Sains Malaysiana 43(1)(2014): 53–63

Process Development for the Production of Protein Hydrolysate from Cockle *(Anadara granosa)* Meat Wash Water

(Pembangunan Proses untuk Penghasilan Hidrolisat Protein daripada Air Basuhan Isi Kerang (*Anadara granosa*))

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ABSTRACT

*A study was carried out to determine the process parameters and optimization for the hydrolysis of protein precipitate from cockle (*Anadara granosa*) meat wash water. Precipitation of the protein in the wash water was done using pH manipulation (pH3-8). The precipitate was hydrolyzed using hydrochloric acid (HCl) and optimized for HCl volume, HCl concentration and hydrolysis time using response surface methodology (RSM) based on a central composite rotatable design. Based on the results, hydrolysis of cockle meat wash water precipitate was carried out by precipitation of the wash water at pH4. Optimum condition for the hydrolysis of 2.0 g of cockle meat wash water precipitate was 25 mL of 1 N HCl for 10 h which resulted in nitrogen content (NC) of 0.7% and degree of hydrolysis (DH) of 55%. NC and DH were significantly influenced only by the hydrolysis time.*

Keywords: Acid hydrolysis; cockle meat wash water; degree of hydrolysis; nitrogen content; protein precipitate

ABSTRAK

*Satu kajian telah dijalankan untuk menentukan parameter dan pengoptimuman proses hidrolisis mendakan protein daripada air basuhan isi kerang (*Anadara granosa*). Pemendakan protein di dalam air basuhan tersebut dilakukan menggunakan pengubahsuaian pH (pH3-8). Mendakan dihidrolisis menggunakan asid hidroklorik (HCl) dan isi padu HCl, kepekatan HCl dan masa hidrolisis dioptimumkan menggunakan Kaedah Respon Permukaan (RSM) berdasarkan reka bentuk pemutaran titik tengah. Berdasarkan keputusan tersebut, hidrolisis mendakan air basuhan isi kerang dijalankan menggunakan pemendakan air basuhan pada pH4. Keadaan optimum bagi hidrolisis 2.0 g mendakan air basuhan isi kerang adalah 25 mL 1 N HCl pada 10 jam dengan menghasilkan kandungan nitrogen 0.7% dan darjah hidrolisis 55%. Kandungan nitrogen dan darjah hidrolisis secara signifikannya dipengaruhi oleh masa hidrolisis sahaja.*

Kata kunci: Air basuhan isi kerang; darjah hidrolisis; hidrolisis berasid; kandungan nitrogen; mendakan protein

INTRODUCTION

Proteins have various biological functions in plants and animals. Protein hydrolysates have been used since the 1940s for the nutritional management of individuals who cannot digest protein. Protein hydrolysates are mixtures of oligopeptides, polypeptides and free amino acids. They may be a source of easily available protein in remedies used to cure metabolic diseases or in dietary/ medicinal preparations (Magdalena et al. 2002). Food protein hydrolysates have a wide range of applications as ingredients in the areas of nutrition, food industry, healthcare and cosmetics (Radha et al. 2007).

Cockles (*Anadara granosa*) are edible bivalves commonly found in South East Asia. Cockles live on open sandy areas of the intertidal zone, feeding in shallow water when submerged and burying themselves just under the ground surface while the tide is out. Cockles feed by filtering huge amounts of seawater. These bivalves are usually consumed whole. These bivalves also contain volatile components which are considered as the most important determinant for their flavor quality (Shahidi 1998). Cockles are consumed fresh and also converted into processed products such as sauces. Commonly, the preparation of cockles involve a washing step. Wash water from this washing step is usually discarded without further processing.

Recycling of wash water from cockles to produce protein hydrolysates may reduce the pollution and give benefit to the food industry. Among the methods available for preparing hydrolysates, dilute acid hydrolysis has been reported to be the best from the economic viewpoint (Herrera et al. 2003). According to Nilsang et al. (2005), the production of seafood flavors from under utilized fish species, using protein hydrolysis, is very challenging in order to ensure a high organoleptic quality. The hydrolysis of protein is often accompanied with flavor defects such as bitterness and off-flavor. Shellfish wastes have a great potential for use as raw material for bioconversion processes, particularly when the ease of collection, the quantities available and the degree of pretreatment

needed are considered. Recovering and utilization of the proteins lost to the waste water can alleviate environmental pollution and generate potential profits (Huang & Michael 1998). Production of protein hydrolysate using acid hydrolysis can be used as some of the enzymatic processes which have been evaluated previously are slow and not cost effective (Ferrer et al. 1996).

Although there is considerable information on the precipitation of proteins from fish and shrimp processing waste effluents (Fabienne et al. 2007; Hang et al. 1980; Nilsang et al. 2005), there is no information dealing with the recovery of proteinaceous materials and the production of hydrolysate from cockle meat wash water. This study had been carried out to improve the functional properties of protein hydrolysate of recycled cockle meat wash water. Thus, the objective of this study was to determine the process parameters and optimize the production of protein hydrolysate from cockle meat wash water.

Materials and Methods

Protocol for cockle meat wash water

Although the actual purpose of the study was to produce hydrolysate from industrial waste water, due to the large variability of its composition, a protocol was needed to produce wash water sample to be used in this study.

Cockles, 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 min. Cockle meats 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 (1000 mL). Distilled water was added at a ratio of 1:3 (minced cockle meat: distilled water) and stirred for different time length (0, 15 and 30 min) at 600 rpm using a stirrer (RW20, Ika Labortechnik, Germany) before being sieved using a siever. Cockle meat wash water, which had a pH of 6.85 were kept directly in freezer at -20°C until further analysis. Before using, the frozen cockle meat wash water was thawed at 4°C until defrosted.

Proximate composition (protein, fat, moisture, ash and carbohydrate) of the raw material and final product were determined according to AOAC methods (1990). All protein measurements (N × 6.25) in the samples were carried out by a standard Kjeldahl method (AOAC 1990) using Kjeltec protein analyzer (Foss–Tecator AB, Sweden). Amino acid content was determined by HPLC using a Amino Acid Analysis Column (C-18 AccQ.Taq™ Column, Waters, Ireland). pH values were measured by pH meter (PHM 210; Radiometer Analytical, France) calibrated using pH4 and pH7 buffers. Data was analyzed using Statistical Analytical System (SAS) version 6.12 for ANOVA test. All experiments were carried out using three replications. The best washing time was selected to be used in the determination of pH for protein precipitation.

Determination of pH for protein precipitation

Precipitation of the protein in the wash water was carried out by pH adjustment using 4N HCl and 0.1 N NaOH. The pH range used was 3 to 8. Frozen cockle meat wash water was thawed and subsequently stirred for 30 min and left to stand for 1 h at room temperature (27°C). Samples were then centrifuged at 7800 × g for 30 min at 4°C. The supernatant was then removed and the precipitate was directly kept frozen at -20°C in freezer before freeze drying (Alpha 1-4 LD Plus, Christ, Germany) for 25 h. The dried samples were analyzed for protein content using Kjeldahl method (AOAC 1990). The pH which produced the highest protein content was selected and used to produce protein precipitate for protein hydrolysis.

Optimization of protein hydrolysate production

Acid hydrolysis was carried out based on Andrews and Alichinidis (1990) with some modifications. Optimization of the hydrolysis conditions were carried out by employing the response surface methodology (RSM) using a central composite rotatable design (CCRD). About 2.0 g of freeze-dried precipitate from cockle meat wash water was defatted using a fat analyzer (Soxtex System, Tecator, Sweden) using hexane. HCl at different concentrations and volume was added to the defatted precipitate. The mixture was then hydrolyzed in an oven for several time at 95°C. After hydrolyzing, the pH was adjusted to 6.0 with 4 N NaOH. The mixture was then divided into two portion which will be analyzed for nitrogen content (NC) and degree of hydrolysis (DH). Then, both of them were centrifuged separately at 7800 g for 30 min at 4°C. The supernatant were analyzed for nitrogen content (NC) and degree of hydrolysis (DH). The remaining supernatant were frozen at -20°C and freeze dried using a freeze-dryer (Alpha 1-4 LD Plus, Christ, Germany) for 24 h.

The range and levels of the experimental factors for HCl volume (x_1), HCl concentration (x_2) and hydrolysis time (x_3) are as shown in Tables 1 and 2, respectively. 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 and r^2 of more than 0.75. The chosen models were subsequently optimized based on the optimization criteria of minimum HCl volume, HCl 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 combined methods described by Hoyle and Merritt (1994) and Fonkwe and Singh (1996). After hydrolyzing, 20 mL of protein hydrolysate was added to 20 mL 20% trichloroacetic acid (TCA) to collect the 10% TCA soluble material as the supernatant. The mixture was left to stand

TABLE 1. Actual and coded levels of HCl volume (x_1), HCl concentration (x_2) and hydrolysis time (x_3) used for optimization of *Anadara granosa* meat wash water hydrolysis

Run Order	x_1	x_2	x_3
1*	20.00 (0.000)	1.75 (0.000)	6.50 (0.000)
2*	20.00 (0.000)	1.75 (0.000)	6.50 (0.000)
3	11.59 (-1.682)	1.75 (0.000)	6.50 (0.000)
4	20.00 (0.000)	1.75 (0.000)	0.61 (-1.682)
5	15.00 (-1.000)	1.00 (-1.000)	10.00 (1.000)
6	25.00 (1.000)	1.00 (-1.000)	10.00 (1.000)
7	20.00 (0.000)	3.01 (1.682)	6.50 (0.000)
8	15.00 (-1.000)	2.50 (1.000)	3.00 (-1.000)
9	15.00 (-1.000)	2.50 (1.000)	10.00 (1.000)
10	28.41 (1.682)	1.75 (0.000)	6.50 (0.000)
11	25.00 (1.000)	2.50 (1.000)	10.00 (1.000)
12	25.00 (1.000)	1.00 (-1.000)	3.00 (-1.000)
13	20.00 (0.000)	0.49 (-1.682)	6.50 (0.000)
14	15.00 (-1.000)	1.00 (-1.000)	3.00 (-1.000)
15*	20.00 (0.000)	1.75 (0.000)	6.50 (0.000)
16*	20.00 (0.000)	1.75 (0.000)	6.50 (0.000)
17	20.00 (0.000)	1.75 (0.000)	12.39 (1.682)
18*	20.00 (0.000)	1.75 (0.000)	6.50 (0.000)
19	25.00 (1.000)	2.50 (1.000)	3.00 (-1.000)
20*	20.00 (0.000)	1.75 (0.000)	6.50 (0.000)
21	11.59 (-1.682)	1.75 (0.000)	6.50 (0.000)
22	20.00 (0.000)	1.75 (0.000)	0.61 (-1.682)
23	15.00 (-1.000)	1.00 (-1.000)	10.00 (1.000)
24	25.00 (1.000)	1.00 (-1.000)	10.00 (1.000)
25	20.00 (0.000)	3.01 (1.682)	6.50 (0.000)
26	15.00 (-1.000)	2.50 (1.000)	3.00 (-1.000)
27	15.00 (-1.000)	2.50 (1.000)	10.00 (1.000)
28	28.41 (1.682)	1.75 (0.000)	6.50 (0.000)
29	25.00 (1.000)	2.50 (1.000)	10.00 (1.000)
30	25.00 (1.000)	1.00 (-1.000)	3.00 (-1.000)
31	20.00 (0.000)	0.49 (-1.682)	6.50 (0.000)
32	15.00 (-1.000)	1.00 (-1.000)	3.00 (-1.000)
33	20.00 (0.000)	1.75 (0.000)	12.39 (1.682)
34	25.00 (1.000)	2.50 (1.000)	3.00 (-1.000)

*replication of the centre point
x_1: HCl volume, x_2: HCl concentration, x_2: time

TABLE 2. Result of composition analysis in cockles (*Anadara granosa*) meat

Component	Percentage, d.b (%)
Protein	62.71±1.21
Fat	7.61±0.68
Ash	11.84±0.25
Carbohydrate	17.84±1.35

for 30 min to precipitate followed by centrifugation (7800 × g for 15 min) with High Speed Centrifuge, Sorvall HS23, USA. The supernatant was analyzed for protein content by Kjeldahl method using a protein analyzer (Kjeltec™ 2000, Foss-Tecator, Sweden). Sample from the hydrolysate was also analyzed for protein content using the same method. DH was determined using the formula below:

$$DH = \frac{\text{soluble N in 10\% TCA, a}}{\text{Total N in the sample, b}} \times 100,$$

where DH is the Degree of hydrolysis and TCA is the Trichloroacetic acid.

Data Analysis

Data was analyzed using Statistical Analytical System (SAS) version 6.12 for ANOVA test and DUNCAN. All experiments were done using three replication. Optimization was carried out using Design Expert Software Version 6.0.10 (StatEase 2003). Validation was done using Root Mean Squared

Deviation (RMSD) as described by Pineiro et al. (2008) as:

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

$\hat{y}_i$ is the observed value and y_i is the predicted value.

Results and Discussion

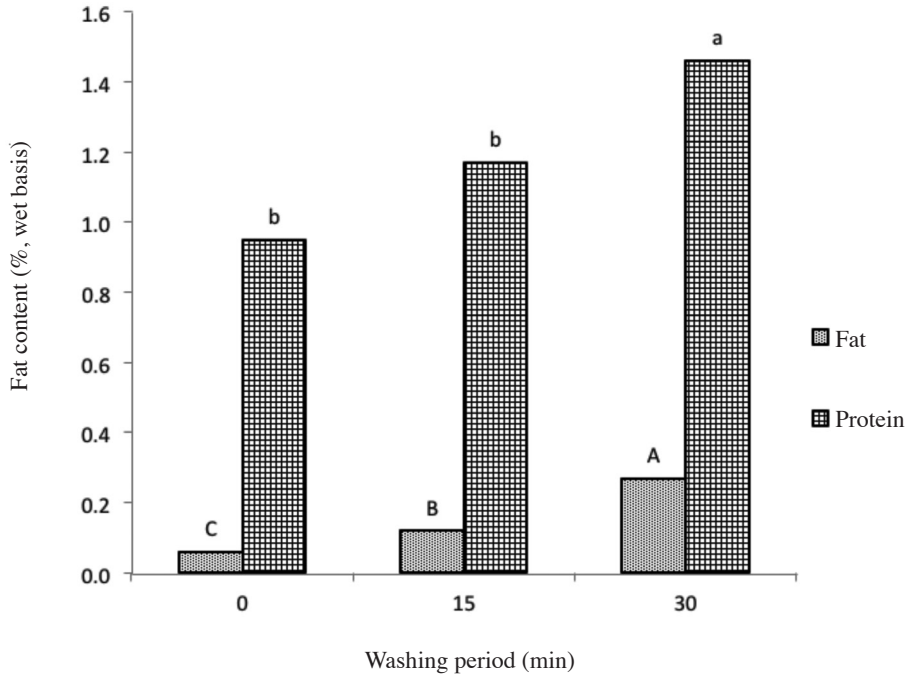
Composition analysis of cockle meat

Table 2 shows the results of proximate analysis for cockle meat. Moisture content for cockle meat was 97.47±1.36% (wet basis). Moisture content in oyster (*Crassostrea gigas*) was reported to be 49.57%, w.b. (Romeroa et al. 2004). The results from Table 2 showed that protein had the highest value at 62.71±1.21% (dry basis). The protein content in cockle meat was found to be much higher if compared to protein content in oyster (*Crassostrea gigas*) (49.57%, d.b.) (Romeroa et al. 2004), Chinese mitten crab (18.9±0.5%, w.b) (Chen et al. 2007) and shrimp waste (51.9±0.72%, d.b.) (Guerard et al. 2007). Fat content of cockle meat had a value of 7.61±0.68%, d.b., not much different when compared to oyster (*Crassostrea gigas*) (8.97%) (Romeroa et al. 2004), but apparently greater than the fat content of Chinese mitten crab (0.9±0.1%, w.b.) (Chen et al. 2007). Ash content in cockle meat was only 11.84±0.25% d.b., lower than oyster (*Crassostrea gigas*) (12.39%) (Romeroa et al. 2004), but the value was higher if compared to Chinese mitten crab which has an ash content of 1.39±0.01%, w.b. (Chen et al. 2007). From the analysis, carbohydrate was found to be 17.84±1.35%, d.b. Carbohydrate content in oyster (*Crassostrea gigas*) (Romeroa et al. 2004) was found to be 70.93%.

Determination of washing period

Figure 1 shows the percentage of protein and fat content in wash water from cockle meat using different washing period. Washing is an essential step in removing water-soluble proteins, primarily sarcroplasmic proteins (Jae & John 2004). Overall, the effect of washing period on total protein content in wash water from cockle meat showed significantly (p<0.05) increasing protein content with increased washing period up to 30 min. When the washing time increased, soluble protein in cockle meat will dissolves, leading to an increase in protein content. The solubility of proteins during washing increased as well when fish were held for a longer period and/or at higher temperature (Jae & John 2004). Babbit (1990) reported that extensive washing might cause myofibrillar proteins to dissolve in water. Antonomanolaki et al. (1999) reported that aqueous washing with tap water resulted in the removal of fat and water soluble proteins. A proper washing process is vital to achieve high-quality surimi with high recovery (Jae & John 2004).

As can be seen in Figure 1, the fat content in cockle meat wash water showed significant difference (p<0.05) when washed using different washing period. Fat content was observed to significantly increase (p<0.05) when the washing period was increased up to 30 min. The results of fat content showed a similar trend to protein content (Figure 1). Similar to protein content, increased exposure



[A-C] Means with the different alphabet denotes significant differences (p>0.05)
[a-b] Means with the different alphabet denotes significant differences (p>0.05)

FIGURE 1. Percentage of protein and fat content in wash water from cockle meat using different washing period

of fat from the minced cockles to the water resulted in the migration of fat into the water system. Based on the results, washing period of 30 min were selected and used for the next step of the process development due to its highest yield of protein content.

DETERMINATION OF PH FOR PROTEIN PRECIPITATION

The weight of precipitate from wash water of cockle meat at different pH is shown in Figure 2. The weight of wet precipitate increased significantly (p<0.05) from pH3 to 4. Then, it decreased significantly when the pH value increased from pH4 to 8 except for pH6. Sample at pH4 showed the highest weight of wet precipitate, (4.98g/100 mL) as compared with other samples. It also shows the weight of precipitate based on dry weight after freeze-drying to remove the moisture. Similar to its wet weight, dry weight of precipitate at pH4 was significantly (p<0.05) the highest at 0.57g/100 mL compared to samples at other pH.

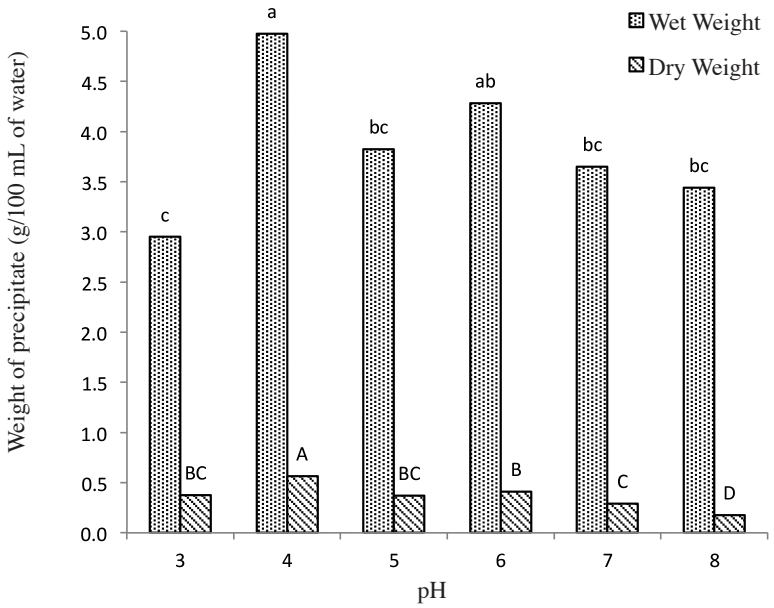
The pH for precipitation of protein from cockle meat wash water was similar to the pH used for other species. Precipitation of fish protein was normally carried out by adjusting the pH at its isoelectric point between the pH range of 3.8 to 4.9 (Hang et al. 1980; Vega & Brennan 1987). According to Kasran (2004), the weight of precipitate from water used for boiling prawns was highest at pH4, (0.2182 g/100 mL). Similarly, Toma and Meyers (1975) had precipitated protein from prawn waste water at pH of 4.5. Thus, based on the results, pH4 was chosen as the precipitation pH and used to prepare protein precipitate for hydrolysis.

PROTEIN AND AMINO ACID CONTENT IN PRECIPITATE OF DIFFERENT pH

Figure 3 shows the protein content of precipitate from wash water of cockle meat significantly increased (p<0.05) when pH was increased from 3 to 4. At pH4, percentage of protein content showed the highest value (68.10%). The value was slightly lower compared to the protein content of precipitate from water used to boil prawns (77.73%) (Kasran 2004) but higher than the protein content of precipitate from prawn waste water (58.98%) (Toma & Meyers 1975). The protein content subsequently shows a decreasing trend when pH was further increased to 7 and 8.

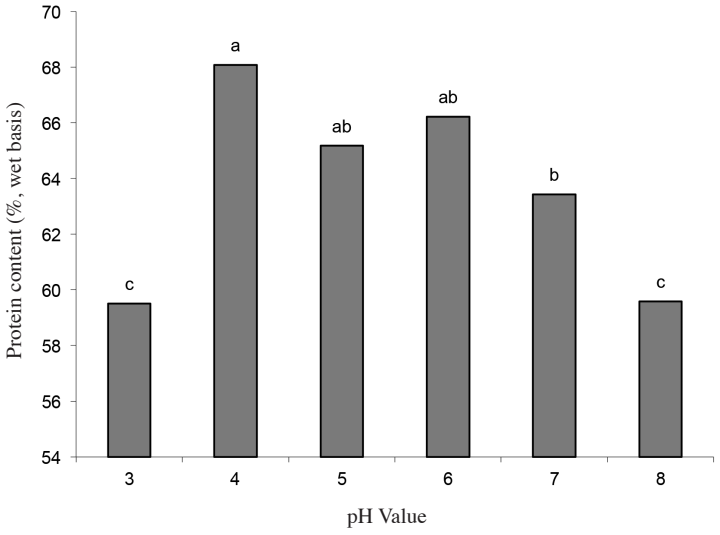
The results from amino acid analysis (Table 3) showed that all amino acid types were present in the precipitate for all pH used. Amino acid is the major contributor of flavor being produced from protein (Weir 1992). The results showed that precipitate of cockle meat wash water have 18 types of amino 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).

From Table 3, almost all amino acids content of precipitate of cockle meat wash water at pH4 showed the highest value compared with precipitate at other pH. Essential amino acids such as histidine, threonine, valine, methionine, isoleucine, leucine, phenylalanine, tryptophan and lysine contributed to 2.176, 2.753, 1.587, 1.802, 1.778 and 2.223 mg/100 g of total protein precipitated at pH3, 4, 5, 6, 7 and 8, respectively. Among the pH analyzed, precipitate from cockle meat wash water at pH4 produced one of the highest essential amino acid content. Amino



[A-B] Means with the different alphabet denotes significant differences (p>0.05)
[a-c] Means with the different alphabet denotes significant differences (p>0.05)a

FIGURE 2. Weight of wet and dry precipitate from wash water of cockle meat at different pH



[a-c] Means with the different alphabet denotes significant differences (p>0.05)

FIGURE 3. Percentage of protein content of precipitate from cockle meat wash water at different pH

TABLE 3. Amino acid composition (%) of precipitate from cockle meat wash water at different pH

Amino Acids	pH3	pH4	pH5	pH6	pH7	pH8
Aspartic acid	0.900^{c}	1.021^{bc}	0.913^{c}	1.230^{a}	1.143^{ab}	1.136^{ab}
Serine	0.436^{a}	0.461^{a}	0.420^{a}	0.456^{a}	0.363^{a}	0.434^{a}
Glutamic acid	0.783^{a}	0.873^{a}	0.912^{a}	0.779^{a}	0.742^{a}	0.857^{a}
Glycine	0.239^{a}	0.292^{a}	0.248^{a}	0.339^{a}	0.300^{a}	0.328^{a}
Histidine *	0.122^{a}	0.160^{a}	0.121^{a}	0.082^{a}	0.101^{a}	0.133^{a}
Arginine	0.379^{ab}	0.465^{a}	0.192^{c}	0.292^{bc}	0.324^{bc}	0.423^{ab}
Threonine*	0.242^{a}	0.280^{a}	0.162^{a}	0.210^{a}	0.201^{a}	0.269^{a}
Alanine	0.428^{a}	0.478^{a}	0.413^{a}	0.476^{a}	0.394^{a}	0.468^{a}
Proline	0.216^{c}	0.345^{bc}	0.562^{a}	0.249^{c}	0.206^{c}	0.415^{b}
Tyrosine	0.211^{a}	0.262^{a}	0.123^{a}	0.180^{a}	0.190^{a}	0.251^{a}
Valine *	0.237^{ab}	0.325^{a}	0.142^{b}	0.173^{ab}	0.165^{ab}	0.204^{ab}
Methionine *	0.191^{a}	0.239^{a}	0.136^{a}	0.175^{a}	0.156^{a}	0.227^{a}
Cystine	0.081^{b}	0.147^{a}	0.056^{b}	0.051^{b}	0.039^{b}	0.027^{b}
Lysine *	0.339^{a}	0.406^{a}	0.263^{a}	0.287^{a}	0.272^{a}	0.343^{a}
Isoleucine *	0.212^{ab}	0.275^{a}	0.122^{b}	0.164^{b}	0.147^{b}	0.188^{ab}
Leucine *	0.553^{abc}	0.662^{a}	0.400^{c}	0.464^{bc}	0.502^{abc}	0.592^{ab}
Phenylalanine *	0.207^{a}	0.252^{a}	0.143^{a}	0.189^{a}	0.185^{a}	0.224^{a}
Tryptophan	0.073^{ab}	0.154^{a}	0.098^{ab}	0.058^{b}	0.049^{b}	0.043^{b}

[a-c] Means with the different alphabet within the same row denotes significant differences (p>0.05)
* Essential amino acid

acids such as arginine, alanine, glutamic acid, serine and glycine are important flavor precursors for crustacean (Hayashi et al. 1981). These amino acids which were important flavor precursors for crustaceans, were found at comparable or higher levels in the precipitate of cockle meat wash water of pH4 compared with other pH.

OPTIMIZATION OF HYDROLYSIS PARAMETERS

The results of nitrogen content (NC) and degree of hydrolysis (DH) for acid hydrolysis of the cockle meat wash water precipitate using central composite rotatable design (CCRD) are shown in Table 4. NC and DH experimental data was fitted using linear, quadratic and cubic models. Statistical results suggested that quadratic model was the most suitable model for both NC and DH.

The response surface equation for the fitting of NC and DH data based on the quadratic models are shown in Table 5. According to the variance analysis, both 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 0.9609 and 0.8854, respectively. The lack-of-fit test was not significant for both NC and DH which also

TABLE 4. Actual levels of independent variables along with the observed values for nitrogen content (NC) and degree of hydrolysis (DH)

Standard Order	x_1	x_2	x_3	NC (%)	DH (%)
1*	20.00	1.75	6.50	0.4324	47.67
2*	20.00	1.75	6.50	0.4402	47.50
3	11.59	1.75	6.50	0.4174	47.61
4	20.00	1.75	0.61	0.3931	47.62
5	15.00	1.00	10.00	0.6172	52.34
6	25.00	1.00	10.00	0.6684	54.47
7	20.00	3.01	6.50	0.6333	52.40
8	15.00	2.50	3.00	0.5027	48.01
9	15.00	2.50	10.00	0.5089	48.06
10	28.41	1.75	6.50	0.6541	54.49
11	25.00	2.50	10.00	0.6832	54.52
12	25.00	1.00	3.00	0.3709	47.00
13	20.00	0.49	6.50	0.3020	45.91
14	15.00	1.00	3.00	0.6432	52.98
15*	20.00	1.75	6.50	0.5860	51.97
16*	20.00	1.75	6.50	0.5897	54.10
17	20.00	1.75	12.39	0.5893	49.77
18*	20.00	1.75	6.50	0.5880	51.98
19	25.00	2.50	3.00	0.5897	52.00
20*	20.00	1.75	6.50	0.5900	52.06
21	11.59	1.75	6.50	0.5500	49.85
22	20.00	1.75	0.61	0.3110	45.52
23	15.00	1.00	10.00	0.656	53.15
24	25.00	1.00	10.00	0.7041	55.43
25	20.00	3.01	6.50	0.4368	47.98
26	15.00	2.50	3.00	0.3870	47.35
27	15.00	2.50	10.00	0.5647	49.87
28	28.41	1.75	6.50	0.5979	51.12
29	25.00	2.50	10.00	0.5249	49.42
30	25.00	1.00	3.00	0.5144	49.86
31	20.00	0.49	6.50	0.6100	52.39
32	15.00	1.00	3.00	0.4128	46.68
33	20.00	1.75	12.39	0.6276	52.59
34	25.00	2.50	3.00	0.3934	47.29

*replication of the centre point

x_1: HCl volume, (ml) x_2: HCl concentration, (N) x_3: time, (h)

TABLE 5. Model equations fitted for nitrogen content and degree of hydrolysis experimental data for hydrolysis of cockles wash water with HCl

Responses	Model equation	Model significance	Lack of fit	r^2
NC	Actual equation $-0.93994 + 0.16443x_1 - 0.10894x_2 + 0.099754x_3 - (7.39598 \times 10^{-3})x_{11} - 0.18196x_{22} - (4.78467 \times 10^{-3})x_{33} - (6.61667 \times 10^{-3})x_{12} - (5.80714 \times 10^{-4})x_{13} - (5.02857 \times 10^{-3})x_{23} + (1.20013 \times 10^{-4})x_{111} - 0.042596x_{222} + (6.69065 \times 10^{-5})x_{333}$	<0.0001 (Significant)	0.4275 (Not significant)	0.9609
	Coded equation $0.59 - 0.014x_1 - 0.021x_2 + 0.090x_3 - (4.879 \times 10^{-3})x_{11} - 0.023x_{22} - 0.043x_{33} - 0.025x_{12} - 0.010x_{13} - 0.013x_{23} + 0.015\ x_{111} - 0.018x_{222} + (2.869 \times 10^{-3})x_{333}$			
DH	Actual equation $-5.13536 + 6.27007x_1 - 0.93944x_2 + 2.52415x_3 - 0.27720x_{11} - 3.54674x_{22} - 0.086168x_{33} - 0.20517x_{12} - 0.012107x_{13} - 0.32976x_{23} + (4.31622 \times 10^{-3})x_{111} - 0.87867x_{222} + (9.81867 \times 10^{-5})x_{333}$	<0.0001 (Significant)	0.3824 (Not significant)	0.8854
	Coded equation $52.00 - 0.38x_1 - 0.72x_2 + 2.09x_3 - 0.46x_{11} - 0.60x_{22} - 1.03x_{33} - 0.77x_{12} - 0.21x_{13} - 0.87x_{23} + 0.54x_{111} - 0.37x_{222} + (4.210 \times 10^{-3})x_{333}$			

Note: x_1 = HCl volume; x_2 = HCl concentration; x_3 = Hydrolysis time

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 6. The results in Table 6 shows that, among the independent variables, only hydrolysis time had a significant effect (p<0.05) for both NC and DH. Both HCl concentration and volume did not show any significant effect on both NC and DH. For the interaction variables, model coefficient for NC showed significance (p<0.05) for $x_{22,}$ $x_{33,}$ $x_{12,}$ x_{111} and x_{222}. Model coefficient for DH showed significance for $x_{22,}$ $x_{33,}$ $x_{12,}$ x_{23} and x_{111}.

From Table 6, interaction between HCl volume (x_1) and HCl concentration (x_2) was shown to be significant (p<0.05). Figure 4 shows the response surface for the interaction between HCl volume and HCl concentration for NC. From Figure 4, NC decreased when HCl concentration was increased at each HCl volume. The decrease in NC might be due to the deamidation process which involved the release of aspartic acid and ammonia (Howell 1996). Chan and Ma (1999) reported that as acid concentration increased, degree of deamidation also increased. They also reported that when the level of HCl concentration increased, the protein content of okara protein isolates decreased slightly. As proteins were broken down as a side reaction during acid hydrolysis, the small peptides may be lost during hydrolysis and this will lead to a decrease in protein content.

At lower HCl concentration, increasing HCl volume produced only a slight increase in NC. However, at higher HCl concentration, increase in HCl volume resulted in a decrease in NC. The results showed that HCl concentration has more influence on NC than HCl volume. Increasing both HCl concentration and volume produced a decrease in NC as suggested by the negative value of the coefficient (-0.025) (Table 6).

Figure 5 shows the DH as a function of HCl volume and concentration for the hydrolysis of cockle meat wash water

TABLE 6. Analysis of coefficients for models used to fit NC and DH experimental data for hydrolysis of cockles meat wash water

	NC			DH		
	Coefficient	F	Prob <F	Coefficient	F	Prob < F
Independent variables						
HCl volume, x_1	-0.014	1.38	0.2524	-0.38	0.56	0.4618
HCl concentration, x_2	-0.021	3.35	0.0815	-0.72	2.03	0.1689
Hydrolysis time, x_3	0.090	59.10	<0.0001*	2.09	16.88	0.0005*
Interactions						
x_{11}	-4.879E-003	0.69	0.4141	-0.46	3.18	0.0888
x_{22}	-0.023	16.03	0.0006*	-0.60	5.52	0.0287*
x_{33}	-0.043	53.00	<0.0001*	-1.03	16.33	0.0006*
x_{12}	-0.025	12.74	0.0018*	-0.77	6.44	0.0192*
x_{13}	-0.010	2.14	0.1578	-0.21	0.49	0.4923
x_{23}	-0.013	3.61	0.0714	-0.87	8.15	0.0095*
x_{111}	0.015	6.45	0.0191*	0.54	4.39	0.0486*
x_{222}	-0.018	9.26	0.0062*	-0.37	2.07	0.1650
x_{333}	2.869E-003	0.24	0.6322	4.210E-003	2.670E-004	0.9871

* significant p<0.05

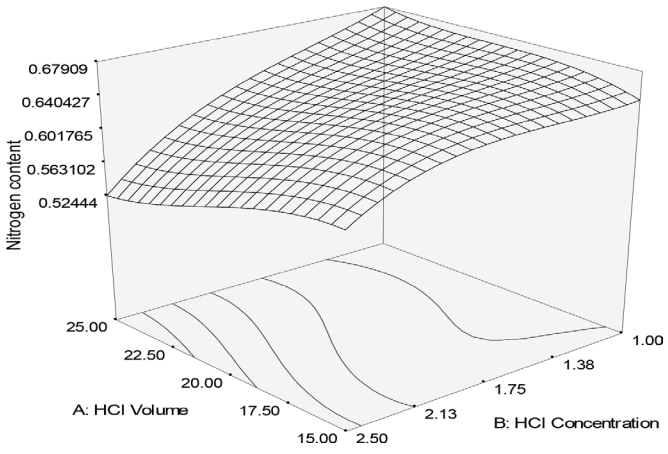


FIGURE 4. Response surface for nitrogen content as a function of HCl volume and concentration during hydrolysis of cockle meat wash water precipitate using HCl

precipitate using HCl. At lower HCl volume, DH value did not change drastically when HCl concentration was increased. However, at higher HCl volume, increasing HCl concentration resulted in a decrease in DH. Similar to NC, the decrease in DH may be due to the loss of protein caused by the deamination process. At higher HCl concentration, DH did not show any appreciable change when HCl volume was increased. However, at lower HCl concentrations, increasing HCl volume resulted in an increase in DH. Higher HCl volume allows more extensive hydrolysis to occur resulting in a higher DH. Increasing both HCl concentration and volume produced a decreasing DH as projected by the negative value of the coefficient (-0.77) (Table 6).

Figure 6 shows the DH as a function of the HCl volume and time during hydrolysis of cockle meat wash water precipitate using HCl. It can be observed that at each HCl volume, DH increased when hydrolysis time was increased. Higher hydrolysis time allowed more extensive hydrolysis to occur resulting in a higher DH. This trend was similar to the result of Dong et al. (2008) on silver carp defatted meat during hydrolysis with Alcalase and Flavourzyme. Increasing the HCl volume at fixed hydrolysis time produced only a slight increase in DH.

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 optimum point was determined based on the highest desirability to the responses. The analysis indicated that optimum NC and DH for hydrolysis of cockle meat wash water precipitate can be achieved using a HCl concentration of 1.00 N, HCl volume of 24.99 mL and

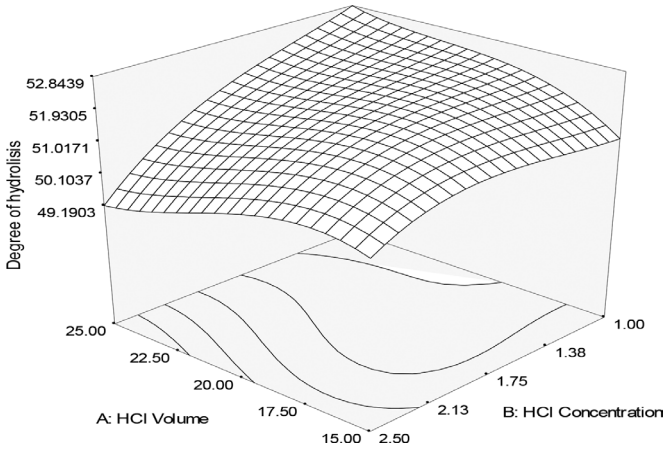


FIGURE 5. Response surface for degree of hydrolysis as a function of HCl volume and concentration during hydrolysis of cockle meat wash water precipitate using HCl

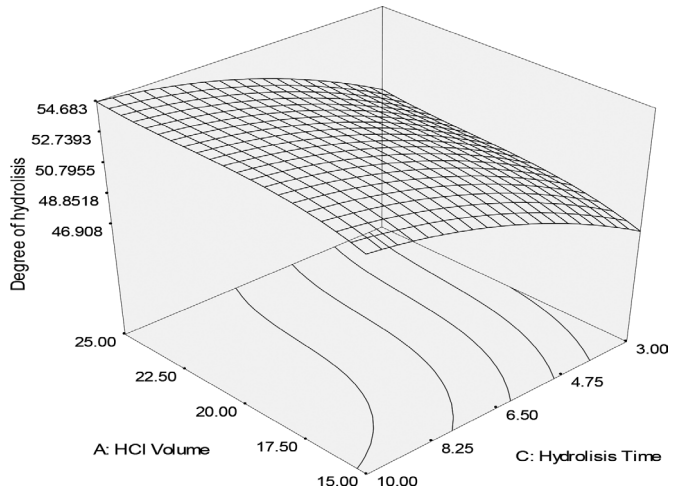


FIGURE 6. Response surface for degree of hydrolysis as a function of HCl volume and time during hydrolysis of cockle meat wash water precipitate using HCl

hydrolysis time of 10 h. From the optimization study, NC and DH predicted were 0.6790 and 54.6813%, respectively, with a desirability value of 0.931.

Experimental runs (3 replications) of the optimum point were carried out and the NC and DH values were compared with the predicted value for validation. Experimental results predicted NC of 0.5957±0.0078 and DH of 52.7067±0.74. The results showed only small RMSD values between the experimental and predicted NC (0.0212) and DH (1.7078) indicating the validity of the model. The low values of RMSD showed that model satisfactorily predicted the response.

CONCLUSION

Based on the results, hydrolysis of cockle (*Anadara granosa*) meat wash water precipitate was carried out by precipitation of the wash water at pH4. Optimum condition for the hydrolysis of 2.0 g of cockle meat wash water precipitate was 25 mL of 1 N HCl for 10 h which resulted in nitrogen content (NC) of 0.7% and degree of hydrolysis (DH) of 55%. The NC and DH are significantly influenced only by the hydrolysis time.

ACKNOWLEDGEMENTS

The authors would like to convey their gratitude to Hajjah Faazaz bt Abdul Latiff of Fishery Research Institute (FRI), Batu Maung, Penang, Malaysia and the Ministry of Agriculture, Malaysia for the financial support of this work under the grant STGL-010-2007.

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Received: 18 July 2012
Accepted: 20 March 2013