

Characterization of green mussel (*Perna viridis*) hydrolysate prepared using alcalase and starfruit (*Averrhoa carambola. L*) protease

*Normah, I. and Asmah, A.H.

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

Article history

Received: 14 October 2015
Received in revised form:
27 November 2015
Accepted: 24 December 2015

Abstract

This study was carried out to evaluate the efficiency of natural protease extracted from starfruit (*Averrhoa carambola. L*) to hydrolyse green mussel (*Perna viridis*). Starfruit juice from fruit of maturity index 2 was extracted and purified using acetone precipitation method. The obtained protease was then used to hydrolyse green mussel and compared with alcalase. The conditions used to produce starfruit protease hydrolysate (SPH) were pH 8, E/S 3%, 60°C for 2 hrs and alcalase hydrolysate (AH) were pH 9, E/S 3%, 60°C for 2 hrs. Yield, degree of hydrolysis (DH), protein concentration, color, solubility, heat stability and molecular weight distribution of the hydrolysates were determined. Results showed that the acetone precipitated protease had higher specific activity (0.31 U/mg) than the crude extract (0.16 U/mg). Alcalase exhibited higher DH (34.82%) than starfruit protease (11.68%), higher yield and protein concentration. The soluble protein content of both hydrolysates were high in pH range of 3 to 10 while AH was more stable at high temperature. The SDS-PAGE profiles of the hydrolysate showed that AH contains molecular weight (MW) lower (<10 kDa) than SPH (<50 kDa), thus it corresponds with the characteristic of AH that was more soluble (91.51%) as compared to SPH (81.21%). It is concluded that hydrolysis using alcalase produced better results in terms of yield, DH, protein concentration and molecular weight distribution. However, in terms of solubility and heat stability both hydrolysates showed high soluble protein content. Thus, starfruit protease has the potential to be used as an alternative source of protease for the production of protein hydrolysate.

Keywords

Starfruit
Protease activity
Hydrolysate
Green mussel
Acetone precipitation

© All Rights Reserved

Introduction

The Asian green mussels (*Perna viridis*) are bivalves belonging to the Mytilidae family that are economically important mussel. Global total production of fish, crustaceans, molluscs and other aquatic animals has continued to increase, reaching 156.2 million tonnes in 2011 and the world mussel productions of all types reached about 93 million tonnes in 2011 (FAO, 2011). In Malaysia, the aquaculture production of fish, crustaceans, molluscs and others by principal producers in 2011 was 287 076 tonnes (FAO, 2011). *Perna perna* are found widely distributed in the South America, Africa, Asia and India while *P. viridis* is distributed widely in the Persian Gulf to the South-west Pacific longitudinally and from Southern Japan to Papua New Guinea latitudinally (Siddall, 1980). They are important for marine ecology and for human diet since they are an important source of nutrients (Fuentes *et al.*, 2009). Farmed marine mussels from the Mytilidae family comprising genera such as *Mytilus* and *Perna* are popular in human diet providing at least 10% proteins,

omega-3 polyunsaturated fatty acids (PUFAs), iodine and carbohydrates (Normah *et al.*, 2013; Grienke *et al.*, 2014).

Protein hydrolysate is a mixture of amino acids prepared by splitting a protein with acid, alkali or enzyme. Alcalase are most favoured for fish hydrolysis due to the high DH that can be achieved in a relatively short time under moderate pH conditions (Aspmo *et al.*, 2005; Bhaskar and Mahendrakar, 2008). Hydrolysates prepared by using alcalase had the highest recovery and the lowest lipid content than those produced using papain and neutrase (Adler-Nissen, 1986). Good foaming properties for capelin protein hydrolysates prepared by alcalase at low DH have been reported (Shahidi *et al.*, 1995). In another study, it was showed a DH of 37.27% was achieved when cockle protein was hydrolysed using alcalase at 65°C, enzyme concentration 2%, pH of 9.5 and hydrolysis time 180 minutes (Amiza and Masitah, 2012).

The great majority of commercial enzymes have been obtained mainly from microbial sources but plant enzymes are becoming increasingly

*Corresponding author.

Email: norismel@salam.uitm.edu.my, normaisma@yahoo.com
Tel: 603-55443876; Fax: 603-55444562

important with applications in industrial processes, biotechnology and pharmacology (González-Rábade *et al.*, 2011). Plant peel could be a potential source of proteases due to the easy purification methods, low levels of interfering substances during purification and good yield of proteases (Tomar *et al.*, 2008). Commonly used methods for protease purification are ammonium sulfate precipitation and acetone precipitation (He *et al.*, 2008; Siti Balqis and Rosma, 2011; Memarpoor Yazdi *et al.*, 2013).

Most studies on the production of protein hydrolysate were conducted using a variety of commercial enzyme such as alcalase, flavourzyme, pepsin and neutrase (Tang *et al.*, 2009; Li *et al.*, 2012; Normah *et al.*, 2013). Several hydrolyzed fish protein foods have been studied, however, not much research has been done on hydrolysate production from shellfish. Furthermore, the study involving production of green mussel hydrolysate using partially purified enzyme from plant sources has never been done before. Therefore, in this study, starfruit protease was partially purified by acetone precipitation method. The efficacy of starfruit protease in hydrolysing green mussel in terms of yield, degree of hydrolysis (DH), protein concentration, color, solubility, heat stability and molecular weight distribution was compared with the commercial protease, alcalase.

Materials and Methods

Materials

Starfruit (*Averrhoa carambola*. L) was purchased from the Selangor Fruit Valley in Kuala Selangor, Selangor, Malaysia. The unripe fruit of maturity Index 2 (green overall with stripes of yellow) was chosen for enzyme extraction. Green mussel (*Perna viridis*) was bought from Pantai Remis, Kuala Selangor, Malaysia and immediately stored in ice before being transported to the laboratory. All chemicals used were of analytical grade purchased from Sigma-Aldrich, UK.

Extraction and purification of proteases from starfruit

Extraction of the protease was done at 4°C. Before grinding, the fruit was cut and the seeds were removed followed by washing. The juice was extracted by using a juice extractor and then centrifuged (Model 320 R, Universal Hettich, Malaysia) at 5000 rpm for 10 minutes to allow separation. The supernatant was filtered through a layer of muslin cloth into a beaker. The collected supernatant, also referred as the crude extract was then purified by acetone precipitation method according to He *et al.*, (2008). Cold acetone (-20°C) was slowly added to the supernatant and

agitated gently at least 30 minutes to allow for precipitation. This was followed by centrifugation in a centrifuge (Model 320 R, Universal Hettich, Malaysia) at 5000 rpm for 15 minutes. The precipitate was dissolved in minimum amount of phosphate buffer (50 mmol/L) and dialyzed against the same buffer at 4°C for 12 hr.

Protein content

Protein content of starfruit pulp and crude extract were measured using Kjeldahl method (AOAC, 2005).

Protease activity assay

Protease activity was measured using casein as a substrate according to Cupp-Enyard (2008). An amount of 5 mL of 0.65% w/v casein in 50 mM potassium phosphate buffer, pH 7.5 and 1 mL sample were added into a vial and incubated at 55°C for 10 minutes. Then, 5 mL of 110 mM trichloroacetic acid was added to stop the reaction. The solutions were incubated at 37°C for 30 minutes to stabilize the reaction. After incubation, the solutions were filtered and 2 mL of the filtrate were mixed with 1 mL of Folin's reagent and 5 mL of 500 mM sodium carbonate. The solution was incubated at 37°C for 30 minutes and then filtered. Absorbance was measured at 660 nm. One unit of protease activity (U) was defined as the amount of protease that can hydrolyze casein to 1 µg of tyrosine in 1 minute at 55°C. The total activity of the protease was determined using the following formula:

$$\text{Total activity (Units/mL)} = \frac{(\mu\text{moles tyrosine equivalents released}) \times (11)}{(1) \times (10) \times (1)}$$

where:

11 = total volume of assay

1 = volume of enzyme

10 = time of assay (in minute) as per the unit definition

1 = volume (in milliliters) used in colorimetric determination

Proteases specific activity

Proteases specific activity was calculated using the following formula:

$$\text{Enzyme specific activity (U/mg)} = \frac{\text{Enzyme units per ml}}{\text{Protein (mg/ml)}}$$

Preparation of green mussel hydrolysate

Green mussel hydrolysate was prepared according to method of Normah *et al.*, (2013). Approximately 507.69 g green mussel was mixed in

564.81 g distilled water and then minced in a blender. Hydrolysis was performed in a 1 L reaction vessel placed in a thermally controlled water bath and stirred continuously at 200 rpm. The pH of the mixture was adjusted to pH 9 by adding 1.0 M NaOH and the temperature was maintained at 60°C. Hydrolysis was initiated by alcalase addition at E/S ratio of 3% and the hydrolysis proceeded at pH 9, 60 °C for 2 hrs. The efficiency of starfruit protease to hydrolyze green mussel was also evaluated to compare with alcalase. Therefore, starfruit protease was added at E/S ratio of 3% at pH 8. DH was calculated based on the amount of NaOH added to keep the pH constant during the hydrolysis. At the end of the 2 hrs hydrolysis, the reaction was terminated by immersing the reaction vessel in water bath set at 95°C for 15 minutes with constant agitation to ensure enzyme inactivation. The resultant slurry was centrifuged (Model 320 R, Universal Hettich, Malaysia) at 5000 rpm, 4°C for 20 minutes. The supernatant was collected and freeze dried (Martin Christ, Alpha 1-4, Germany).

Degree of hydrolysis (DH)

The hydrolysis was carried out using the pH-stat method which allowed the estimation of DH based on the consumption of alkali to maintain a constant pH at the desired value (Adler-Nissen, 1986). The DH (%) was calculated from the volume and molarity of alkali used to maintain a constant pH.

DH was determined based on the following formula:

$$DH (\%) = \frac{\beta \times N\beta \times 100}{\alpha \times MP \times htot}$$

where:

β = volume of NaOH

$N\beta$ = molarity of NaOH

α = average degree of dissociation of the NH₂ groups

MP = mass of protein

htot = total number of peptide bonds in the protein substrate in mmol/g protein

Yield

The yield obtained by hydrolysis of green mussel using either alcalase or starfruit protease was determined using the ratio of the mass of hydrolysate to the total weight of the raw green mussel flesh using the following calculation:

$$Yield (\%) = \frac{\text{weight of powdered hydrolysate}}{\text{wet weight of green mussel flesh}} \times 100$$

Protein concentration

Protein concentration was measured with bovine

serum albumin as a standard according to Lowry *et al.* (1951).

Color measurement

Hydrolysate color was measured by using Hunterlab Ultrascan Sphere Spectrocolorimeter (Model Minalto, CR-400, Malaysia). L*, a*, and b* parameters indicate brightness, redness and yellowness, respectively. The instrument was calibrated with white tile CM-A101.

Solubility

Hydrolysate solubility was determined by using the method of Li *et al.*, (2012). An amount of 100 mg hydrolysate was dispersed in 10 mL deionised water at room temperature and the pH of the solution was adjusted to pH 3 to 10. The solution was centrifuged (Model 320 R, Universal Hettich, Malaysia) at 5000 rpm for 15 minutes. Total protein concentration in the sample was determined after dissolving in 0.5 mol/L NaOH. Protein concentration in the supernatant was determined using the Biuret method.

$$\text{Soluble protein (\%)} = \frac{\text{Protein concentration in supernatant}}{\text{Protein concentration in sample}} \times 100$$

Heat stability

Heat stability of the hydrolysate was determined according to Li *et al.*, (2012). An amount of 100 mg hydrolysate was dispersed in 10 mL of deionised water and the pH of the solution was adjusted to pH 3, 5, 7 and 10 with 0.1 mol/L HCl or 0.1 mol/L NaOH. After heating at 60°C and 90°C for 30 minutes, the solution was kept in ice-water (0°C) for about 10 minutes. Heat stability was calculated as the soluble protein after heat treatment at 60°C and 90°C. Total protein concentration in the sample was determined after dissolving in 0.5 mol/L NaOH.

$$\text{Soluble protein (\%)} = \frac{\text{Protein concentration in supernatant}}{\text{Protein concentration in sample}} \times 100$$

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted to determine the molecular weight based on Normah *et al.*, (2013). Samples were prepared by mixing 19.5 μ L of sample in 7.5 μ L of 2x sample buffer and 3 μ L of reducing agent which is then heated at 70°C for 10 minutes. 10 μ L of the solution was then loaded into each well on the gel (10 cm x 10 cm). Benchmark™ protein ladder with molecular weight ranging from 10 to 220 kDa was used as a marker.

Electrophoresis was performed by XCell Surelock electrophoresis cell (Consort, EV231,

Germany) and run for 50 minutes at 100-125 mA/gel. Subsequently, the gel was washed in distilled water, heated in microwave oven at 180°C for one minute and then gently shaken for about 2 minutes. The process was repeated twice. The gel was stained in Coomassie brilliant blue (G-250) and destained in ultra pure water.

Statistical analysis

All the tests were conducted in triplicate and data were averaged. Statistical analysis was performed using the SAS Version 9.1.3 for Windows (SAS Institute Inc., Cary, NC, 2009). The data was subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests at significant difference of 95% confidence interval ($p < 0.05$).

Results and Discussion

Proteolytic activity of starfruit protease

Starfruit protease was purified using acetone precipitation method and the proteolytic activity was analyzed. The protein contents were 1.02 g per 100 g for the pulp and 0.37 g per 100 g for the crude extract. Results showed that the crude extract had higher total activity (231.43 U) and total protein (1484.52 mg) than protease produced by acetone precipitation (Table 1). However, the specific activity of starfruit protease purified using acetone (0.31 U/mg) is higher than the crude (0.16 U/mg). Several studies showed that acetone precipitation resulted in higher yield, specific activity and recovery (Michail *et al.*, 2006; He *et al.*, 2008; Negi *et al.*, 2011). Using trout heads as the source of protease, a successful precipitation was achieved with cold acetone in the ratio of 1:1.25 (acetone : crude extract) recording 99% recovery and specific activity of 1559 U/mg which is higher than the crude (47.6 U/mg) (Michail *et al.*, 2006). A related study also showed the specific activity of garlic protease purified using acetone increased with the increase in acetone concentration (He *et al.*, 2008). The range used was between 0 to 1.5 where the highest specific enzyme activity (527.4 U/mg) was achieved with 50% acetone concentration. Starfruit protease prepared from both the ripe (stage 7) and unripe (stage 2) fruit using acetone exhibited proteolytic activity which was higher than the crude ranging from 100 to 710 CDU/mg whereas the crude extract specific activity was in the range from 15 to 110 CDU/mg (Normah and Ezzana Zuraini, 2015). A study on the purification of cysteine protease from the rhizomes of *Curcuma longa* (Linn.) by using acetone showed 28% recovery with six purification fold

Table 1. Proteolytic activity of starfruit (*Averrhoa carambola*. L) crude extract and protease purified using acetone precipitation

Stage	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	1484.52	231.43	0.16	1.00	100
Acetone precipitation	595.17	184.19	0.31	1.94	79.59

(Nagarathnam *et al.*, 2010). Acetone precipitation provides superior recovery and efficiently removes the detergents, protease inhibitor, lipids and other buffer with no introduction of salts or strong acids to the sample (Duan *et al.*, 2009).

Yield, color, DH and protein concentration

The efficiency of starfruit protease and alcalase in hydrolysing green mussel protein was compared. Green mussel was hydrolyzed for 2 hrs using two different enzymes; alcalase and starfruit protease. Yield of starfruit hydrolysate (SPH) was significantly lower ($p < 0.05$) (5.27%) than alcalase hydrolysate (AH) (8.66%) (Table 2). The yield of other shellfish hydrolysate which is from angelwing clam produced using alcalase were 11.03% (Normah and Nurfazlika Nashrah, 2013).

The hydrolysate produced using starfruit protease (SPH) and alcalase (AH) were characterized by strong fishy odor. SPH was significantly lighter ($L = 72.06$, $a = 0.24$, $b = 25.52$) than AH ($L = 63.67$, $a = 1.37$, $b = 24.87$). The slight differences in color between AH and SPH might be due to the utilization of different types of enzymes and effect of freeze drying. Hydrolysate's color is affected by factors such as species, processing, fat content, moisture, hemoglobin and myoglobin content (Bueno-Solano *et al.*, 2008). Enzymatic reactions are assumed to have contributed to the reduction in the luminosity giving a darker appearance at high DH (Wasswa *et al.*, 2007).

DH of AH was significantly higher ($p < 0.05$) than SPH (Table 2). During hydrolysis, the amount of NaOH added to maintain the pH in AH is higher than in SPH. It was stated that the amount of peptide bond cleaved is proportional to the volume of titrant consumed for maintaining constant pH in the pH-stat method (Adler-Nissen, 1986). Thus, the higher the amount of peptide bond cleaved by the enzyme, the

Table 2. Yield (%), degree of hydrolysis (DH) % and protein concentration (%) of green mussel hydrolysate produced using starfruit protease and alcalase

Sample	Yield (%)	DH (%)	Protein concentration (%)
Raw green mussel	-	-	12.28 ± 0.056 ^C
AH	8.66 ± 0.1788 ^A	34.82 ± 0.1300 ^A	51.13 ± 0.097 ^A
SPH	5.27 ± 0.2409 ^B	11.68 ± 0.1456 ^B	39.09 ± 0.022 ^B

*Values within the same column followed by different superscript A, B or C are significantly different ($p < 0.05$). Alcalase Hydrolysate (AH); Starfruit Protease Hydrolysate (SPH).

higher is the DH (Seniman *et al.*, 2014). Alcalase has been used for the studies of protein hydrolysates from cockle, blue mussel and angelwing clam (Amiza and Masitah, 2012; Normah and Nurfazlika Nashrah, 2013; Wang *et al.*, 2013). Alcalase is most favoured for fish hydrolysis due to the high DH that can be achieved in a relatively short time under moderate pH conditions (Aspmo *et al.*, 2005; Bhaskar and Mahendrakar, 2008).

Protein concentration of raw green mussel and its hydrolysates were quantified as shown in Table 2. Raw mussel contains 12.28% protein followed by AH (51.13%) and SPH (39.09%), respectively. AH with higher DH also showed higher protein concentration. Due to proteolytic enzyme activity, protein content was higher since more peptides are released (Seniman *et al.*, 2014). The high protein is also due to solubilization of proteins during hydrolysis and removal of insoluble solid matter by centrifugation and the partial removal of lipid after hydrolysis (Benjakul and Morrissey, 1997; Liceaga-Gesualdo and Li-Chan, 1999; Thiansilakul *et al.*, 2007; Chalamaiah *et al.*, 2010).

Protein solubility

The soluble protein of green mussel hydrolysate was expressed as the percentage of protein content in the supernatant to the total protein content in the sample. Figure 1 shows the solubility of AH and SPH produced at 3% E/S ratio at pH 9 and at 3% E/S ratio at pH 8, respectively. AH was soluble over a wide pH range in which more than 38% solubility was obtained at the DH of 38.42% and the highest solubility was achieved at pH 9 (91.51%). SPH showed solubility from 48.79% at 11.68% DH, pH 3 and highest solubility was at pH 6 (81.21%).

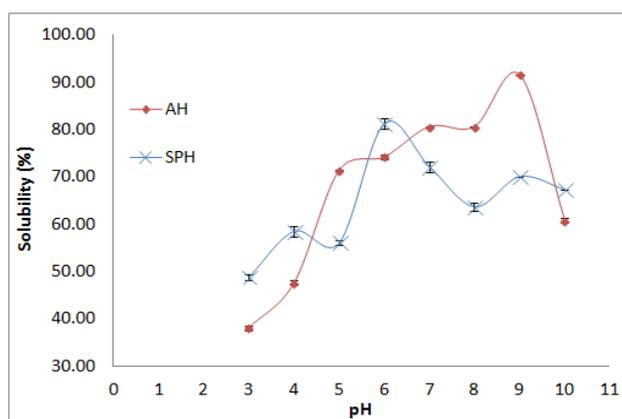


Figure 1. Solubility (%) of green mussel hydrolysate at different pH; alcalase hydrolysate (AH), starfruit protease hydrolysate (SPH)

A relationship between solubility and DH value was observed where higher DH values showed higher solubility. Smaller peptides are expected to have proportionally more polar residues with the ability to form hydrogen bonds with water and increasing solubility (Gbogouri *et al.*, 2004). Hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) flesh hydrolysed by alcalase and flavourzyme at DH between 5 to 25% showed high solubility with more than 85% solubility in the pH range of 2 to 12 (Klompong *et al.*, 2007). In this study, approximately 90 and 80% solubility was achieved at pH 9 and pH 6 for AH and SPH, respectively. Salmon head hydrolysate produced using Alcalase 2.4 L showed more than 75% solubility at pH ranging from 3 to 11 and 11.5 to 17.3% DH (Gbogouri *et al.*, 2004).

Green mussel hydrolysates were generally soluble in alkaline pH to a greater extent except at pH 10 where the solubility slightly decreased. The hydrolysate has lower solubility in the acidic than in the alkaline condition with the lowest solubility value of 41.59% was recorded at pH 3. Grass carp hydrolysates produced using alcalase and papain showed solubility of more than 81% over pH range of 3 to 8 with the lowest values occurring at pH 4 (Li *et al.*, 2012). This showed that at lower pH some of the protein content in the hydrolysate might be denatured due to acidic condition. pH influences the charge on the weakly acidic and basic side-chain groups, thus protein and hydrolysates display low solubility at their isoelectric point (Chobert *et al.*, 1988; Linder *et al.*, 1996). Many proteins in solution generally precipitate at their isoelectric points and have the minimum solubility because the net charge is zero and thus charge repulsions of similar molecules are at minimum. Hydrolysis of protein released both the hydrophilic and hydrophobic soluble peptides where the proportion

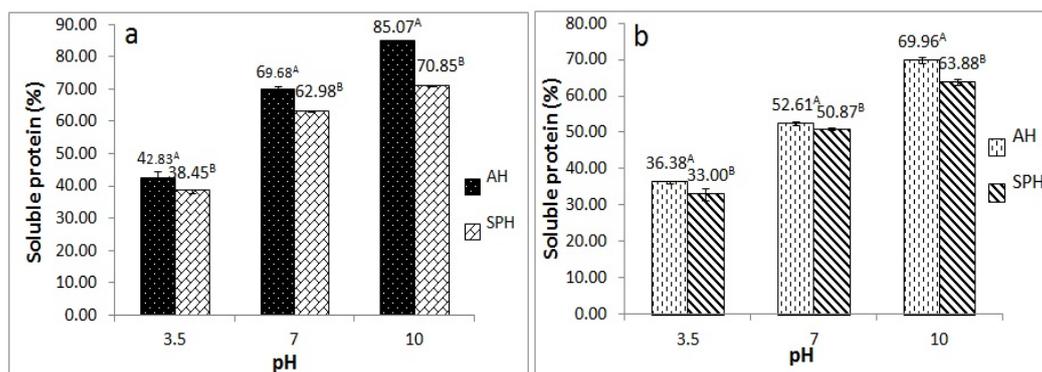


Figure 2. Heat stability of green mussel hydrolysate at a) 60°C and b) 90°C; alcalase hydrolysate (AH), starfruit protease hydrolysate (SPH)

of these peptides depends on the DH (Linares *et al.*, 2000). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity as well as polar and ionizable group of protein hydrolysates (Turgeon *et al.*, 1992; Mutilangi *et al.*, 1996). Previous study on angelwing clam hydrolysate prepared using alcalase showed that the hydrolysate had solubility value of 92.32% and was presumed to have a low molecular weight and was hydrophilic in nature (Normah and Nurfazlika Nashrah, 2013). Solubility is necessary to allow protein to be able to absorb water and then thicken the protein dispersion (Kinsella, 1984; Nakai and Li-Chan, 1993). Hydrolysates containing smaller peptides with proportionally more polar residues are able to form hydrogen bonds with water and increase the solubility (Kristinsson and Rasco, 2000). In general, the degradation of protein into smaller peptides will lead to more soluble products (Gbogouri *et al.*, 2004).

Heat stability of hydrolysates at different heat treatment and pH

The heat stability of AH and SPH based on different heat treatment at different pH was studied and the results are shown on Figure 2 a and b. Soluble protein in AH and SPH increased with increase in pH. However, as the temperature increased, soluble protein decreased. In general, soluble protein in AH is more stable than those from SPH. At acidic pH, sedimentation was observed which indicate protein degradation. In hydrolysates obtained using alcalase 2.4L and papain, a good heat stability was exhibited with more than 80 % solubility after heating at 63°C for 30 minutes or at 93°C for 30 s (Li *et al.*, 2012). However, in this study at high temperature (90°C) both AH and SPH showed a decrease in soluble protein due to longer treatment time (30 minutes).

At 60°C the hydrolysates produced using alcalase and starfruit protease had the highest amount of soluble protein at pH 10 and the lowest at pH 3.5.

Soluble protein of AH was significantly higher ($p < 0.05$) than SPH. Similar trend was observed at 90°C. This might be due to denaturation of protein after exposure to high temperature thus there is no significant effect observed at certain pH. Therefore, both pH and temperature affected the amount of soluble protein in the hydrolysates.

Alcalase has great ability to solublize fish protein and is nonspecific with an optimum temperature ranged from 50 to 70°C (Chabeaud *et al.*, 2009). Proteins were vulnerable to heat treatment leading to the aggregation of protein and the exposure of hydrophobic domain (Sikorski and Naczka, 1981). This showed that both pH and temperature affected the solubility of the hydrolysates. Temperature and pH are the two major factors affecting denaturation of proteins. At high temperatures, increased hydrophobicity leads to denaturation-aggregation which causes loss of functional properties, mainly solubility (Poulter *et al.*, 1985; Tornberg, 2005).

During analysis, the hydrolysates prepared in acidic pH were observed to produce sedimentation. This might be due to the degradation of protein that occurs as the protein bond is broken down when subjected to low pH. Therefore, the lowest soluble protein was obtained at pH 3.5. Based on the result, this showed that SPH was not suitable to be used in food that is high in acid as the protein will be reduced rapidly. The hydrolysates may be used in production of food that is either in neutral or alkaline condition. The heat stability may be attributed to enzymatic hydrolysis improving the balance of hydrophilic and hydrophobic forces of peptide which can inhibit the aggregation of peptides during heat treatment (Betancur-Ancona *et al.*, 2009). Thermal treatment of protein hydrolysates significantly affects protein solubility (Poulter *et al.*, 1985; Tornberg, 2005; Betancur-Ancona *et al.*, 2009). The thermal effect is particularly important because mussel hydrolysate will have to undergo thermal treatment in real

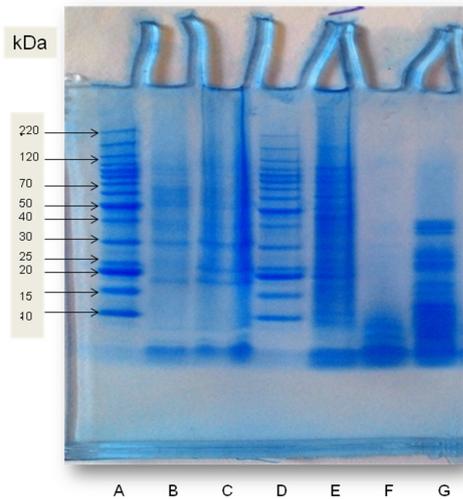


Figure 3. Electrophoretic pattern of starfruit crude extract (*Averrhoa carambola* L.), starfruit protease, raw green mussel (*Perna viridis*) and hydrolysates. From left : (A) protein marker, (B) crude extract, (C) starfruit protease, (D) protein marker, (E) raw green mussel, (F) alcalase hydrolysate and (G) starfruit protease hydrolysate

food systems as the foods are in basic, acidic or alkaline condition. Mussel hydrolysates prepared from alcalase and starfruit protease has the highest solubility at neutral and alkaline pH as compared with acidic condition. However, in terms of heat treatment on the hydrolysate, the soluble protein present is lower when subjected to high temperature treatment. This might be due to the effect of pH that alters the solubility after heating.

Molecular weight distribution

Figure 3 showed that starfruit protease purified using acetone have clear bands from 15 to 50 kDa while the crude extract showed bands in the range of 15 to 220 kDa. Fewer bands were identified in starfruit protease as compared to the crude extract. This might be due to the effect of dialysis during enzyme preparation where excess salts and constituents were removed through cellulose tube during dialysis. Thus, only a certain size of protein that remains in the enzyme produced from the starfruit. A single band in the ion exchange isolated purified bromelain protein inferred the integrity and purity of bromelain protein as the band appeared at approximately 30 kDa (Gautam *et al.*, 2010). Besides, an intense band at 23 to 25 kDa for papain corresponded to papain content in the sample (Thomas *et al.*, 2009).

Raw mussel showed bands ranging from 10 to 220 kDa. In addition, AH with protein concentration of 51.13% has smear band at molecular weight <10 kDa while the SPH with protein concentration of 39.09% showed band at <50 kDa suggesting the hydrolyzing effect by enzyme. AH showed lower

molecular weight as compared to SPH as most of the high molecular weight proteins in the hydrolysate have been hydrolyzed by alcalase. Molecular weight distribution was also related to the solubility of the hydrolysate. DH of AH was 34.82% which is higher than SPH (11.68%). Previous alcalase hydrolysates showed molecular weight of <38 kDa for mussel and <8 kDa from fish viscera (Bhaskar and Mahendrakar, 2008; Normah *et al.*, 2013). Hydrolysates with low molecular weight peptides (<8 kDa) could be of high nutritive value and can also be used effectively as food flavoring agent (Bhaskar and Mahendrakar, 2008). Thus, based on the molecular weight distribution AH which is characterized by higher protein concentration and yield a larger quantity of small peptides as compared to SPH. Hence, by hydrolysis using alcalase it improves the AH solubility better than using the starfruit protease.

AH has better characteristics as compared to the SPH based on yield, DH, protein concentration and molecular weight distribution. However, in terms of solubility and heat stability both hydrolysates showed high soluble protein. Thus, it is suggested that starfruit protease might be able to replace commercial enzyme for the production of hydrolysates which can be applied in food such as beverages for protein supplement.

References

- Adler-Nissen, J. 1986. Enzymatic hydrolysis of food proteins. New York: Elsevier Applied Science Publishers.
- Amiza, M. A. and Masitah, M. 2012. Optimization of enzymatic hydrolysis of blood cockle (*Anadara granosa*) using alcalase®. Borneo Science 31: 1-8.
- AOAC. 2005. Official Method of Analysis, 15th ed. Virginia, Association of Official Analytical Chemistry International.
- Aspmo, S. I., Horn, S. J. and Eijsink, V. G. H. 2005. Enzymatic hydrolysis of Atlantic cod (*Gadus morhua* L.) viscera. Process Biochemistry 40: 1957-1966.
- Benjakul, S. and Morrissey, M. T. 1997. Protein hydrolysates from Pacific whiting solid wastes. Journal of Agricultural and Food Chemistry 45: 3423-3430.
- Betancur-Ancona, D., Martínez-Rosado, R., Corona-Cruz, A., Castellanos-Ruelas, A., Jaramillo-Flores, M. E. and Chel-Guerrero, L. 2009. Functional properties of hydrolysates from Phaseolus lunatus seeds. International Journal of Food Science and Technology 44: 128-137.
- Bhaskar, N. and Mahendrakar, N. S. 2008. Protein hydrolysate from visceral waste protein of Catla (*Catla catla*): Optimization of hydrolysis conditions for a commercial neutral protease. Bioresource Technology 99: 4105-4111.
- Bueno-Solano, C., López-Cervantes, J., Campas-

- Baypoli, O. N., Lauterio-García, R., Adan-Bante, N. P. and Sánchez-Machado, D. I. 2008. Chemical and biological characteristics of protein hydrolysates from fermented shrimp by-products. *Food Chemistry* 112: 671-675.
- Chabeaud, A., Dutournié, P., Guérard, F., Vandanjon, L. and Bourseau, P. 2009. Application of response surface methodology to optimise the antioxidant activity of a saithe (*Pollachius virens*) hydrolysate. *Marine Biotechnology* 11: 445-455.
- Chalamaiah, M., Rao, G. N., Rao, D. G. and Jyothirmayi, T. 2010. Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: A review. *Food Chemistry* 120: 652-657.
- Chobert, J. M., Bertrand, H. C. and Nicolas, M. G. 1988. Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. *Journal of Agriculture and Food Chemistry* 36: 883-892.
- Cupp-Enyard, C. 2008. Sigma's non-specific protease activity assay-casein as a substrate. Sigma-Aldrich.
- Duan, X., Young, R., Straubinger, R. M., Page, B. J., Cao, J., Wang, H., Yu, H., Jr, J. M. C. and Qu, J. 2009. A Straightforward and Highly Efficient Precipitation/On-pellet Digestion Procedure Coupled to a Long Gradient Nano-LC Separation and Orbitrap Mass Spectrometry for Label-free Expression Profiling of the Swine Heart Mitochondrial Proteome. *Journal of Proteome Research* 8: 2838-2850.
- FAO. 2011. Food and Agricultural Organization (FAO), FAO Yearbook. *Fishery Statistics*. 1-79.
- Fuentes, A., Fernandez-Segovia, I., Escrichel, I. and Serra, J. A. 2009. Comparison of physico-chemical parameters and composition of mussels (*Mytilus galloprovincialis* Lmk.) from different Spanish origins. *Food Chemistry* 112: 295-302.
- Gautam, S. S., Mishra, S. K., Dash, V., Goyal, A. K. and Rath, G. 2010. Comparative study of extraction, purification and estimation of bromelain from stem and fruit of pineapple plant. *Thailand Journal of Pharmaceutical Sciences* 34: 67-76.
- Gbogouri, G. A., Linder, M., Fanni, J. and Parmentier, M. 2004. Influence of hydrolysis degree on the functional properties of salmon byproduct hydrolysates. *Journal of Food Science* 69: 615-622.
- González-Rábade, N., Badillo-Corona, J. A., Aranda-Barradas, J. S. and Oliver-Salvador, M. D. C. 2011. Production of plant proteases in vivo and in vitro - A review. *Biotechnology Advances* 29: 983-996.
- Grienke, U., Silke, J. and Tasdemir, D. 2014. Bioactive compounds from marine mussels and their effects on human health. *Food Chemistry* 142: 48-60.
- He, N., Li, Q., Sun, D. and Ling, X. 2008. Isolation, purification and characterization of superoxide dismutase from garlic. *Biochemical Engineering Journal* 38: 33-38.
- Kinsella, J. E. 1984. Milk proteins: physicochemical and functional properties. *Critical Review in Food Science Nutrition* 21: 197-262.
- Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry* 102: 1317-1327.
- Kristinsson, H. G. and Rasco, B. A. 2000. Fish protein hydrolysates. Production, bio-chemical and functional properties. *Critical Review in Food Science and Nutrition* 40: 43-81.
- Li, X., Luo, Y., Shen, H. and You, J. 2012. Antioxidant activities and functional properties of grass carp (*Ctenopharyngodonidellus*) protein hydrolysates. *Journal of the Science of Food and Agriculture* 92: 292-298.
- Liceaga-Gesualdo, A. M. and Li-Chan, E. C. Y. 1999. Functional properties of fish protein hydrolysate from herring (*Clupea harengus*). *Journal of Food Science* 64: 1000-1004.
- Linares, E., Larre, C., Lemeste, M. and Popineau, Y. 2000. Emulsifying and foaming properties of gluten hydrolysates with an increasing degree of hydrolysis: role of soluble and insoluble fractions. *Cereal Chemistry* 77: 414-420.
- Linder, M., Fanni, J. and Parmentier, M. 1996. Functional properties of veal bone hydrolysates. *Journal of Food Science* 61: 712-716.
- Lowry, O. H., Resebrough, N. J. and Farr, A. I. 1951. Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry* 193: 265-275.
- Memarpoor Yazdi, M., Mahaki, H. and Zare Zardini, H. 2013. Antioxidant activity of protein hydrolysates and purified peptides from *Zizyphus jujuba* fruits. *Journal of Functional Foods* 5: 62-70.
- Michail, M., Vasiliadou, M. and Zotos, A. 2006. Partial purification and comparison of precipitation techniques of proteolytic enzymes from trout (*Salmo gairdnerii*) heads. *Food Chemistry* 97: 50-55.
- Mutilangi, W. A. M., Panyam, D. and Kilara, A. 1996. Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. *Journal of Food Science* 61: 270-303.
- Nagarathnam, R., Rengasamy, A. and Balasubramanian, R. 2010. Purification and properties of cysteine protease from rhizomes of *Curcuma longa* (Linn.). *Journal of the Science of Food and Agriculture* 90: 97-105.
- Nakai, S. and Li-Chan, E. 1993. Recent advances in structure and function of food proteins: QSAR approach. *Critical Review in Food Science and Nutrition* 33: 477-499.
- Negi, S., Gupta, S. and Banerjee, R. 2011. Extraction and purification of glucoamylase and protease produced by *Aspergillus awamori* in a single-stage fermentation. *Food Technology Biotechnology* 49: 310-315.
- Normah, I. and Ezzana Zuraini, Z. 2015. Effects of precipitation methods on the properties of protease extracted from starfruit (*Averrhoa carambola* L.) of different maturity index. *Scientific Research Journal* 12(1): 1-12.
- Normah, I. and Nurfazlika Nashrah, M. P. 2013. Evaluation

- on the properties of mentarang (*Pholas orientalis*) protein hydrolysate. *Pertanika Journal of Tropical Agricultural Science* 36: 199–210.
- Normah, I., Siti Hafisah, M. S. and Nurul Izzaira, A. 2013. Bitterness of green mussel (*Perna viridis*) hydrolysate as influenced by the degree of hydrolysis. *International Food Research Journal* 20: 2261-2268.
- Poulter, R. G., Ledward, D. A., Godber, S., Hall, G. and Rowlands, B. 1985. Heat stability of fish muscle proteins. *Journal of Food Technology* 20: 203–217.
- SAS Institute 2009. The SAS system for Windows. Release 9.2. SAS Institute Inc., Cary, NC, USA.
- Seniman, M. S. M., Yusop, S. M. and Babji, A. S. 2014. Biochemical properties and proximate composition of catfish enzymatic protein hydrolysates made using subtilisin. *International Journal of Technical Research and Applications* 2: 15-19.
- Shahidi, F., Han, X-Q. and Synowiecki, J. 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). *Food Chemistry*, 53: 285-293.
- Siddall, S. E. 1980. A clarification of the genus *Perna* (Mytilidae). *Bulletin of Marine Science* 30: 858-870.
- Sikorski, Z. E. and Naczek, M. 1981. Modification of technological properties of fish protein concentrates. *Critical Reviews in Food Science and Nutrition* 14: 201–230.
- Siti Balqis, Z. and Rosma, A. 2011. Artocarpus integer leaf protease: Purification and characterisation. *Food Chemistry* 129: 1523–1529.
- Tang, C. H., Wang, X. S. and Yang, X. Q. 2009. Enzymatic hydrolysis of hemp (*Cannabis sativa* L.) protein isolate by various proteases and antioxidant properties of the resulting hydrolysates. *Food Chemistry* 114: 1484–1490.
- Thiansilakul, Y., Benjakul, S. and Shahidi, F. 2007. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry* 103: 1385–1394.
- Thomas, G.E., Rodolfo, H.G., Juan, M.D., Georgina, S.F., Luis, C.H., Ingrid, R.B. and Santiago, G.T. 2009. Proteolytic activity in enzyme extracts from *Carica papaya* L. cv. *Maradol* harvest by-products. *Process Biochemistry* 44: 77-82.
- Tomar, R., Kumar, R. and Jagannadham, M. V. 2008. A stable serine protease, wrightin, from the latex of the plant wrightia tinctoria (Roxb.) R. Br.: Purification and biochemical properties. *Journal of Agricultural and Food Chemistry* 56: 1479-1487.
- Tornberg, E. 2005. Effects of heat on meat proteins – Implications on structure and quality of meat products. *Meat Science* 70: 493–508.
- Turgeon, S. L., Gauthier, S. F., Molle, D. and Leonil, J. 1992. Interfacial properties of tryptic peptides of b-lactoglobulin. *Journal of Agricultural and Food Chemistry* 40: 669–675.
- Wang, B., Li, L., Chi, C. F., Maa, J. H., Luo, H. Y. and Xu, Y. F. 2013. Purification and characterisation of a novel antioxidant peptide derived from blue mussel (*Mytilus edulis*) protein hydrolysate. *Food Chemistry* 138: 1713–1719.
- Wasswa, J., Tang, J., Gu, X. H. and Yuan, X. Q. 2007. Influence of the extent of enzymatic hydrolysis on the functional properties of protein hydrolysate from grass carp (*Ctenopharyngodon idella*) skin. *Food Chemistry* 104: 1698-1704.