extent of proteolysis was quantified as the DH, which refers to the percentage of peptide bonds cleaved (Jin *et al*., 2007). The method used to evaluate DH of the peptide bonds is based on the amount of nitrogen released by protein hydrolysis in the presence of a precipitate agent, such

Table 2. Actual levels of independent variables along with the observed values for the response variables, nitrogen content (NC) and degree of hydrolysis (DH) of cockle meat precipitate hydrolyzed using bromelain

Run #	X_1	X_2	X_3	NC (%)	DH (%)
1	40.00	3.11	32.00	0.6983	48.73
2	50.00	0.70	48.00	0.6319	44.19
3*	40.00	1.60	32.00	0.6714	50.33
4*	40.00	1.60	32.00	0.6901	51.97
5*	40.00	1.60	32.00	0.6691	51.82
6	40.00	1.60	5.09	0.3924	36.49
7	30.00	0.70	16.00	0.3048	32.26
8	50.00	0.70	16.00	0.4621	38.78
9	30.00	2.50	48.00	0.6817	50.26
10	23.18	1.60	32.00	0.5826	40.17
11	30.00	0.70	48.00	0.4328	51.94
12	40.00	1.60	58.91	0.7318	51.84
13	30.00	2.50	16.00	0.5937	40.37
14	50.00	2.50	16.00	0.5937	44.23
15	40.00	0.09	32.00	0.3948	36.76
16	56.82	1.60	32.00	0.6623	51.27
17*	40.00	1.60	32.00	0.6837	52.22
18	50.00	2.50	48.00	0.7314	55.18
19*	40.00	1.60	32.00	0.6689	55.12
20*	40.00	1.60	32.00	0.7217	52.73

*replication of the centre point
X_1: Temperature, (°C) X_2: Enzyme concentration, (%) X_3: Hydrolysis time, (h)

Table 3. Model equations fitted for nitrogen content (NC) and degree of hydrolysis (DH) experimental data for enzymatic hydrolysis of cockle meat wash water with bromelain

Responses	Model Equation	Model Significance	Lack of Fit	R^2
NC	Actual Equation $-0.76962 + 0.028724X_1 + 0.50047X_2 + 0.014810X_3 - [2.53010E-004]X_{11} - 0.064365X_{22} - [1.82180E-004]X_{33} - [4.25972E-003]X_{12} + 7.14844E-004X_{13} - [6.25868E-003]X_{23}$ Coded Equation $0.68 + 0.040x_1 + 0.094x_2 + 0.080x_3 - 0.025x_{11} - 0.052x_{22} - 0.047x_{33} - 0.038x_{12} + 0.011x_{13} - [9.013E-003]x_{23}$	<0.0001 (Significant)	0.0899 (Not significant)	0.9698
DH	Actual Equation $-26.81027 + 1.91346X_1 + 16.15504X_2 + 1.00375X_3 - 0.021519X_{11} - 3.95516X_{22} - 0.010553X_{33}$ Coded Equation $52.33 + 1.92x_1 + 3.15x_2 + 5.25x_3 - 2.15x_{11} - 3.20x_{22} - 2.70x_{33}$	<0.0001 (Significant)	0.0519 (Not significant)	0.8872

Note: X_1/x_1 = Temperature; X_2/x_2 = Enzyme concentration; X_3/x_3 = Hydrolysis time

as, trichloroacetic acid (Hoyle and Merritt, 1994).

The response surface equation for the fitting of NC and DH data based on the quadratic models are shown in Table 3. According to the variance analysis, both of the models were significant. The R^2 values for both models were higher than 0.75, indicating a good fit. The R^2 values for NC and DH were 0.9698 and 0.8872, respectively. The lack-of-fit tests were not significant for both NC and DH which also showed a good fit between the experimental data and the model.

Analysis of coefficients for each model used to fit the data of NC and DH are as shown in Table 4. Results in Table 4 shows that, all of the independent variables which were temperature, enzyme concentration and hydrolysis time had significant effects ($p < 0.05$) for both NC and DH. For the interaction variables, model coefficient for NC showed significance ($p < 0.05$) for x_{11}, x_{22}, x_{33} and x_{12}. Model coefficient for DH showed significance ($p < 0.05$) for x_{11}, x_{22} and x_{33}. The coefficients for all independent variables (temperature, enzyme concentration, hydrolysis time) were in positive values both for NC and DH. These showed that when the variables were increased, the NC and DH values also increased.

Table 4. Analysis of coefficients for coded models used to fit nitrogen content (NC) and degree of hydrolysis (DH) experimental data for enzymatic hydrolysis of cockle meat wash water

	NC			DH		
	Coefficient	F	Prob < F	Coefficient	F	Prob < F
Independent variables						
Temperature, x_1	0.040	22.12	<0.0001	1.92	6.19	0.0272
Enzyme concentration, x_2	0.094	124.09	0.0008	3.15	16.65	0.0013
Hydrolysis time, x_3	0.080	90.79	<0.0001	5.25	46.34	<0.0001
Interactions						
x_{11}	-0.025	9.55	0.0114*	-2.15	8.21	0.0133*
x_{22}	-0.052	40.56	<0.0001*	-3.20	18.19	0.0009*
x_{33}	-0.047	32.46	0.0002*	-2.70	12.93	0.0033*
x_{12}	-0.038	12.17	0.0058*			
x_{13}	0.011	1.08	0.3224			
x_{23}	-9.013E-003	0.67	0.4312			

*significant $p < 0.05$

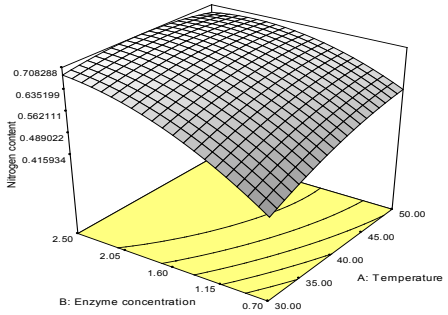

Figure 1. Response surface graph for NC as a function of enzyme concentration and temperature during hydrolysis of cockle meat wash water precipitate using bromelain

Figure 1 shows the response surface for the interaction between enzyme concentration and temperature during hydrolysis of cockle meat wash water precipitate using bromelain for NC. From Figure 1, NC increased when enzyme concentration was increased up to the optimum point at each temperature. Subsequently, increase in enzyme concentration showed a slight decrease in NC. At lower bromelain concentration, only a small amount of peptides were produced resulting in a low NC value. Increasing bromelain concentration allowed the occurence of hydrolysis at a higher degree thus producing a higher NC value in the supernatant. It was reported that increased proteolysis resulted in an increase in the content of soluble forms of nitrogen in hydrolysates during hydrolysis (Jin *et al*., 2007). Increasing the temperature at each enzyme

concentration showed an increase in NC especially at lower enzyme concentration. Increasing both enzyme concentration and temperature produced a response surface with a maximum point for NC as suggested by the negative value of the coefficient (-0.038) (Table 4).

A high DH is needed to produce flavor from protein hydrolysate where it may reduce bitterness and increase the effectiveness of flavor production (Nielsen, 1995). In addition, DH is an important method in controlling proteolytic reaction (Addler-Nissen, 1984). As reported by Rozenn *et al.* (2000), the amount of DH depends on the number of peptide bonds which are present in the preparation. The positive coefficient for temperature (x_1) showed that DH increased when temperature was increased. Nielsen (1995) reported that heat treatment caused the exposure of peptide bonds during enzymatic hydrolysis leading to the increase of DH.

Coefficient for hydrolysis time was also positive indicating that as hydrolysis time increased, DH also increased. Higher hydrolysis time allowed more extensive hydrolysis to occur resulting in a higher DH. This trend was also reported by Dong *et al.* (2008) on silver carp defatted meat during hydrolysis with Alcalase and Flavourzyme. Vijaya *et al.* (2002) also reported an increase in DH when incubation time was increased. The increasing in DH was caused by the increased cleavage of peptide bonds thus increasing the peptides solubility in TCA (Montecalvo *et al.*, 1984). A longer incubation time allowed bromelain to act more extensively on the protein resulting in increased DH. In addition, the value of DH has also been reported to increase with increasing incubation time and temperature during the hydrolysis of protein from *Persian sturgeon* (*Acipenser persicus*) viscera (Mahmoudreza *et al.*, 2009).

DH increased when enzyme concentration was increased as indicated by the positive coefficient of 3.15. With an increase in bromelain concentration, more peptides were hydrolyzed by the enzymes into amino acids and smaller peptides. James *et al.* (2005) reported that increased enzyme concentration generally had a greater effect on reducing hydrolysis time than did increased temperature. Guerard *et al.* (2001) also reported that DH increased with increasing enzyme concentration according to their study on enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacores*) wastes.

The optimum point was determined based on the highest desirability to the responses. The analysis indicated that optimum NC and DH for hydrolysis of cockles wash water precipitate can be achieved using a bromelain concentration of 1.45% (E/S), temperature of 33.7°C and hydrolysis time of 28.42 hr. From the optimization study, NC and DH predicted were 0.6101% and 48.3675%, respectively with a desirability value of 0.682. The observed values from experimental runs were used to evaluate the validity of the optimum point using Root Mean Squared Deviation (RMSD). The small RMSD values (0.0356 for NC) and (4.5724 for DH) indicated the validity of the model.

Table 5. Flavour compounds in protein hydrolysate of cockle meat wash water (*Anadara granosa*) as detected using GC-MS which are also found in other seafoods

Method of hydrolysis	Flavor compounds detected (GC-MS)	Flavor compounds in seafood
Enzymatic hydrolysis	• 1-Butanol, 3-methyl	• Clam
	• 1-Pentanol	• Clam , oyster

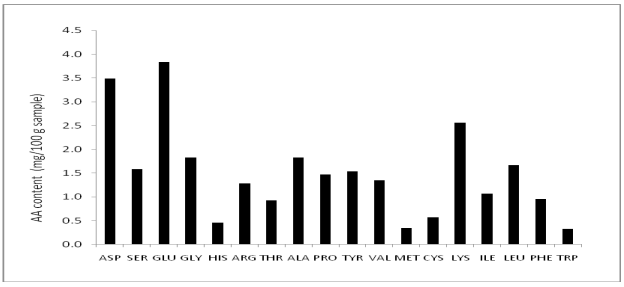

Figure 2. Amino acid composition in protein hydrolysate of cockle (*Anadara granosa*) meat wash water precipitate produced at optimum condition (1.45% bromelain concentration, 28.42h, 33.7˚C)

Figure 2 shows the amino acid composition in the protein hydrolysate from cockle meat wash water precipitate produced using the optimum condition. Amino acid is the major contributor of flavor being produced from protein (Weir, 1992). Results showed that hydrolysate of cockle meat wash water precipitate have 18 types of amino acids, namely aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cystine, isoleucine, leucine, phenylalanine, tryptophan and lysine as reported by Mandiville *et al.* (1992). Amino acids such as arginine, alanine, glutamic acid, serine and glycine are important flavor precursors for crustacean (Hayashi *et al*., 1981). From Figure 2, it can be observed that glutamic acid had the highest value compared to other amino acids.

Table 5 shows the flavour compounds in protein hydrolysates of cockle (*Anadara granosa*) meat wash water precipitate as detected using Gas Chromatography-Mass Spectrometry (GC-MS) (Shimadzu GCMS-QP5050, Japan) which are also found in other seafood. The flavor compounds that was detected were 3-methylbutanol and 1-pentanol. These compounds were known flavor compounds found in clam and oyster (Shahidi, 1998). Analysis of 3-Monochloropropanediol (3-MCPD) in the

hydrolysate also found that the compound was not present in protein hydrolysate using bromelain hydrolysis.

Conclusion

Based on the results, optimum conditions for the hydrolysis of cockle (*Anadara granosa*) meat wash water precipitate carried out using bromelain were 1.45% (E/S) of bromelain concentration at 33.73°C for 28.42 hrs. At the optimum condition, hydrolysis of cockle meat wash water precipitate using bromelain resulted in NC of 0.6% and DH of 48%. The NC and DH were significantly influenced by temperature, enzyme concentration and hydrolysis time. Increased bromelain concentration, hydrolysis time and temperature resulted in increased values of NC and DH. Hydrolysate produced contained flavor compounds found in clam and oyster whic were 3-methylbutanol and 1-pentanol. The compound 3-MCPD was not found in the hydrolysate.

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