

Evaluation on the Properties of Mentarang (*Pholas orientalis*) Protein Hydrolysate

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ABSTRACT

Mentarang (*Pholas orientalis*) protein hydrolysate was produced by hydrolysis with Alcalase 2.4L using pH-stat method. The muscle was initially hydrolysed for 2 hours at 3% enzyme-substrate ratio, 60°C and pH 8.5, centrifuged and freeze dried. The yield, composition and functional properties of the resulting hydrolysate were determined. A reasonably high yield was achieved which is 11.03%. The hydrolysate was characterized by high protein content (43.0%) and yellowish in colour ($L^* = 72.98$, $a^* = 0.42$, $b^* = 15.15$). It contains high amount of essential amino acids (45.62%) with alanine and serine as the dominant amino acids. The protein hydrolysate had an excellent solubility (92.32%) and an emulsifying stability index of 36.13 min. Foaming properties decrease significantly ($p < 0.05$) with increasing time of foam. In view of these beneficial properties, mentarang (*Pholas orientalis*) hydrolysate has the potential for application as a natural additive in food.

Keywords: Mentarang (*Pholas orientalis*), hydrolysate, alcalase, solubility, colour, hydrolysis

INTRODUCTION

Mentarang (*Pholas orientalis*) is a bivalve species enclosed by two thin elongated shells. In western countries it is also known as 'anglewing' clam (Jesse *et al.*, 2006). It

can be found in muddy shore and live in low temperature water for example in estuary. In Malaysia, this species can be found easily in Sabak Bernam, Selangor. *Pholas orientalis* is highly favoured because of its excellent flavour and attractive milky white shell.

Protein hydrolysate refers to compound produced by the hydrolysis of high protein food such as milk, egg, fish and meat with acid, alkali or enzyme. In general, protein

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hydrolysate is obtained by mixing the protein raw material with water and incubated at specific time and temperature before the enzyme, acid or alkaline is introduced. Hydrolysate contains a mixture of amino acids and peptides. Some studies have shown that hydrolysate can contribute to water holding, texture, gelling and whipping properties when added to food (Kristinsson, 2007; Wasswa *et al.*, 2007b; Wasswa *et al.*, 2008). Addition of hydrolysate from salmon reduced water loss after freezing (Kristinsson, 2000a). Hydrolysates have also been proven to have good foaming and emulsifying properties. Therefore, it may be used as emulsifying and emulsion stabilizing ingredients in a variety of products as well as aids in the formation and stabilisation of foam-based products (Kristinsson, 2007).

Most hydrolysate was produced using sea species because sea species are easy to find, breed in thousands, and almost all sea species are edible and 'halal'. Many bivalve species including mentarang has limited uses and only introduced as seafood meal. In Malaysia, they are only sold and consumed in some areas and not very popular among many people, even though this seafood species contains a very rich source of protein. Thus, hydrolysate was prepared in this study to expand the utilization of bivalves. In addition, the determination of compositional and functional properties of hydrolysate derived from this bivalve species may lead to the discovery of new ingredient to food or pharmaceutical product. The objective of this study is to determine the compositions and the functional properties of mentarang

(*Pholas orientalis*) hydrolysate produced by hydrolysis using alcalase.

MATERIALS AND METHOD

Materials

Mentarang was bought from Pantai Remis, Selangor, Malaysia and immediately placed in ice and transported to the laboratory. Upon arrival, the flesh was removed manually from the shell, washed and then minced using a blender.

Alcalase (with a declared activity of 2.4 AU/g and a density of 1.18 g/ml) is a bacterial endoproteinase from a strain of *Bacillus Licheniformis* was purchased from Science Technic Sdn. Bhd. Malaysia.

Preparation of Mentarang (Pholas orientalis) Protein Hydrolysate

Mentarang hydrolysate was prepared according to the method by Adler-Nissen (1986), with a slight of modification. Thirty grams of minced mentarang meat was suspended in 120 ml of distilled water. The mixture was incubated in a circulated water bath at 60°C. The pH of the mixture was adjusted to pH 8.5 and constantly maintained during hydrolysis using 1.0 N NaOH. Once the pH and temperature have stabilized, alcalase at enzyme-substrate ratio of 3% was added and the reactions continued for 2 hours. The enzymatic reaction was terminated by placing the samples in a water bath at 90°C for 15 min with occasional agitation. This was followed by centrifugation at 14000g for 10 min. Supernatants obtained were freeze-dried using the SANYO-Biomedical freeze dryer.

Determination of the Percent Degree of Hydrolysis (DH %)

Degree of hydrolysis (DH) is defined as the percentage ratio between the number of peptide bonds cleaved (h) and the total number of peptide bonds in the substrate studied (h_{tot}) (Adler-Nissen, 1986). The degree of hydrolysis was determined based on the consumption of base necessary for controlling the mixture's pH during the batch assay as in the equation below:

$$DH\% = \frac{\beta \times N_{\beta}}{\alpha \times M_p \times h_{tot}} \times 100$$

Where:

β = Volume of 1.0N NaOH

N_{β} = Normality of NaOH

α = Average degree of dissociation of the NH_3 groups

M_p = Mass of protein in g

h_{tot} = Total number of peptide bonds in the protein substrate (mmol/g protein)

Yield

The yield was determined by the ratio of the mass of hydrolysate and the total weight of the fresh mentarang muscle. The yield obtained was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of powdered hydrolysate} \times 100}{\text{Wet weight of fresh mentarang muscle}}$$

Determination of Mentarang (Pholas orientalis) Protein Hydrolysate Compositions

Moisture Content

Moisture content was determined according to the (AOAC, 2005) by placing approximately 2 g of minced mentarang or hydrolysate sample into a pre-weighed aluminium dish. The sample was dried in a forced-air convection oven at 105°C until a constant weight was achieved. Moisture content was calculated as follows:

$$\% \text{ Moisture (wt / wt)} = \frac{\text{Weight of wet sample} - \text{Weight of dry sample}}{\text{Weight of wet sample}} \times 100$$

Determination of Fat Content

The extraction and determination of the fat content from the minced muscle or the hydrolysate sample were performed using the Soxhlet extraction method (AOAC, 2005).

Protein Concentration

Protein concentration was determined using the Lowry method based on the modified procedure of Hartree (1972).

Colour Measurement

The colour of powdered hydrolysate was measured by chromameter CR400 (Konica Minalto). L^* , a^* , and b^* parameters indicate brightness, redness and yellowness, respectively. The measurement was performed in triplicate.

Amino Acid Analysis

Sample preparation was conducted by hydrolysis with 6 M HCl at 110°C for 24 hours and derivatisation using phenyl isothiocyanate prior to AccQ Tag HPLC analysis. The total amino acid was analysed by the AccQ Tag method using an AccQ Tag column (3.9 x 150mm) at a flow rate of 1 ml/min with UV detection. The mobile phase used is AccQ Tag Eluent A that consists of 100ml Eluent A and 1000ml deionized water, while AccQ Tag Eluent B consists of 60% acetonitrile and 40% deionized water or 60% acetonitrile. The total running time per injection was 50 minutes.

Determination of Functional Properties

Solubility

Solubility was determined following the procedure of Morr (1985) with slight modification. Protein hydrolysate (0.5 g) was dispersed in 50 ml of 0.1 M NaCl. The mixture was stirred for 1 hour at room temperature and then centrifuged using a Kubota 5420 centrifuge at 2600g for 30 min. The supernatant was filtered through Whatman filter paper No.1 and the nitrogen content in the total fraction and in the soluble fraction was analysed by Lowry method. Solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Total protein content in the sample}} \times 100$$

Emulsifying Stability

The emulsion stability index (ESI) was determined according to the method of Pearce and Kinsella (1978) with a slight

modification. Soybean oil (10 ml) and 30 ml of protein hydrolysate solution (3g in 100ml) was mixed and homogenised using homogenizer at a speed of 20,000 rpm for 1 min using IKA T25 digital ULTRA-TURRAX homogenizer. Aliquot of the emulsion (50 µl) was pipetted from the bottom of the container at 0 and 10 min after homogenisation and diluted 100-fold using 0.1% SDS solution. The absorbance of the diluted solution was measured at 500 nm. The absorbance, measured immediately (A_0) and 10 min (A_{10}) after emulsion formation was used to calculate the emulsion stability index (ESI), as follows:

$$\text{ESI (min)} = A_0 \times \Delta t / \Delta A$$

Where $\Delta A = A_0 - A_{10}$ and $\Delta t = 10$ min.

Foaming Stability

Foaming stability of protein hydrolysate was analysed according to the method of Shahidi *et al.* (1995). Twenty millilitres of protein hydrolysate solution was homogenized in a 50 ml cylinder at a speed of 16,000 rpm to incorporate the air for 1 min. The total volume was measured at 0, 0.5, 5, 10, 40, and 60 min after whipping. Foaming stability was expressed as foam expansion at 60 min. Foam stability was calculated according to the following equation (Sathe & Salunkhe, 1981):

$$\text{Foam stability (\%)} = [(A - B) / B] \times 100$$

Where;

A = volume after whipping (ml) at different time (0, 0.5, 5, 10, 40, 60)

B = volume before whipping (ml)

Water Holding Capacity

Water-holding capacity (WHC) was determined using the centrifugation method according to Diniz and Martin (1997). The samples (0.5 g) of hydrolysate was dissolved with 20 ml water in centrifuge tubes and dispersed with a vortex mixer for 30s. The dispersion was allowed to stand at room temperature for 6 hours, and it was subsequently centrifuged at 2800g for 30 min. The supernatant was filtered through Whatman filter paper No. 1 and the volume recovered was accurately measured. The difference between the initial volume of distilled water added to the protein sample and the volume of the supernatant were determined. The results were reported as ml of water absorbed per gram of protein sample.

$$\text{WHC (ml/g)} = \frac{\text{Initial volume of distilled water} - \text{Volume of supernatant (ml)}}{\text{Weight of hydrolysate (g)}}$$

RESULTS AND DISCUSSION

Percent Degree of Hydrolysis (%DH)

During hydrolysis, mentarang muscle mixture changed into brownish red. After centrifugation, the supernatant was clear, reddish in colour and had a sticky characteristic. After freeze drying the hydrolysate had a light yellowish and strong fishy odour. The freeze-dried hydrolysate turned into dark yellowish powdered when stored for long period at room temperature for more than a month. The degree of hydrolysis using alcalase at 60°C was 9.44%. Shahidi *et al.* (1995) reported a higher degree of hydrolysis (22%) during

the hydrolysis of capelin protein at 65°C using alcalase. According to Guerard *et al.* (2002) a reduction in the reaction rate may be due to the limitation of the enzyme activity by formation of reaction products at high degrees of hydrolysis. However, the decrease in hydrolysis rate may also be due to a decrease in the concentration of peptide bonds available for hydrolysis, enzyme inhibition and enzyme deactivation. The research by Mahmudreza *et al.* (2009) revealed that the degree of hydrolysis increase at increasing hydrolysis time and temperature. Bhaskar *et al.* (2008) also observed higher degree of hydrolysis at elevated temperatures. In addition, alkaline proteases like alcalase have been reported to exhibit higher activities than neutral or acid proteases, such as papain or pepsin (Rebeca *et al.*, 1991; Sugiyama *et al.*, 1991).

Compositions, Yield and Colour

The compositions of mentarang (*Pholas orientalis*) and its hydrolysate are given in Table 1. The yield obtained was reasonably high (11.03%). The yield can be improved by properly monitoring the hydrolysis conditions. The low moisture content of the hydrolysate enhanced the stability of the hydrolysate to be stored for a prolonged period of time. Hydrolysis of mentarang muscle in this study increased the protein concentration in hydrolysate. The increasing protein content was a result of the solubilisation of protein during hydrolysis and the removal of insoluble undigested non-protein substance (Benjakul & Morrissey, 1997). A previous report has

shown that the sample containing high amount of lipids contained low percentages of solubilised protein (Slizyte *et al.*, 2005). Mentarang muscle contains only 2.82% fat content, thus the protein content obtained in the hydrolysate was considerably high.

Hydrolysate contains lower fat content than the mentarang muscle. The low fat content could be due to centrifugation, where some of the fats are separated while others may have entrapped in the pellet after the centrifugation. Decreasing lipid content in the protein hydrolysates might significantly increase stability of the materials towards lipid oxidation, which may also enhance the product stability (Kristinsson & Rasco, 2000b).

Colour influences the overall acceptability of food products. Hydrolysis of mentarang (*Pholas orientalis*) produced hydrolysate that is light yellow in colour. Wasswa *et al.*, (2007a) stated that increased hydrolysis time resulted in increased enzymatic browning reaction. Enzymatic reactions are assumed to have contributed to the reduction in the luminosity, giving a darker appearance at high degree of hydrolysis (Wasswa *et al.*, 2007b).

Amino Acid Composition

The amino acids composition of the freeze-dried mentarang hydrolysate is presented in Table 2. The hydrolysate contains high amount of alanine followed by serine, histidine, threonine and glutamine. Mentarang hydrolysate may probably exhibit some antioxidant activity. This was due to the high content of histidine and alanine, the amino acids known to contribute to antioxidant activity (Mendis *et al.*, 2005). High antioxidant activity of histidine-containing peptide was attributable to the imidazole ring of histidine that enables histidine to chelate metal ions and trap lipid radicals (Uchida & Kawakishi, 1992). The higher content of total hydrophilic amino acids (aspartic acid, glutamic acid, glycine, alanine, threonine and serine) as compared to total hydrophobic amino acids is consistent with the high solubility characteristic of the hydrolysate. The ratio of essential amino acid to non-essential amino acid is 0.75. Thiansilakul *et al.* (2007) obtained the ratio of 0.92. Fish and shellfish have been reported to contain high essential amino acid to non-essential amino acid ratio

TABLE 1
Yield, compositions and colour of mentarang (*Pholas orientalis*) hydrolysate

Composition	mentarang (<i>Pholas orientalis</i>)	hydrolysate
Yield (%)		11.03 ± 1.38
Moisture content (%)	79.60 ± 4.37	9.51 ± 1.51
Fat content (%)	2.82 ± 1.84	0.81 ± 0.56
Protein (%)	13.72 ± 0.56	43.0 ± 0.04
Colour		
L*		72.98 ± 0.06
a*		0.42 ± 0.01
b*		15.15 ± 0.01

(Iwasaki & Harada, 1985). Based on the amino acids profile, mentarang hydrolysate is high in nutritional value.

Functional Properties

Solubility

Solubility is one of the most important physicochemical and functional properties of protein hydrolysates (Kristinsson & Rasco, 2000a). In general, the degradation of protein into smaller peptides leads to more soluble products (Gbogouri *et al.*, 2004). The smaller peptides are expected to have more polar residues, increasing hydrophilicity through an increased ability to form hydrogen bonds with water (Sathivel *et al.*, 2005). The balance of hydrophilic and hydrophobic forces of peptides is another crucial influence on solubility increments (Gbogouri *et al.*, 2004). Protein hydrolysate from *Pholas orientalis* shows high solubility at pH 8.5 (Table 3). Salmon head hydrolysate produced using Alcalase 2.4L was reported to show more than 75% solubility at 11.5% to 17.3% DH

(Gbogouri *et al.*, 2004). Due to its high solubility, *Pholas orientalis* hydrolysate was presumed to have a low molecular weight and was hydrophilic in nature and with 92% solubility this suggested that the hydrolysate is very soluble in aqueous system. This shows that protein hydrolysate derived from *Pholas orientalis* can be a good food additive because it is easy to dissolve. The high nitrogen solubility of protein hydrolysate indicates potential applications in formulated food systems by providing attractive appearance and smooth mouth feel to the product (Peterson, 1981).

Emulsifying Properties

Emulsifying stability index of protein hydrolysate ten minutes after homogenization was 39.18 min (Table 3). This value shows that protein hydrolysate produced from *Pholas orientalis* at 9.44% degree of hydrolysis can slightly emulsify with oily food. Emulsifying stability of hydrolysates decreased with the increase in hydrolysis due to the presence of small peptides,

TABLE 2
Amino acids composition of mentarang (*Pholas orientalis*) hydrolysate

Essential amino acid	Content (%)	Non-essential amino acids	Content (%)
Val	3.39 ± 1.23	Asp	4.22 ± 2.08
His	14.10 ± 0.71	Ser	15.71 ± 0.42
Met	3.29 ± 2.15	Glu	9.24 ± 1.32
Thr	13.02 ± 0.87	Gly	8.57 ± 3.98
Lys	1.58 ± 0.33	Ala	17.97 ± 1.01
Ile	5.75 ± 4.00	Pro	2.19 ± 1.40
Leu	4.49 ± 2.32	Cys	0.97 ± 1.10
		Tyr	2.30 ± 0.64
Total	45.62	Total	61.17

which are less effective in stabilizing emulsions (Wasswa *et al.*, 2007a; Wasswa *et al.*, 2007b). In addition, an increase in hydrolysis also reduced the hydrolysate emulsifying capacity and stability due to lesser surface hydrophobicity (Kristinsson & Rasco, 2000b). A peptide should have a minimum length of more than 20 amino acid residues in order to possess good emulsifying and interfacial properties and it has been shown that large peptides of about 20,000 Da contributed to the high emulsifying capacity of hydrolysate (Lee *et al.*, 1987; Kristinsson & Rasco, 2000b). The pH of protein solutions during emulsification affects their emulsifying properties via charge effects (Nielsen, 2001). Meanwhile, an addition of salt improves the emulsion properties of peptide fractions (Turgeon *et al.*, 1992). Several previous studies have reported that excessive hydrolysis brings about the loss of emulsifying properties (Gbogouri *et al.*, 2004; Kristinsson & Rasco, 2000b; Klompong *et al.*, 2007), while at limited degree of hydrolysis, the hydrolysates have an exceptional emulsifying stability (Kristinsson & Rasco, 2000b). Several factors such as blending speed, protein source, temperature, pH, type of oil added and water content influence emulsion capacity. In this study the degree of hydrolysis obtained was reasonably low (9.44%), and thus, good emulsifying properties was achieved. Nalinanon *et al.* (2011) obtained emulsifying stability index of 14.1 min at 10% DH using pepsin. This shows that the specificity of enzyme may also influence emulsifying properties.

TABLE 3
Functional properties of mentarang
(*Pholas orientalis*) hydrolysate

Functional properties	
Solubility (%)	92.32 ± 2.10
Emulsifying stability (min)	39.13 ± 0.72
Foaming properties (%)	
0 min	78.00 ^a ± 1.15
0.5 min	76.00 ^a ± 7.21
5 min	56.67 ^b ± 1.15
10 min	26.67 ^c ± 1.15
40 min	22.00 ^c ± 2.00
60 min	6.67 ^d ± 3.06
Water holding capacity (ml/g)	3.92 ml/g ± 0.98

Foaming Properties

Foaming properties are physicochemical characteristics of proteins to form and stabilise foams (Thiansilakul *et al.*, 2007). Enzymatic hydrolysis of protein can improve foaming characteristics (Adler-Nissen, 1986). However, this depends on the degree of hydrolysis achieved which is influenced by several factors including the type of enzyme, pH, duration of hydrolysis and temperature. Foam stability for powdered mentarang (*Pholas Orientalis*) hydrolysate at different times after homogenization is shown in Table 3. In general, there was a significant decrease ($p < 0.05$) in foaming properties with time. Thiansilakul *et al.* (2007) who studied on the effect of different protein concentration observed that foam stability increases with the increasing in protein concentration. High degree of hydrolysis has been shown to reduce foaming properties of hydrolysate. Klompong *et al.* (2007), who studied on the effect of 5% to 25% degree of hydrolysis (DH) during the production of yellow-

striped trevally hydrolysate, found that the highest foaming capacity was at 5% DH, while the lowest at 25% DH. Shahidi *et al.* (1995) reported that a good foaming for capelin protein hydrolysate was produced at 12% DH. This finding suggested that a prolonged hydrolysis could reduce foaming stability since microscopic peptides do not have the strength needed to maintain stable foam. In order for a hydrolysate to present good foamability, it must have among other factors, a good balance of surface, a molecular hydrophobicity, as well as net charge and charge distribution (Damodaran, 1997; Pacheco-Aguilar *et al.*, 2008). Molecular weight and charge of peptides may be different for hydrolysate produced at different conditions which will affect the foaming properties (Van Der van *et al.*, 2002; Klompong *et al.*, 2007).

Water Holding Capacity

Water holding capacity (WHC) for powdered mentarang hydrolysate is 3.92mg/ml (Table 3). In general, water holding capacity of partly unfolded and hydrolysed proteins is greater than that of the native proteins due to an increase in surface area to mass ratio with an exposure of some previously buried hydrophobic groups (Damodaran, 1997). Several studies have shown that hydrolysates have excellent water holding capacity and can increase the cooking yield when added to minced meat (Kristinsson & Rasco, 2000b; Shahidi *et al.*, 1995). The presence of polar groups such as COOH and NH₂ that increased during enzymatic hydrolysis had a substantial effect on the

amount of adsorbed water (Kristinsson & Rasco, 2000b). Wasswa *et al.* (2007) showed a similar result of water holding capacity at 10% degree of hydrolysis with 3.8 ml/g. WHC increased with an increased in degree of hydrolysis (Wasswa *et al.*, 2007). This shows that protein hydrolysate derived from *Pholas Orientalis* has the ability to hold water molecules well.

CONCLUSION

The protein hydrolysate derived from mentarang (*Pholas orientalis*) muscle serve as a good source of desirable amino acids. This protein hydrolysate could be used as an emulsifier, foaming agent as well as water holding agent. Mentarang hydrolysate has the potential to be used as natural additive, possessing functionality properties in food systems. Based on the findings of this study, it can be stated that the functionalities and compositions of protein hydrolysate derived from *Pholas Orientalis* using alcalase are reasonably acceptable.

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