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Production and Characterization of Spray-Dried Swamp Eel (*Monopterus albus*) Protein Hydrolysate Prepared by Papain

(Pengeluaran dan Pencirian Hidrolisat Protein Belut Paya Semburan Kering (*Monopterus albus*) disediakan melalui Papain)

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ABSTRACT

Protein hydrolysate from swamp eel (Monopterus albus) has been prepared by enzymatic hydrolysis process using papain enzyme. Evaluation of the extent of protein hydrolysis was conducted by measuring the degree of hydrolysis (DH). The optimization of protein hydrolysate production has been carried out by analyzing the influences of papain enzyme concentration, temperature, and time of hydrolysis on the degree of hydrolysis (DH) using RSM design. The optimized product was spray-dried and analyzed the proximate (moisture, lipid, protein) content and the yield (%). The fish protein hydrolysate (FPH) powder product was characterized by the foaming capacity and stability, and also by FTIR, DSC and PSA methods. The optimum condition of enzymatic hydrolysis of swamp eel protein was obtained by an addition 0.49 % of papain enzyme at 45 °C for 9 hours. The degree of hydrolysis (DH) of this product was 7.96 % with a yield of 14.72 %. The foaming capacity of swamp eel protein hydrolysate powder was between 12.5 - 62.5 % and the foaming stability was between 3.22 - 31.25 %. The highest foaming capacity and stability of this product was reached at pH 4.0. Based on the spectrum FTIR analysis, the FPH product contained amines, aromatics, aliphatics, amide B and amide II groups. DSC analysis of the FPH product showed two peaks (Tm) at 65 °C and 108.5 °C. The particle size of the FPH powder product was distributed within 100 nm-1500 nm range with the highest intensity was 8.91 %. This study shows the potential usage of swamp eel for the production of FPH by enzymatic hydrolysis using papain enzyme with high yield and serves as a protein supplement.

Keywords: *Monopterus albus; papain; protein hydrolysate*

ABSTRAK

Hidrolisat protein daripada belut paya (Monopterus albus) telah disediakan melalui proses hidrolisis enzim dengan menggunakan enzim papain. Penilaian tahap hidrolisis protein telah dijalankan dengan mengukur darjah hidrolisis (DH). Pengoptimuman pengeluaran hidrolisat protein telah dijalankan dengan menganalisis kepekatan enzim papain, suhu dan masa hidrolisis pada darjah hidrolisis (DH) menggunakan reka bentuk RSM. Produk yang dioptimumkan adalah semburan-kering dan analisis kandungan proksimat serta hasil (%). Produk serbuk protein hidrolisat (FPH) dicirikan melalui kapasiti buih dan kestabilan serta kaedah FTIR, DSC dan PSA. Keadaan yang optimum untuk enzim hidrolisis daripada protein belut paya diperolehi dengan penambahan 0.49 % enzim papain pada 45 °C untuk 9 jam. Darjah hidrolisis (DH) produk ini adalah 7.96 % dengan hasil sebanyak 14.72 %. Kapasiti buih serbuk hidrolisat protein belut paya adalah antara 12.5-62.5 % dan kestabilan buih adalah antara 3.22-31.25 %. Kapasiti buih tertinggi dan kestabilan produk ini telah dicapai pada pH4.0. Berdasarkan analisis spektrum FTIR, produk FPH mengandungi kumpulan amina, aromatik, alifatik, amida B dan amida II. Analisis DSC produk FPH menunjukkan dua puncak (Tm) pada 65 °C dan 108.5 °C. Saiz zarah produk serbuk FPH diagihkan dalam julat lingkungan 100 nm-1500 nm dengan keamatan tertinggi adalah pada 8.91 %. Kajian ini menunjukkan potensi penggunaan belut paya untuk pengeluaran FPH melalui hidrolisis enzim menggunakan enzim papain dengan hasil yang tinggi dan berfungsi sebagai protein tambahan.

Kata kunci: *Hidrolisat protein; Monopterus albus; papain*

INTRODUCTION

Asian swamp eels are usually found in slowly moving freshwater, especially in rice field regions. They often burrow into a slump and small spaces. Asian Swamp Eel feed a wide variety of aquacultures and categorized as a sequential hermaphrodite to native fishes, frogs, and aquatic invertebrates (Hilles 2018). Swamp eels are known

has good nutrition, tasty and used for medicinal purposes (Rosli & Sarbon 2015). The protein content of swamp eel flesh was 16.88%, moisture content was 83.87%, and fat content was 3.41% (Halim & Sarbon 2017). Protein hydrolysate from fish has been studied and reported with the aim for commercial purposes included food, feed, agriculture, microbiology, and medicinal formulation.

Hydrolysis of protein by strong acids, strong bases or proteolytic enzymes resulting in the form of amino acids and peptides. Hydrolysis with a strong acid is nonspecific and attack all peptide bonds, produces a large number of fragments (Al-Bahri et al. 2009). Enzymatic hydrolysis of protein is the alternative process to improve its functional properties without influencing the nutrition value. The nutritional and functional properties such as solubility, foaming and emulsion stability were improved by enzymatic hydrolysis. Some studies reported the advantage of hydrolyzed protein in human health such as less allergenic, easy digested and absorbed (Kain et al. 2009). Hydrolysis of protein increased the number of peptides and the hydrophobic of amino acid residues would contribute to the formation of the emulsion. Fish protein hydrolysate products have been used as nutritional supplements (Prabha et al. 2016). The functional of protein influences by its molecular size, structure and amino acid content (Chabanon et al. 2007). The particle size distribution of soy protein is also important as a factor that contributing to food formulation that influences the solubility and texture of food (John et al. 2018). The molecular weight of FPH is a factor affecting the functional properties, a study showed that with a low molecular weight of peptides resulting high impact on functional properties of FPH (Samsudin et al. 2018). The functional properties of fish protein hydrolysates can be optimized by partial hydrolysis using the specific enzymes at certain conditions such as time, pH, temperature and enzyme concentration (Salwanee et al. 2013). Some proteolytic enzymes can be used for the production of fish protein hydrolysate (FPH) such as alcalase, papain, pepsin, and trypsin. The production of FPH should be used as food-grade and non-pathogenic enzymes (Halim & Sarbon 2017; Salwanee et al. 2013). The functional properties of rapeseed protein have been improved by using different enzymes. Nitrogen solubility, foaming properties, water and fat adsorption capacity of rapeseed meal are improved after hydrolysis by Ficin enzyme. Meanwhile, oil adsorption capacity, foaming and emulsifying properties of this protein can be improved after hydrolysis with alcalase (Chabanon et al. 2007). If compared to other enzymes such as alcalase, papain widely available in the market and also very stable and active enzyme under wide range conditions. Papain enzyme as cysteine proteases has activity towards proteins, short-chain peptides, amino acid esters and amide links (Amri & Mamboya 2012). Halim and Sarbon (2019) reported the characterization of Asian swamp eel protein hydrolysate functional properties prepared using Alcalase. The characterization was carried out by using a powder sample obtained from the freeze-dried process. Freeze drying can induce conformational instability and result in the degradation of protein (Heller et al. 1999). Lee et al. (2002) have shown that spray drying is a suitable drying process for producing a dried formulation of peptide. Therefore, the objective of this study was to produce the fish protein hydrolysate (FPH) from swamp eels using papain enzyme and to characterize

the spray-dried of FPH product both physical and chemical properties.

MATERIALS AND METHODS

MATERIALS

Swamp eels were purchased at a traditional market in Bandung, Indonesia. The eels were filleted and frozen until further used at -20 °C. Commercial papain enzyme (brand Xian Arisun ChemParm Co. Ltd, CAS no. 2323.627-2, Shaanxi, China) in the form of powder. All chemicals used were of analytical grade.

PREPARATION OF FISH PROTEIN HYDROLYSATE (FPH)

FPH preparation was carried out by using a modification of Anissa et al. (2017) and Priatni et al. (2017) methods. The frozen of eel fish was thawed and mixed with distilled water with a ratio of 1:4. Samples were blended and pH adjusted to 7.0. The optimization of FPH production was carried out in a water bath by using 0.3-0.7 % of papain at 50-60 °C for 6-10 h. The enzymatic hydrolysis process was stopped at 85 °C and allowed to stand for 15 min. FPH extract was vacuum-filtrated and the filtrate was stored at -20 °C. For product characterization purposes, the filtrate sample was spray dried at 160 °C (inlet) and 80 °C (outlet).

OPTIMIZATION OF ENZYMATIC HYDROLYSIS CONDITION OF FPH BY RSM-CCD METHOD

RSM-CCD (Response Surface Methodology - Central Composite Design) was used to predict the optimal hydrolysis condition for FPH using papain enzyme. Optimization of enzymatic hydrolysis was used three factors i.e. the influences of enzyme concentration, temperature and hydrolysis time. Percentage of degree hydrolysis (% DH) was used as a parameter of hydrolysis. Twenty hydrolysis trials were randomly run per CCD. The center value was selected according to references which are 0.3% papain enzyme, 55 °C and 6 h (Annisa et al. 2017; Saputra & Nurhayati 2016). Design Expert 7.0 software was used in this experimental design. The optimum condition was used for FPH powder production that prepared by spray-dried the supernatant of FPH with inlet temperature 160 °C and outlet temperature 80 °C.

PROXIMATE ANALYSIS AND YIELD OF FPH POWDER

The moisture, protein, fat and ash content of FPH powder were determined according to AOAC (1995). Soluble protein content was analyzed by using a modification of Lowry method. The yield of FPH powder was calculated by using the following formula:

$$\text{yield (\%)} = \frac{W_2}{W_1} \times 100\%$$

where W2 is the mass of FPH powder; and W1 is the mass of eel fish fillet.

DETERMINATION OF DEGREE HYDROLYSIS

Degree hydrolysis (DH) of peptone extract was calculated using the relationship between α -amino nitrogen (AN) and total nitrogen (TN) according to (10):

$$\%DH = \frac{\alpha\text{-amino nitrogen (AN)}}{\text{Total nitrogen}} \times 100$$

Total nitrogen was determined by Kjeldahl method. α -Amino nitrogen was analyzed using a modification of Wang et al. (2012) method. The concentration of α -AN was calculated using the following equation:

$$\alpha\text{-AN (\%)} = \frac{V}{W \times 10} \times N_{NaOH} \times 14.008$$

where V is the titration volume; and W is the weight of the sample.

FOAMING CAPACITY AND STABILITY

The foaming capacity and stability of FPH from the powder sample were determined according to Naqash and Nazeer (2013) method with modification. The foaming capacity was calculated as,

$$\text{foaming capacity (\%)} = \frac{(A - B)}{B} \times 100\%$$

where A is the volume after whipping (mL); and B is the volume before whipping (mL).

Foam stability was calculated as follows:

$$\text{foaming stability (\%)} = \frac{(A - B)}{B} \times 100\%$$

FOURIER TRANSFORMS INFRARED SPECTROSCOPY (FTIR)

The functional group of FPH powder sample has been analyzed by FTIR (Thermo Scientific, Nicolet iS5 iD5 ATR) technique according to Rosli and Sarbon's (2015) method with modification. The FTIR spectra were obtained from discs contained FPH powder in potassium bromide (KBr). Duplicates samples were analyzed and spectra from 4000 to 550 cm^{-1} were obtained at a data acquisition rate of 4 cm^{-1} at room temperature. The functional group of FPH was monitored from the spectra and compared to references data. The peaks obtained from the spectra of samples were assigned based on functional groups present in the sample.

DETERMINATION OF MELTING TEMPERATURE

FPH powder sample melting temperature (T_m) was determined by a differential scanning calorimetry (DSC) technique (Netzsch, DSC214), according to Ren et al. (2010) method with modification. The samples were filled in a pan, weighed and pressed by a hydraulic handle. Samples were analyzed at a heating rate of 10 $^{\circ}\text{C}/\text{min}$ ranging from 20 to 12 $^{\circ}\text{C}$. The transition midpoint (T_m , or the melting temperature) is the temperature at which half the protein molecules are folded and half are unfolded.

PARTICLE SIZE DISTRIBUTION

The particle size distribution of FPH powder was determined using a particle size analyzer (Zetasizer Nano ZS Malvern), according to John et al. (2018) method with modification. The sample was dispersed in 0.9 % of NaCl solution and centrifuged at 5.000 rpm for 30 min. Particle size distribution has analyzed the percentage of intensity (%).

RESULTS AND DISCUSSION

The optimization of the hydrolysis process of FPH from swamp eels was determined according to the percentage of the degree hydrolysis (%DH). The optimization of enzymatic hydrolysis was analyzed by RSM-CCD design. Analysis of variance (ANOVA) of the response surface quadratic model for DH of FPH was presented in Table 1. Overall data showed that the model had significant ($p < 0.05$) with R^2 value was 0,8639.

Table 1 shows that enzyme concentration, time and the quadratic coefficients A^2 (enzyme concentration) had significantly ($p < 0.05$) affected the DH of FPH. Meanwhile, temperature, the quadratic coefficients B^2 , C^2 and all interactions were not significant. In this study, the temperature was not significantly for DH of FPH, we assumed due to the papain is the stable and active enzyme at some condition, including temperature. The equation for DH and the response variable (Y) of FPH was derived using the regression coefficient of intercept, linear, enzyme concentration, time and quadratic terms to fit a full response surface model. The equation was given as follow:

$$Y = 5.5465 + 0.9886 A + 0.5317 C - 0.5539 A^2$$

Analysis data by RSM showed that the optimum condition of the hydrolysis process of FPH from swamp eels was obtained by using 0.49% of papain enzyme, 45 $^{\circ}\text{C}$ for 9 h with DH prediction was 7.3005% \pm 0.5962%. Effect of interaction between two factors on the degree of hydrolysis (DH) of FPH products was presented in a 3D curve (Figure 1), the orange color and contour in curve indicated the highest of DH. The 3D response surface plot of the temperature and enzyme concentration effect on DH of FPH is shown in Figure 1(a). The hydrolysis time was kept constant as suggested by RSM at 9 h. The 3D curve showed that the highest of DH (6.51%) was obtained at

TABLE 1. Analysis of variance (ANOVA) of degree hydrolysis FPH

Source	Sum of squares	df	MeanSquare	FValue	p-valueProb > F	
Model	24.61576	9	2.735084	7.052566	0.0026	significant
A-Concentration	13.34635	1	13.34635	34.4143	0.0002	
B-Temperature	1.828926	1	1.828926	4.715986	0.0550	
C-Time	3.861327	1	3.861327	9.956645	0.0102	
AB	0.000802	1	0.000802	0.002067	0.9646	
AC	0.02004	1	0.02004	0.051675	0.8248	
BC	0.501005	1	0.501005	1.29187	0.2822	
A2	4.420911	1	4.420911	11.39956	0.0070	
B2	0.341565	1	0.341565	0.880744	0.3701	
C2	0.028203	1	0.028203	0.072723	0.7929	
Residual	3.878141	10	0.387814			
Lack of Fit	3.148944	5	0.629789	4.318375	0.0671	not significant
Pure Error	0.729197	5	0.145839			
Cor Total	28.4939	19				

R² = 0.8639, A = enzyme concentration (%), B = temperature (°C), C = time (min)

0.5% of enzyme concentration at 45 °C. The graph shows the DH of FPH was significantly increased with the increase in enzyme concentration. Figure 1(b) shows the 3D response surface plot of the effect of hydrolysis time and enzyme concentration with the temperature fixed at 45 °C. The result showed that the highest of DH (6.52 %) obtained at 0.5 % of enzyme concentration for 9 h. Meanwhile, Figure 1(c) shows the highest of DH (6.8 %) was achieved when the interaction between temperature (45 °C) and time (9 h) is used. This data has shown that DH of FPH was decreased by increasing the temperature. Fish protein was degraded by a proteolytic enzyme such as papain resulting soluble and insoluble protein fraction. The degree of hydrolysis (DH) values is important in producing protein hydrolysate that will improve its functional properties and the indication of protein hydrolysis efficiency (Prabha et al. 2016; Wisuthiphaet & Kongruang 2015). Our previous study reported the enzymatic hydrolysis of boso fish (*Oxyeleotris marmorata*) by papain at 50 °C for 7 h with DH value 5.47 %. Fish protein hydrolysate or peptone was produced using enzymatic hydrolysis of silver carp by-products by alcalase and trypsin (at 55 °C for alcalase and 37 °C for trypsin) with DH value 4.94 %, 4.6 %, respectively (Fallah et al. 2015).

According to RSM data, fish protein hydrolysate (FPH) was prepared by using the optimized condition was 0.49% of papain enzyme, 45 °C for 9 h. Results of DH analysis shown that the Degree hydrolysis (DH) of FPH product (7.96 %) slightly higher than DH predicted by RSM (7.30 %). The results indicated that the same treatments during FPH production was very important and should be reproducible. Hydrolysate from eel was then spray-dried and the yield FPH powder i.e. 14.72 % ± 0.41 %. Meanwhile, the moisture, protein and fat content of the spray-dried FPH product were 7.11 %, 62.56 %, and 8.05

%, respectively. Production of FPH from fish carp meat and viscera had been conducted by using 0.26 % of papain enzyme at 60 °C for 3 h. The study reported the solubility of FPH from viscera was higher than meat of fish carp (Saputra & Nurhayati 2016). Halim and Sarbon (2017) reported that the yield of eel protein hydrolysate (EPH) obtained was 6.97 % by using 2.26 % of alcalase enzyme. Other studies have reported the yield of extracted gelatin from skin eel was 12.75 % (Rosli & Sarbon 2015).

Foaming properties is one of the important functional properties of protein products. The foaming stability of food materials will determine the shelf life and appearance of its products (Kempka et al. 2015). To have good foaming, a protein can migrate rapidly to interface and then peptide bonds will undergo unfolding and rearranging (Hassan et al. 2018). Figure 2 shows the profiles of the foaming capacity (a) and foaming stability (b) at different pH values of FPH from the swamp eel sample. The data showed that at different pH, the foaming capacity of FPH was between 18.75 - 60 % and the foaming stability was between 4.68 - 28.12 %. The highest foaming capacity and stability were found at pH 4. Foaming properties of FPH samples were then decreased from pH 4 to pH 6 and slightly increased by increasing the pH of the solution (from pH 6 to pH 8). The data shows that the isoelectric point of FPH from swamp eel was around pH 4. The protein hydrolysate obtained from *Leiognathus bindus* fish prepared at pH 5 gave the highest foaming capacity. A good foaming capacity might affect the surface activity as the result of partial proteolysis that produced a high number of polypeptide chains (Prabha et al. 2016). Amino acid content contributes to the functional properties of protein. Halim and Sarbon (2017) reported that swamp eel flesh contains high glutamic acid (13.88 %) and aspartic acid (8.55 %). The small size of peptides derived from hydrolyzed protein products influences the foaming properties resulting in

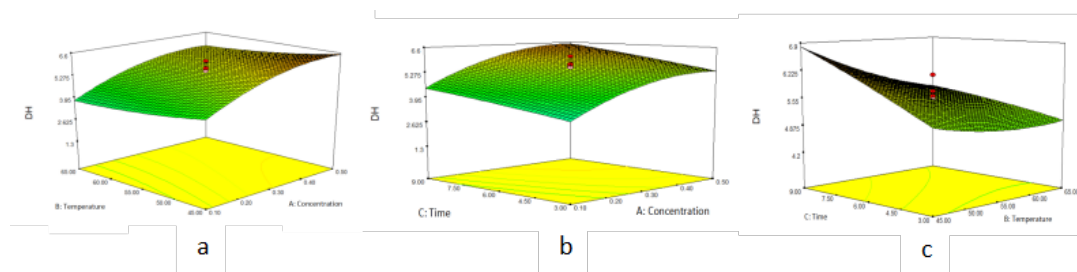


FIGURE 1. 3D surface plot of interaction between temperature and concentration (a), time and concentration (b), temperature and time (c) factors on degree hydrolysis (DH) of FPH from swamp eel sample

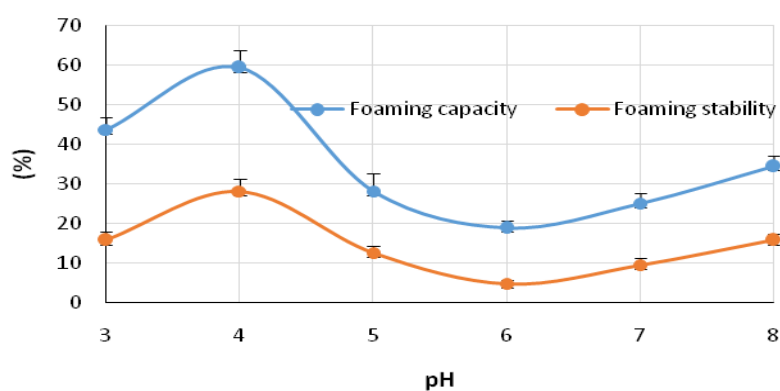


FIGURE 2. Profiles of the foaming capacity (a) and foaming stability (b) at different pH values of FPH from swamp eel sample

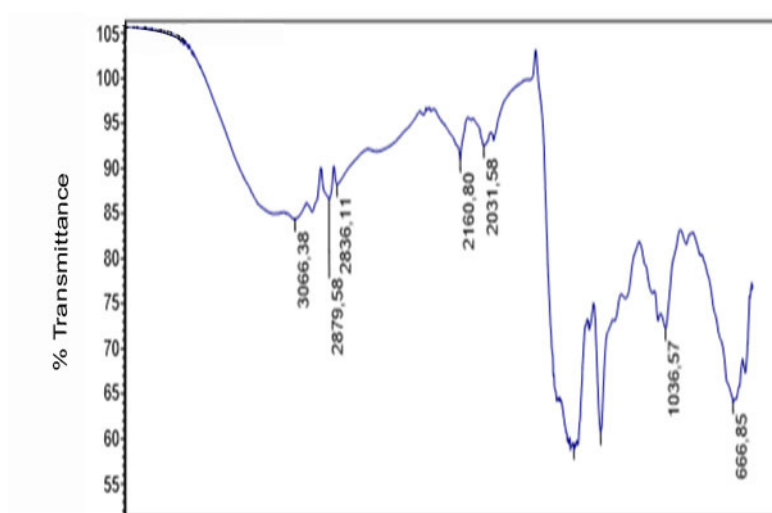


FIGURE 3. FTIR spectra of FPH from swamp eel sample

weak forms of the medium. The pH of the medium is important to foam stability and correlates with the isoelectric point of the protein (Hassan et al. 2018).

The FTIR spectrum of fish protein hydrolysate (FPH) from swamp eel was shown in Table 2. Based on Table 2, it can be seen that the aromatic compound of the carbonyl group (C-H) in FPH was absorbed by infrared spectra at 666.85 cm^{-1} , containing C-OH or C-O with strong intensity and stretch vibration mode at 1036.57 cm^{-1} , C-H bending vibration mode at 1392.17 cm^{-1} . The C=C found at 1538.04

cm^{-1} was functional groups with aromatic rings, C-H stretching bands indicating the amide group found at 2879.58 cm^{-1} and NH_3^+ group with stretch vibration mode at 3066.38 cm^{-1} . FTIR spectra of FPH from swamp eel had a similar pattern with the FTIR spectra of fish peptone was procured from HiMedia Laboratories, India (Trivedi et al. 2015). Halim and Sarbon (2019) reported the characterization of freeze-dried swamp eel by FTIR method. The data showed that EPH (eel protein hydrolysate) contained proteins with secondary and tertiary amides and

TABLE 2. FTIR spectra peak wavenumber $^{-1}$ with their assignment based on the functional group of FPH from swamp eel sample

FTIR spectra peak cm^{-1}	Assignment	Assignment spectra peak cm^{-1}
666.85	C-H, aromatic, bending	1000 - 650
1036.57	C-OH/-CO, acids, stretching	1260 - 1000
1392.17	CH ₃ , C-H bending	1395 - 1385
1538.04	C=C, aromatic, stretching	1500 - 1400
2879.58	C-H, amide, stretching	3000 - 2850
3066.38	N-H, NH ₃ ⁺ , stretching	3100 - 2600

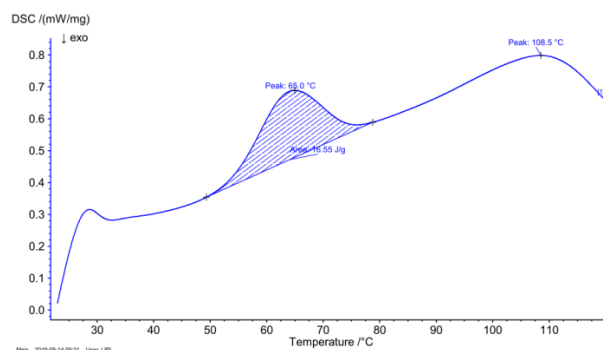


FIGURE 4. DSC thermogram of FPH from swamp eel sample

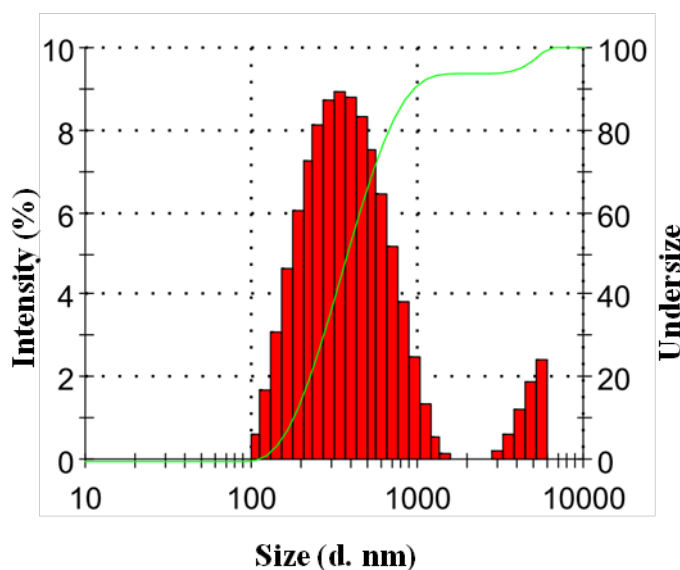


FIGURE 5. Particle size distribution of FPH from swamp eel sample

aromatic compounds of carbonyl group which corresponds to the aromatic of amino acids. This data was shown similar to our study except the containing of free amino acids (NH_3^+) that indicated at 3066.38 cm^{-1} in our FPH sample.

DSC is a measurement technique of the heat effects correlates with intra or inter-macromolecular processes of materials that considered as the thermodynamic parameter of protein unfolding. Therefore, DSC data is possible to determine the optimum conditions for protein formulation (Gill et al. 2010). In DSC thermogram (Figure 4), it shows

the thermal transition temperature (T_m) of FPH was 65°C and 108.5°C . This data indicated about 50% of the protein in native conformation and the rest of 50% was denatured. Levitsky et al. (2008) were reported the thermal unfolding of actin by DSC method. Actin is a protein that very dynamic structure and exists with some conformational states. Actin monomer will form polar filaments (F-actin) in neutrals salts condition. In this study, the interaction of protein forming a complex protein that stabilized and denatured resulting in a new form of protein. We predicted

FPH from the swamp eel majority contained actin which has thermal transition 65 °C. According to Rosli and Sarbon (2015), the thermal transition (T_m) of eel skin gelatin was 35 °C, this significantly lower than T_m of FPH which obtained from swamp eel fillets.

Particle size and distribution of FPH powder product was determined by particle size analyzer is presented in Figure 5. The data shows that a greater percentage of particle size was distributed within 100 - 1500 nm range with the highest intensity was 8.91 %. Carić (1994) reported that spray-dried milk powder particles are usually spherical with diameters are between 10 and 250 µm. This study suggested that rapid dispersion requires a particle size of about 150 to 200 µm. The particle size distribution of particle is an important factor contributing to the properties and behavior of food formulations. Particle size also contributes to the solubility and texture of final food preparations (John et al. 2018). The hydrolysis process influences the particle size distribution of protein as the result of degradation into small peptide fragments (Kain et al. 2009). Laval and Pak (in John et al. 2018) reported the particle size of the spray-dried product of milk. The study shows that the particle size of protein products was affected by its original's characteristics, preparation, and equipment for the drying process. Moreover, there is a correlation between the droplet size and the size of the powder particle of the spray-dried product.

CONCLUSION

The outcome of this study shows the potential usage of swamp eel for the production of FPH through the enzymatic hydrolysis using papain enzyme with high yield and serves as a protein supplement. The yield of spray-dried FPH hydrolyzed by papain shows higher than FPH hydrolyzed by other enzymes such as alcalase. The structural analysis of the FPH product found the presence of aromatic, amide groups related to the existence of amino acids with an aromatic ring and contain free amino acids. The hydrolysis of eel protein using papain resulting in the change of its functional properties includes foaming properties, conformation and particle size. The functional properties of spray-dried of swamp eel protein hydrolysate products were highly comparable to freeze-dried products.

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