Antioxidant Activity and Solubility of Green Mussel (*Perna Viridis)* Hydrolysate as Influenced by Degree of Hydrolysis.

***Normah Ismail and Fadilah Mohd Hasni**

Department of Food Technology, Faculty of Applied Sciences,
Universiti Teknologi MARA, 40450 Shah Alam, Selangor Darul Ehsan

**Corresponding author: norismel@salam.uitm.edu.my*

Abstract

Antioxidant activity and solubility of green mussel *(Perna viridis)* hydrolysate prepared by hydrolysis with Alcalase 2.4L under two different conditions (pH7, enzyme substrate ratio (ES) 5% and pH 9, ES 3%) were investigated. Green mussel hydrolysate exhibited free radical scavenging effect where pH7, ES 5% hydrolysate had higher radical scavenging effect than pH9, ES 3% hydrolysate. The degree of hydrolysis (DH) achieved were 43.81% and 28.33% for hydrolysis at pH9, ES 3% and pH7, ES 5%, respectively. Results revealed that antioxidant activity and solubility of green mussel hydrolysate were influenced by DH where the antioxidant activity was higher at low DH and the hydrolysate was more soluble at high DH. Antioxidant activity was more stable when the hydrolysate was stored at 4°C compared to 25°C but gradually decreased during the seven day storage period at both temperatures. Based on the solubility and antioxidant properties, green mussel hydrolysate has the potential to be used in several food applications as natural food additive.

Keywords : antioxidant, green mussel (*Perna viridis),* hydrolysate, degree of hydrolysis, alcalase, solubility

Introduction

Green mussel *(Perna viridis)* which is abundantly found in most Asian region is an inexpensive source of protein with high biological value, essential minerals and vitamins (Ismail *et al*., 2004; Fuentes *et al*., 2009). It has unique flavor and aroma, therefore suitable to be added in food as flavoring agent such as in soup, beverages and jellies. Due to increasing market demands on protein ingredients, novel proteins with diversified and improved functional properties have been purified from various sources by hydrolysis (El Nasri & El Tinay, 2007; Lamsal *et al*., 2007; Lokra *et al.,* 2007; Li *et al*., 2010). This will enhance their use in different food and non-food applications (Li *et al*., 2010).

Enzymatic hydrolysis is known to improve the functional properties of dietary protein without affecting its nutritional value by converting into peptides with desired size, charge and surface properties (Kristinsson & Rasco, 2000a; Panyam & Kilara, 1996; Moure *et al.,* 2006). Enzymatic hydrolysis has also been reported to be helpful in removing or reducing antinutritional factors such as tannins and phytic acid in rapeseed protein isolates and trypsin inhibitors in chickpea protein

hydrolysates (Chabanon *et al.*, 2007; Clemente *et al.*, 1999). The hydrolysis of protein, which is measured in terms of degree of hydrolysis (DH) is an important parameter in determining the functional properties such as solubility, foaming and emulsifying properties of protein hydrolysate preparations (Chabanon *et al.*, 2007; Klompong *et al.*, 2007; Normah & Nurfazlika Nashrah, 2013). The functional properties have been defined as "those physical and chemical properties that influence the behaviour of proteins in food systems during processing, storage, cooking and consumption" (Kinsella & Melachouris, 1976). DH affects the size and hence the amino acid composition of the peptides, which can affect the taste, solubility and antioxidant properties of protein hydrolysate (Kristinsson & Rasco, 2000a). Hence, by applying several ranges of DH with various manipulated variables including time, enzyme substrate ratio (ES) and pH control, soluble hydrolysate with reduce

bitterness can be obtained. Information regarding antioxidant activity and solubility of protein hydrolysate from green mussel (*Perna viridis)* due to different DH and ES used are still very limited. Therefore, this study is aimed to determine the antioxidant activities and solubility of protein hydrolysate from green mussel *(Perna viridis*) produced under different DH.

Conditions	Temperature	pH	ES	Time
1	60°C	7	5%	2hr
2	60°C	9	3%	2hr

Materials and method

Materials

Green mussel (*Perna viridis*) was obtained from a local supplier in Pantai Morib, Selangor. The mussel was kept in ice and immediately brought to the laboratory before further treatment. Alcalase 2.4L was purchased from Science Technic Sdn Bhd, Selangor. Alcalase 2.4L is an endopeptidase extracted from *Bacillus licheniformis*, with subtilisin carlsberg as the major enzymic component, having a specific activity of 2.4 Anson Units (AU) per gram. All chemicals used were of analytical grade.

Preparation of green mussel hydrolysate

Green mussel hydrolysate was prepared according to Normah *et al.,* (2013). Two hydrolysed samples with varying degree of hydrolysis (DH) were produced using the conditions described in Table 1. Fresh green mussel was mixed with distilled water at 1:1 ratio and homogenized in a blender for one minute at room temperature. The mixture was then poured into a 1L beaker and placed in a thermostatically controlled water bath set at 60°C. The mixture was stirred constantly using a four blade propeller at an agitation speed of 200 rpm. The pH was adjusted to the desirable value of either pH 7 or pH 9 using 4N NaOH or 4N HCl. Once the pH and temperature were consistent, 25 ml alcalase at enzyme substrate ratio (ES) 5% (7.2 AUg^{-1}) or 3% (4.32 AUg^{-1}) for pH 7 or pH 9, respectively, was added and the reaction was allowed to proceed. Throughout the process, the desirable pH was maintained by the addition of 4N NaOH. The amount of NaOH added to keep the pH constant was recorded and used to calculate the DH. The reaction was terminated after 2 hours by immersing the beaker into water bath set at 95°C for 15 minutes with occasional stirring in order to ensure complete inactivation of the enzyme. The resulting slurry was allowed to directly cool to room temperature followed by centrifugation at 5000 rpm for 20 minutes at 4°C. The supernatant containing the hydrolysate was collected, freeze dried and kept in a desiccator until further analysis.

Table 1: Hydrolysis conditions for producing green mussel (*Perna viridis*) hydrolysate.

Estimation of the degree of hydrolysis (DH)

The degree of hydrolysis was determined according to Adler-Nissen (1986) based on the consumption of NaOH necessary for controlling the pH. The formula used for the calculation of DH was as follows:

$$DH = V_ß N_ß (1/\alpha) \times (1/MP) \times (1/h_{tot}) \times 100 \quad (1)$$

where,
$V_ß$ = base consumption (mL)
$N_ß$ = base normality
MP = mass of protein substrate (g);
h_{tot} = total number of peptide bond (meq α-amino/g protein)

Determination of antioxidant activities of green mussel hydrolysate

DPPH free radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity was determined according to Thiansilakul *et al.,* (2007) with slight modification. Hydrolysate (0.04 g) was diluted with 10ml distilled water. 1.0 ml of the diluted sample was then mixed with 1.5ml of 0.15mM DPPH in 75% ethanol and vortexed. The mixture was then incubated in the dark for 30min at room temperature. At the end of the incubation, the mixture was centrifuged (Kubota Centrifuge 5420, Japan) for 15min at 3500rpm followed by filtration through Whatman filter paper No. 41. Filtrate was collected and the absorbance was read at 517nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Blank was prepared in the same manner except that distilled water was used instead of sample. The scavenging effect was calculated as follows:

$$\text{Radical scavenging activity (\%)} = \frac{B-A}{B} \times 100 \quad (2)$$

where A is A_{517} of sample and B is A_{517} of the blank.

Determination of Nitrogen Solubility Index (% NSI)

Nitrogen Solubility Index (% NSI) was used to determine the solubility of green mussel hydrolysate following the procedure of Morr *et al.,* (1985). 0.5g hydrolysate was dispersed in 50ml of 0.1M NaCl and the pH was adjusted to pH 7. The mixture was stirred

at room temperature for 1 hour, centrifuged at 2560g for 30 minutes (Sowell Model RC-5B plus Newtown, Ct, USA) and the supernatant collected was then filtered through Whatman filter paper No. 41. Nitrogen content was analysed using Kjeldhal method (AOAC, 2000). Nitrogen solubility index (NSI) was calculated using the following formula:

$$NSI = A/B \times 100 \quad (3)$$

where A= protein content in supernatant and B is protein content in the sample.

Determination of protein solubility at different concentrations of sodium chloride

Protein solubility was determined according to the method by Morr (1985). 0.2g hydrolysate was added into 20ml of 0 to 6M NaCl and the pH was immediately corrected to pH 8 with 1N NaOH and centrifuged at 7500g for 15 minutes. The soluble protein in the collected supernatants was determined by Kjeldhal method according to AOAC (2000).

$$\text{Solubility (\%)} = \frac{\text{protein content in supernatant}}{\text{total protein in sample}} \times 100 \quad (4)$$

Determination of the stability of green mussel hydrolysate during storage

The freeze dried hydrolysate (2.5g) was kept at 4 and 25°C for seven weeks in amber vial tightly closed with screw-cap. At the end of the storage period, the samples were taken out for antioxidative activity analysis as described previously.

Statistical analysis

All experiments were run in triplicate. Data were subjected to Analysis of Variance (ANOVA). Mean comparisons to evaluate significance at 95% confidence level were carried out by using Duncan's multiple range tests. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for windows: SPSS Inc. Chicago, II., USA, 2006).

Results and discussion

DPPH radical-scavenging activity of green mussel hydrolysate

The stability of DPPH radical-scavenging activity (%) in the green mussel hydrolysates produced at pH9, ES 3% and pH7, ES 5% after seven week storage at 4 and 25°C was observed to decrease gradually with the increase in storage time (Figure 1). This observation was in agreement with Thiansilakul *et al.,* (2007) who suggested that prolonged storage of the hydrolysate could destroy the antioxidative compounds leading to losses in antioxidative activity. The percentage DPPH was significantly higher ($p<0.05$) at 4°C compared to 25°C throughout the seven week storage. This implied that antioxidant activity of green mussel hydrolysate was more stable when stored at 4°C compared to 25°C. In fruits, the antioxidant activities has been associated with phenolic compounds, however, in fish protein hydrolysates this has been attributed to its constituents peptides and / or amino acids (Margetts & Buttriss, 2003; Anusha *et al.,* 2008).

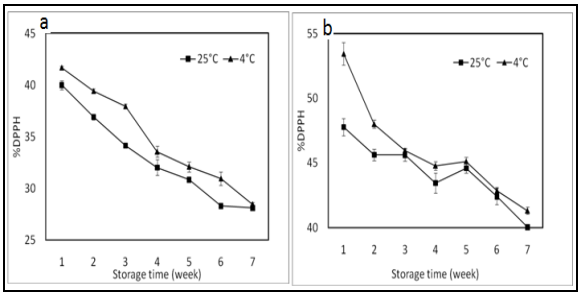

Figure 1:%DPPH radical-scavenging activity of green mussel *(Perna viridis)* hydrolysate produced at a) pH9, ES 3% and b) pH7, ES 5% during seven weeks storage at 4°C and 25°C.

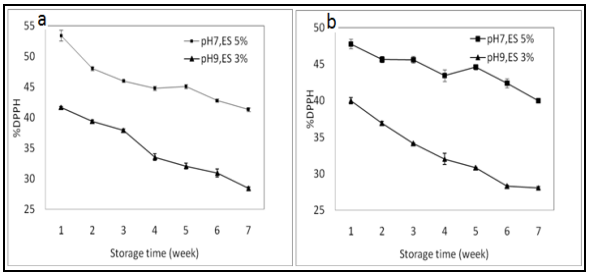

Figure 2:%DPPH radical-scavenging activity of green mussel *(Perna viridis)* hydrolysate produced at pH9, ES 3% and pH7, ES 5% during seven week storage at a) 4°C and b) 25°C.

Generally, aromatic amino acids are considered to be effective radical scavengers due to their ability to

donate proton easily to electron-deficient radicals (Rajapakse *et al.,* 2005). Rissom *et al.,* (1980) stated that amino acids such as histidine and tryptophan exhibited higher antioxidative activity than methionine, cysteine, glycine and alanine. Histidine residues in the peptide contain imidazole ring which enable it to chelate and trap free radicals whereas tyrosine may act as a potent hydrogen donor (Je *et al.,* 2005).

Hydrolysate with lower DH (28.33%) produced at pH7, ES 5% showed higher DPPH radical-scavenging activity than hydrolysate produced at pH 9, ES 3% (43.81%) (Figure 2). This indicated that DH can strongly influence the antioxidant properties of a hydrolysate. The higher DPPH radical-scavenging value at low DH was in agreement with Klompong *et al.,* (2007) who obtained a higher DPPH radical-scavenging activity at 5% DH compared to that of 25% DH. Similar findings were also obtained by Jun *et al.,* (2004) in yellowfin sole hydrolysate produced using pepsin where at lowest DH (22%), the antioxidant activity was higher compared to those produced using α-chymotrypsin, papain, pepsin, pronase E, neutrase and trypsin. Their hydrolysate was characterised by peptides with 1300 Da. Studies by Li *et al.,* (2007) showed that radical scavenging effect initially increased when the DH was below 85%, however, when the DH exceed 85% where most of the peptides have been converted into free amino acid, the radical-scavenging activity decreased. They concluded that small peptides with molecular weight 265 to 560 Da had a high level of scavenging effect. In contrary, Li *et al.,* (2008) reported that chickpea protein hydrolysate (CPH) peptide characterised by molecular weight of 200 to 3000 Da had high antioxidant activity while Wu *et al.,* (2003) studies showed that peptide from mackerel hydrolysate with molecular weight of 1400 Da are more potent antioxidant than those with 900 and 200 Da. Peptides and proteins in the hydrolysate react with radicals and convert them into more stable products thereby terminating the radical chain reaction (Khanthaphant & Benjakul, 2008). The peptides in hydrolysate produced under different DH might differently scavenge with DPPH radicals due to variations in terms of the peptide chain length and amino acid sequence (Nalinanon *et al.,* 2011). Chen *et al.,* (1996) indicated that six peptides comprising of 5 to 16 amino acids residues had strong antioxidant activities. Others include short peptides such as Leu-Pro-His-Ser-Gly-Tyr (672 Da) (Je *et al.,* 2005) and His-Gly-Pro-Leu-Gly-Pro-Leu (797 Da) (Mendis *et al.,* 2005). High degree of hydrolysis could lead to the loss of scavenging effect of the contributing peptides and usually DH increased with time (Li *et al.,* 2007). Wu *et al.,* (2003) observed a dramatic increased in peptides when the hydrolysis time increased from 5 to 10 hrs, however, the peptides declined at 15 hrs of hydrolysis in-line with the antioxidant activity. They suggested that the peptides were degraded into free amino acids. Although in general, the DH had influence on the radical scavenging effect, the radical-scavenging activity of a protein hydrolysate would also depend on a variety of other factors such as the size of peptides and their composition and the composition of free amino acids (Wu *et al.,* 2003).

Effect of DH on solubility of green mussel hydrolysate

Nitrogen Solubility index (NSI)

Solubility is one of the most important physicochemical and functional properties of protein hydrolysates (Kinsella, 1976). Solubility of green mussel hydrolysate was expressed as the percentage of soluble nitrogen to total nitrogen in sample. Table 2 shows the DH and NSI of the freeze-dried green mussel hydrolysate produced at pH 9, ES 3% and pH 7, ES 5%. NSI ranged between 18 to 35%.

Table 2: Degree of hydrolysis (DH) and nitrogen solubility index (NSI%) of green mussel (*Perna viridis*) hydrolysate produced at pH7, ES 5% and pH9, ES 3%.

Hydrolysis condition	DH (%)	**NSI (%)**
pH 7, ES 5%	28.33±1.05	17.96±0.32
pH 9, ES 3%	43.81±0.60	34.71±0.76

Table 3: Solubility of green mussel (*Perna viridis*) hydrolysate produced at pH7, ES 5% and pH9, ES 3% at different concentrations of NaCl.

Concentration of NaCl (M)	**pH7, ES 3%**	**pH9, ES 5%**
0	6.94 ± 0.31^{aB}	16.30 ± 0.07^{aA}
1	6.85 ± 0.11^{aB}	14.34 ± 0.30^{bA}
2	6.38 ± 0.26^{bB}	11.51 ± 0.02^{cA}
3	6.23 ± 0.12^{bB}	10.86 ± 0.22^{dA}
4	5.48 ± 0.00^{dB}	8.63 ± 0.02^{eA}
5	5.79 ± 0.09^{cB}	7.50 ± 0.05^{fA}
6	5.08 ± 0.04^{eA}	2.84 ± 0.03^{gA}

Values are expressed as means± standard deviation from triplicate determinations.

Values with different lower cases within columns and upper cases within rows are significantly different at $p < 0.05$.

Hydrolysate produced at pH 9, ES 3% with 43.81% DH showed approximately 50% higher NSI compared to hydrolysate produced at pH 7, ES 5% with 28.33% DH. Several authors have also shown that hydrolysates produced at high DH had higher solubility (Dong *et al.,* 2008; Klompong *et al.,* 2007; Gbogouri *et al.,* 2004). Klompong *et al.,* (2007) who hydrolysed yellow stripe trevally using up to 10% (w/w) Alcalase 2.4L obtained as high as 40% DH. The solubility reported was at least 85% which increased with DH. Gbogouri *et al.,* (2004) who hydrolysed salmon head protein at 11.5 to 17.3% DH using Alcalase 2.4L at pH 7.5 to 8 achieved more than 75% solubility where the solubility was highest at 17.3% DH. Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity as well as polar and ionizable group of protein hydrolysates (Mutilangi *et al.,* 1996; Turgeon *et al.,* 1992). According to Dong *et al.,* (2008), at higher DH, solubility increases due to the presence of protein fraction characterised by low molecular mass less than 5000 Da. Hydrolysis of protein released both the hydrophilic and hydrophobic soluble peptides where the proportion of these peptides depends on the DH (Linares *et al.,* 2000). Hydrolysates containing smaller peptides with proportionally more polar residues are able to form hydrogen bonds with water and increase the solubility (Kristinsson & Rasco, 2000b). Good solubility protein is required in many functional applications especially for emulsions, foams and gels (Zayas, 1997).

Effect of different sodium chloride concentrations on the solubility of green mussel hydrolysate

The effects of sodium chloride at 0 to 6M NaCl on the solubility of green mussel *(Perna viridis)* hydrolysate are depicted in Table 3. Increased in NaCl concentration resulted in the decreased in solubility of both hydrolysates. Agyare *et al.,* (2009), observed a decrease in surface hydrophobicity of transglutamase-treated wheat gluten hydrolysate in the presence of NaCl as compared to control samples without NaCl treatment. Solubility enhancement was attributed to deamination or formation of ionized carboxyl groups and reduction in surface hydrophobicity (Agyare *et al.,* 2008). Linares *et al.,* (2001) who concluded that gluten hydrolysates solubility was influenced by salt concentration and pH found that the hydrolysates were highly soluble at pH 4, 0.2% NaCl and solubility decreased as NaCl concentration was increased to 2%. Na^+ and Cl^- ions probably masked charges on the hydrophobic peptides via electrostatic interaction (Linares *et al.,* 2000). Solubility was significantly higher ($p<0.05$) when the hydrolysate was produced at pH 9, ES 3% compared to those produced at pH 7, ES5% except at 6M NaCl. Liu *et al.,* (2010) obtained higher solubility of porcine protein hydrolysate when the DH was increased from 6.2% to 17.6%. Similar findings were also reported by Klompong *et al.,* (2007). Linares *et al.,* (2000) found that solubility was lower at pH 4 compared to pH 6.5 with 2% NaCl. These studies were in agreement with the current observation where the solubility of green mussel hydrolysate was higher at higher DH (43.81% DH) and at higher pH.

Conclusion

Protein hydrolysate derived from green mussel exhibited antioxidant activities, high solubility and was more stable when stored at low temperature (4°C). Therefore, it has a wide range of potential application and can be used in food systems as natural additive possessing functionalities and antioxidant properties.

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