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The effects of enzyme concentration, temperature and incubation time on nitrogen content and degree of hydrolysis of protein precipitate from cockle (*Anadara granosa*) meat wash water

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Abstract: A study was carried out to determine the effect of enzyme concentration, temperature and incubation time of bromelain on nitrogen content (NC) and degree of hydrolysis (DH) of hydrolysate from cockle (Anadara granosa) meat wash water. Protein precipitation of cockle meat wash water was conducted at pH 4. The precipitate was then hydrolyzed using bromelain at concentrations of 0.5, 1.5 and 2.5 % (enzyme/substrate). The best enzyme concentration was subsequently used to study the effect of incubation temperature at 30, 45 and 60°C. The best temperature was then used to determine the effect of incubation time at 0, 24 and 48 hours. Increasing bromelain concentration from 0 to 2.5% produced an increase in NC and DH. Similarly, increasing the incubation time from 0 to 48 hours also increased the value of NC and DH. However, while the increasing of incubation temperature from 30 to 60°C produced an increase in NC, no significant difference was observed for DH.

Keywords: Protein hydrolysate, cockle meat wash water, bromelain, nitrogen content, degree of hydrolysis

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. Food protein hydrolysates have a wide range of applications as ingredients in the areas of nutrition, food industry, health care and cosmetics (Radha *et al*., 2007). Protein hydrolysates can be used to improve the taste of food.

Cockles (*Anadara granosa*) are edible bivalve commonly found in South East Asia. 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. Commonly, the preparation of cockles involves 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 contribute to reduced pollution and give benefit to the food industry. According to Nilsang *et al*. (2005), the production of seafood flavors 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 (Ferrer *et al*., 1996). Enzymatic hydrolysis have the advantage of not producing chlorohydrins and they contain little salt (Weir, 1992).

Several proteases can be used for the process of enzymatic hydrolysis, such as bromelain (plant protease). Bromelain is one of the endoproteases which has been used mainly for meat tenderization (Melendo *et al*., 1997; Kolle *et al*., 2004). Many studies had been carried out regarding the use of protein to improve functional and physicochemical properties of protein, focusing on emulsion formation, water holding capacity (Karakaya and Ockerman, 2002) and production of umami chicken flavor (Maehashi *et al*.,1999). Critical parameters in enzymatic hydrolysis are temperature, hydrolysis time, pH and degree of hydrolysis (Bjoern *et al*., 2000).

Although there is considerable information on the precipitation of proteins from fish and shrimp processing waste effluents (Hang *et al*., 1980; Nilsang *et al*., 2005; Fabienne *et al*., 2007), there is still no information dealing with the recovery of proteinaceous materials and the production of

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hydrolysates from cockles meat wash water. The objective of this study was to determine the effect of enzyme concentration, temperature and incubation time for the production of protein hydrolysate from cockle *(Anadara granosa)* meat wash water through enzymatic hydrolysis using bromelain.

Materials and Methods

Extraction of protein from cockle meat wash water

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 cockle 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.

Protein precipitation

Precipitation of the protein in the cockle meat wash water was carried out by pH adjustment using 4N HCl and 0.1N NaOH. The pH range used was 3 to 8. The pH values were measured by a pH meter (WTW pH 422; pH 1-14) which was calibrated using pH 4 and pH 7 buffers. 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. Protein content was analyzed using Kjeldahl method (AOAC, 1990). Sample at pH 4 (the highest protein content) was selected for protein precipitation.

Production of protein hydrolysate

Protein hydrolysate was produced through enzymatic hydrolysis using bromelain. The hydrolysis process was carried out based on Montecalvo et al. (1984) method. Cockles meat wash water precipitate (2.0g) from the previous step was defatted using a fat analyzer (Soxtex System, Tecator, Sweden) according to the Soxhlet method (AOAC 1990). Distilled water (50ml) was added to the defatted precipitate in a conical flask (100 ml). The mixture was then hydrolyzed in the oven (95°C) and cooled down to room temperature before pH was adjusted to pH 6 using 4N NaOH. Then, enzyme (bromelain) at different concentrations (0.5, 1.5 and 2.5%) was added, along with 2ml of 0.1M L-Cysteine to activate the enzyme. Bromelain Food Grade which have been used is a endopeptidase produced from pineapple. The mixture was incubated at 200 rpm in a shaker incubator (Environ-Shaker) for 0, 24 and 48 hr, at 30, 45 and 60°C. Samples were then heated at 95°C for 15 min to deactivate enzyme. Protein hydrolysates produced were centrifuged at 7800 x g for 15 min in order to separate any impurities and enzyme from hydrolysate. The supernatant (protein hydrolysate) were frozen at -20°C before freeze-drying.

Determination of nitrogen content in protein hydrolysate

Nitrogen content was analyzed by Kjeldahl standard method using protein analyzer (Kjeltec™ 2000, Foss-Tecator, Sweden). Nitrogen content was determined by the formula below.

$$\% N = \frac{0.1 \times \text{Titration volume (ml)} \times 14 \times 10}{\text{Sample weight (g)} \times 1000}$$

Where, N = nitrogen

Determination of degree of hydrolysis in protein hydrolysate

Degree of hydrolysis was calculated according to percent of trichloroacetic acid (TCA) ratio method as described by Hoyle and Merritt (1994) and Fonkwe and Singh (1995). After hydrolysis, 20 ml of protein hydrolysate was added to 20 ml of 20% (w/v) TCA to produce 10% TCA soluble material. The mixtures were left to stand for 30 min to allow precipitation, followed by centrifugation (7800 x g for 15 min) (High Speed Centrifuge, Sorvall HS23, USA). The supernatant was analyzed for protein content by Kjeldahl method (AOAC, 1990). Sample from the hydrolysate was also analyzed for protein content. Degree of hydrolysis (DH) was calculated using the formula below:

$$\%DH = \frac{Soluble\ N\ in\ TCA\ 10\% \left(\frac{w}{v}\right) \times 100}{Total\ N\ in\ the\ sample}$$

Where DH = Degree of hydrolysis; TCA= trichloroacetic acid

Data analysis

Data was analysed using Statistical Analytical System (SAS) version 6.12 for ANOVA test and DUNCAN. All experiments were done using three replication.

Results and Discussions

Bromelain was selected based on the study by Stein et al. (2005) which showed that it gave the highest total yield of α-amino groups compared to other proteases such as alcalase, neutrase and papain. 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 as, TCA (Hoyle and Merritt, 1994).

Enzymatic hydrolysis that were carried out involved three different enzyme concentration which were 0.5, 1.5 and 2.5 % (enzyme/substrate). Percentage of nitrogen in protein hydrolysate of cockle meat wash water (Figure 1) showed significant differences ($p<0.05$) at each concentration with an increasing trend when the concentration of bromelain was increased. NC showed the highest percentage at 2.5% bromelain concentration. At lower bromelain concentration (0.5%), only a small amount of peptides were produced resulting in a low NC value. Increasing bromelain concentration to 2.5% 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).

DH increased when the percentage of bromelain was increased. This observation was due to some of peptides released were hydrolyzed by the enzymes into amino acids and smaller peptides as the bromelain concentration increased. DH do not show any significant difference ($p>0.05$) between bromelain concentration of 0.5 and 1.5%. This might be due to the small percentage of bromelain concentration used thus did not significantly influence the hydrolysis process. However, the similar trend in NC and DH proved that increasing bromelain concentration resulted in increased hydrolysis process of the proteins. James *et al*. (2005) reported that increased enzyme concentration generally had a greater effect on reducing hydrolysis time than did increased temperature. This was supported by the result of DH at 2.5% bromelain concentration which significantly ($p<0.05$) produced the highest percentage compared to the other enzyme concentrations. Subsequently, bromelain at 2.5% concentration was chosen to determine the effect of temperature.

Figure 2 shows percentage of NC and DH in protein hydrolysate of cockle meat wash water at temperatures of 30, 45 and 60°C. According to the results, percentage of NC (Figure 2) significantly increased ($p<0.05$) when temperature was increased from 30°C to 45°C and 60°C. There were no significant difference for NC at 45°C compared to 60°C. As shown in Figure 2, there were no significant difference in percentage of DH between all temperatures used. Increasing the temperature from 30 to 45°C increased the peptides content in the supernatant. In a different study done by Taha et al. (2002) on enzymatic hydrolysis of soybean, sesame oilseed and rice bran meal protein it was shown that the optimum temperature for enymatic hydrolysis using bromelain was 45°C. NC data (Figure 2) from this study corroborates well with those data reported by Taha *et al*. (2002) where NC could be seen as stating to level off at 45°C.

However, DH did not show a similar trend as illustrated by NC, where no significant differences ($p>0.05$) were observed between all incubation temperatures used. The observed trend for DH was contrary to the report by Nielsen (1995) where heat treatment caused the exposure of peptide bonds during enzymatic hydrolysis leading to the increase of DH. The observed insignificant difference in DH may be due to limiting factors during hydrolysis such as the time of reaction which was constant. The limited reaction time may resulted in an increased amount of soluble peptides but with less extensive hydrolysis of its peptide bonds. However, further studies are needed to determine the validity of this possibility. From the above results, temperature at 45°C have been used to determine the effect of time.

Figure 3 shows percentage of NC in protein hydrolysate of cockle meat wash water obtained from different incubation time of 0, 24 and 48 hr. Percentage of NC (Figure 3) was observed to increase significantly ($p<0.05$) when incubation time was increased up to 48 hr. The longer the incubation time took place, a higher percentage of NC was obtained from protein hydrolysate of cockle meat wash water. Increasing the incubation time allowed increased

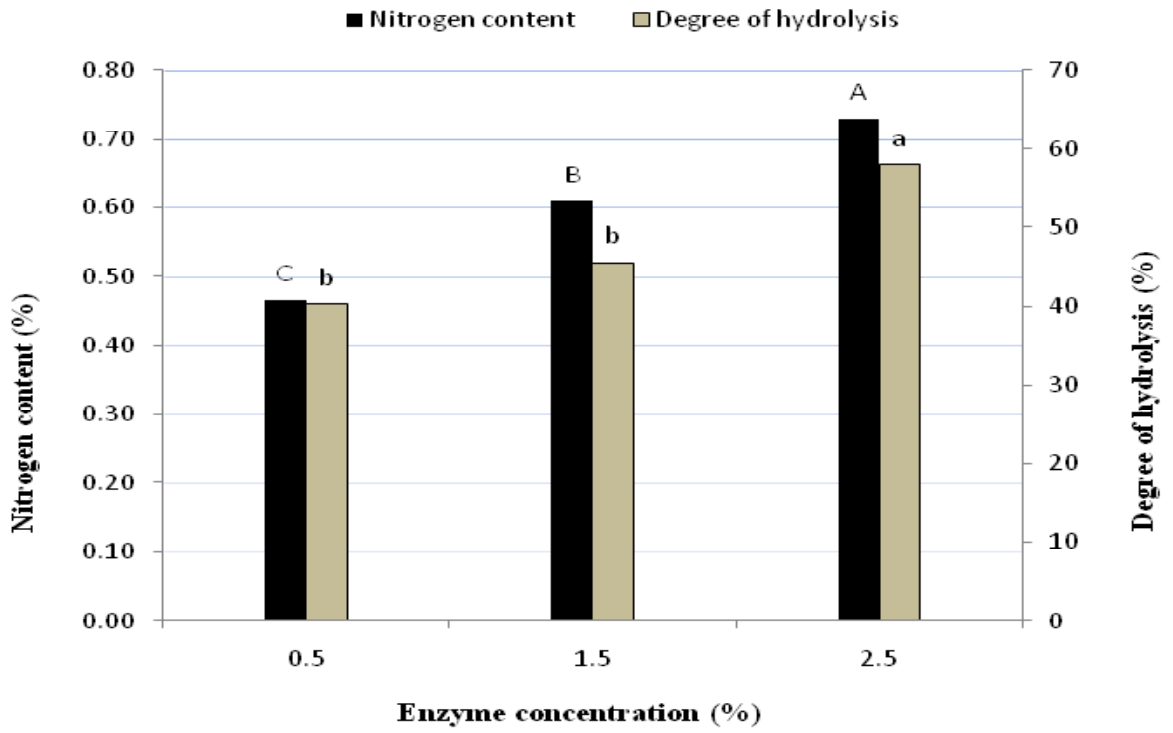

Figure 1. Nitrogen content (NC) and degree of hydrolysis (DH) in protein hydrolysate of cockle meat wash water at different enzyme concentration (enzyme/substrate). A-C or a-c: means with different letters indicate significant difference ($p<0.05$).

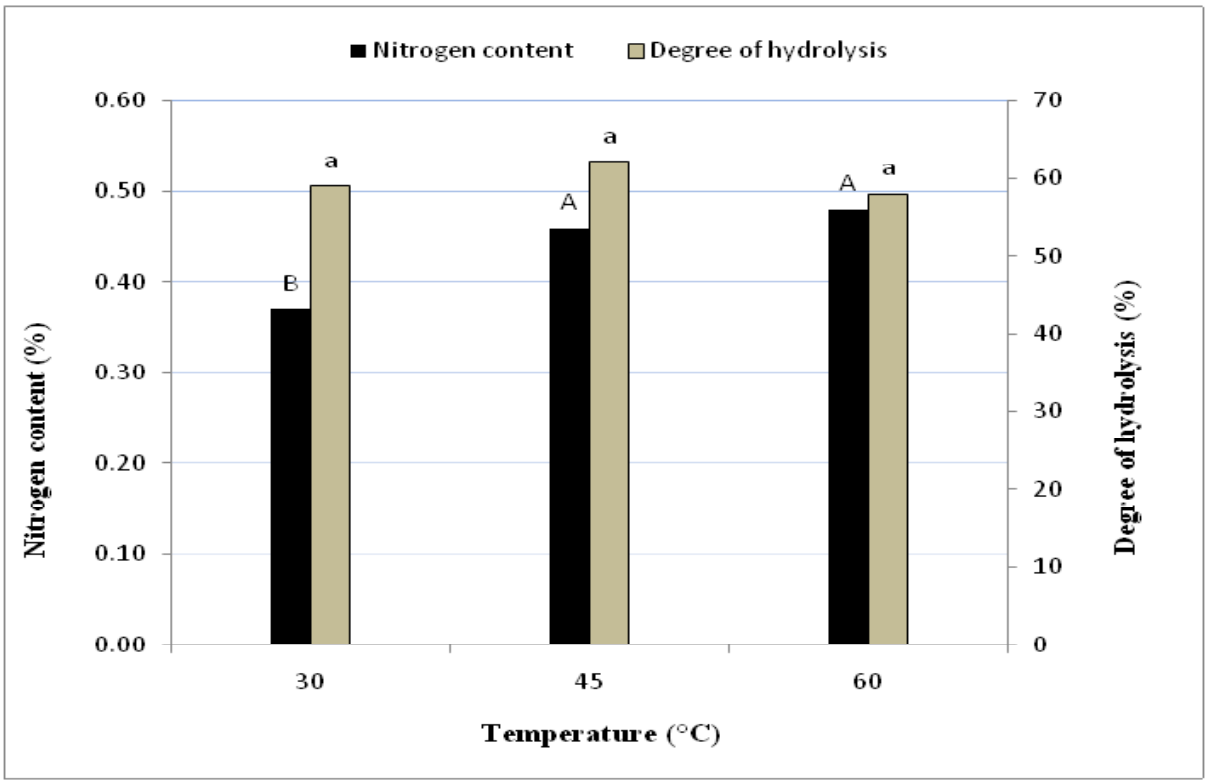

Figure 2. Nitrogen content (NC) and degree of hydrolysis (DH) in protein hydrolysate of cockle meat wash water at different temperature. A-C or a-c: means with different letters indicate significant difference ($p<0.05$).

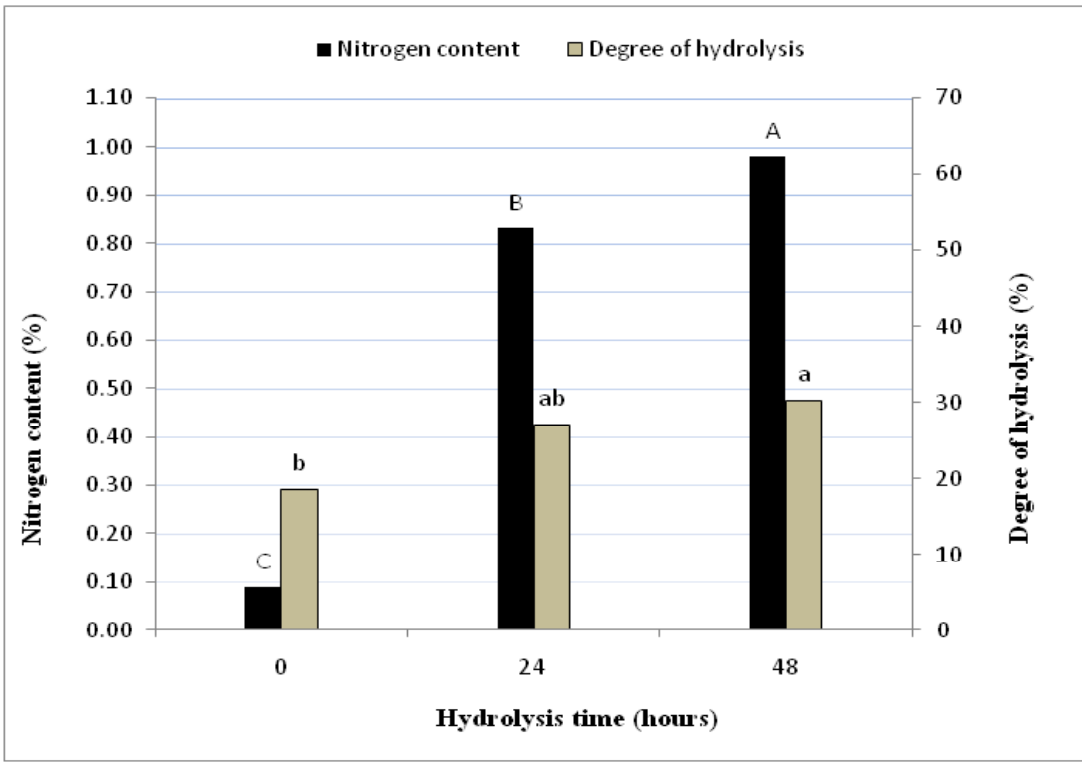

Figure 3. Nitrogen content (NC) and degree of hydrolysis (DH) in protein hydrolysate of cockle meat wash water at different incubation time. A-C or a-c: means with different letters indicate significant difference ($p<0.05$).

proteolysis. DH also showed an increasing trend from 0 to 48 hr. There was no significant difference ($p<0.05$) for NC when incubation was carried out at 24 hr compared to 0 and 48 hr. This result was similar to those reported by Vijaya *et al*. (2002) which indicated 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.

Conclusion

Based on the results, factors which significantly influenced the percentage of NC and DH of protein hydrolysate from cockle meat wash water were enzyme concentration and hydrolysis time, while incubation temperature only slightly influenced the NC. Increasing bromelain concentration from 0 to 2.5% produced an increase in NC and DH. Similarly, increasing the incubation time from 0 to 48 hours also increased the value of NC and DH. However, while the increasing of incubation temperature from 30 to 60°C produced an increase in NC, no significant change was observed for DH.

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