Optimization of Hydrolysis Time for Food Protein Hydrolysate from Food Waste (Fish, Shrimp & Okara)

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***Abstract*- Every year, a huge amount of food waste such as fish, shrimp and soybean waste are generated by their food processing industries. Fish, shrimp and soybean (okara) waste such as skin, head, tail fins, and shell are being discarded or simply dumped without further processing. The aim of this study was to extract the bioactive peptide from food waste by using enzymatic hydrolysis and to optimize the hydrolysis time for food waste from fish, shrimp and okara by variable time constraint between 2 until 5 h with 1 hr interval. Enzymatic hydrolysis of proteins was carried out using alcalase enzyme at 2 % (w of enzyme/ w of substrate), 60 ℃ at four hydrolysis times (2, 3, 4, and 5 h).The food waste (fish, shrimp and okara) was blended using a Mastar blender to obtain the slurry types of sample. The highest degree of hydrolysis (DH) for fish, shrimp and okara are at 2% enzyme concentration, 60 ℃ after 4 h of hydrolysis. The protein determination and analysis were determined by using several laboratory equipments such as UV-Vis Spectrometer, and Fourier Transform Infrared (FTIR) Spectroscopy.**

Keywords- bioactive peptide, enzymatic hydrolysis, degree of hydrolysis.

I. INTRODUCTION

In this era, the abundantly of food waste and their by-products have become a crucial issue in terms of economic waste and environment pollution worldwide. Producing value added product from food waste such as protein hydrolysate has become the most popular choice to consumers because it is convenient and environmentally friendly. The global production of fish and shrimp has been steadily increasing over the last decade and this trend are expected to continue. According to Food and Agriculture Organization of the United Nations in 2016, the demand for seafood industry has been increasing over a year and it stated that the global supply of aquatic products was increasing by 3.2% per year over the world's population for the past 50 years[1].

Enzymatic hydrolysis of food protein has become one of the alternatives to utilize these food waste into value added products such as protein hydrolysate. The production of protein hydrolysate from food waste is a good effort to reduce the abundant food waste thus will help to preserve the environment. Other food waste such as sea cucumber, porcine liver and cereal grains (wheat, oat and rice) also have been used as a source in the extraction of food waste into protein hydrolysate by enzymatic hydrolysis due to their nutritional and functional properties that have beneficial effects on human health and other purposes[2]. However, this study focused on fish waste, shrimp waste and okara (soybean waste) as a raw material. This is because these raw materials have been highly produced in every country over the year. For instance, a huge quantity of okara about 700 000 tons were produced in Japan, Korea and China due to the high demand of the tofu industry every year[3].

The work in this study involves a laboratory scale enzymatic hydrolysis of food waste from fish, shrimp and okara for protein hydrolysate. Commonly, the commercial enzyme used in hydrolysis highly depends on the source of the substrate to be extracted. In this study, alcalase has been used as an enzyme to extract the food protein from selected food waste (fish, shrimp and okara) as it can work effectively to extract the soluble hydrolysates of marine protein and soy protein due to its ability to act on collagen directly[4, 5]. Moreover, the amount of protein generated during the hydrolysis reaction was indicated by the value of Degree of hydrolysis (DH) in this study. The functional group and amount of protein extracted were analyzed by using the UV-Vis Spectrometer, and Fourier Transform Infrared (FTIR) Spectroscopy.

II. MATERIALS AND METHODS

A. *Sample source*

The food by-product used in this study were fish, shrimp and soybean. The selected fish was Leptobarbus hoevenii (Hoven's Carp) and the shrimps used was Indian prawn (Fenneropenaeus indicus). Okara was prepared using soybeans. All of these food sources were obtained from Giant supermarket Seksyen 7 Shah Alam. The fresh wet food source (fish and shrimp) were stored in a freezer at -20℃ meanwhile soybean were kept at room temperature before further processing.

B. Chemicals sources

The enzyme alcalase used in this study was obtained from SIQMA Company. The chemicals used in this study included: Potassium Dihydrogen Phosphate (KH_2PO_4), Di-Potassium Hydrogen Phosphate (K_2HPO_4), Sodium Hydroxide (NaOH)

and Sulphuric Acid (H_2SO_4). All the chemicals were obtained from R&M Chemicals (Chemistry Laboratory).

C. *Preparation of the food samples*

i. *Fish*

The total weight of 750 g fish waste was prepared and left in a freezer at -20℃ overnight. Then, the frozen fish sample were blended with a Mastar (MAS-160BL(A)-I) blender for 15 minutes to obtain a relatively fine slurry mixture. The wastes was blended with a ratio of (1:1) consist of the mass of food waste and distilled water. Next, the blended food waste sample is then heated in a water bath at 80-90°C for 30 minutes to inactivate the endogenous enzyme activities[6]. The pH of the sample were recorded. The 750 g of the fish were then divided into 15 portions of the same quantity, which indicating 50 g of waste mass each of the sample portion. The blended materials was used in this research. The same procedure was then applied for the shrimp sample preparation.

ii. *Okara*

1500 g of soybeans was cleaned and soak in water overnight to get a soft texture of beans. Next, it was cooked in a cooker by using a ratio of (3:1) of soybean to water. The cooked and soften soybean were blended with a Mastar (MAS-160BL(A)-I) blender for 15 minutes to obtain a relatively fine slurry mixture. Then, the residue (okara) were filtered by using a fine cloth in order to remove any excess water. The residue was dried in an oven at 30 ℃ for overnight. Next, the dry waste was blended by a ratio of 1:3 consist of 750 gram mass of food waste and 2250 ml of distilled water. Then, the blended sample was heated in a water bath at 80-90°C for 30 minutes to inactivate the endogenous enzyme activities, the pH of the sample was then recorded and the slurry mixture is divided into 15 portions of the same quantity, which indicating 50 g of waste mass each of the sample portion. The residue (okara) were used in this research.

D. *Enzymatic hydrolysis*

Before enzymatic hydrolysis was started, the buffer solution was added to each of the samples, to stabilize the pH of the samples throughout the hydrolysis reaction. The phosphate buffer solutions was prepared and added into the samples. The value added was 3 ml. The pH of the mixtures are then adjusted to pH 7-7.5 using Sodium hydroxide and Sulfuric acid.

i. *Variables of hydrolysis time*

Hydrolysis temperature was fixed at 60 °C as the optimum temperature to extract the protein hydrolysate from food waste based on previous research done by V.v Ramakrishan[7] . Therefore, the temperature of 60 °C was used and kept maintained during the hydrolysis reaction occur.

Each sample of food waste (50 g) was heated in a water bath at temperature 60 °C. The temperature was maintained before the enzyme was added. Then a 2 % of alcalase was added to the sample and left of 2 hours for hydrolysis. The temperature and pH were kept controlled. After 2 hours of hydrolysis time, the temperature increased to 80 °C for 15 minutes to stop the reaction by denaturing the enzyme. The steps was repeated with different time of hydrolysis time at 3, 4 and 5 hours. The amount of base used in this experiment to maintain the pH 7.5 of the sample was recorded.

E. *Degree of Hydrolysis Determination*

i. *Degree of hydrolysis method and equation*

Degree of hydrolysis (DH) is the amount of protein generated during the hydrolysis reaction. The DH is calculated using the equation[8]:

$$DH = B \times NB \times 1/\alpha \times 1/MP \times 1/htot \times 100\% \quad (1)$$

where B (ml) is the volume of base consumed, NB is the normality of the base, 1/α is the average degree of dissociation of the α-amino groups related to the pK of the amino groups at particular pH and temperature, MP (g) is the amount of the protein in the reaction mixture, and htot is the sum of the millimoles of individual amino acids per gram of protein associated with the source of the protein used in the experiment.

ii. *Protein Content Determination using Kjeldahl Method.*

Kjeldahl method consists of 3 steps in overall which is digestion, distillation and titration. This method used to determine the nitrogen content in organic and inorganic samples. To obtain the mass of protein in a substrate, a conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein).

- *Digestion*

2 g of dried sample was weighed and placed into a digestion flask. 10 g copper sulphate ($CuSO_4$) was added into the digestion flask as a catalyst. 20 mL of sulphuric acid (H_2SO_4) was added as well and the sample is suspended by gently swirl the flask. The mixture was then heated at (350-380) °C by heating block for 90 minutes until white fumes can be seen. The digestion was completed when the sample was finally in a clear, transparent with a slightly blue colour due to reaction with the catalyst[9]. The sample was then allowed to cool and left at room temperature.

$$\text{Sample Protein} + H_2SO_4 \longrightarrow (NH_4)_2SO_4 + CO_2 + H_2O$$

- *Distillation*

The sample that already digested was taken for the distillation. An excess sodium hydroxide (NaOH) was added into the solution to convert the ammonium ion into ammonia. While the distillation process occurs, the ammonia form was received by excess sulphuric acid (H_2SO_4) at the distillate.

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3\,(gas) + Na_2SO_4 + 2H_2O$$

$$H_2SO_4\,(excess) + 2\,NaOH \longrightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

- *Titration*

The mixture of distillate and sulphuric acid (H_2SO_4) was titrated with a known volume of sodium hydroxide (NaOH) to capture the concentration of ammonium ions in sample. The excess H_2SO_4 which is not reacted to the NH_3 will react with the NaOH. Phenolphthalein was added as an indicator. Once the solution is neural, the color of sample is changed, and the

titration is stopped. The volume of NaOH used to neutralize the sample was calculated. By this formula, the volume reacted H_2SO_4 with NH_3 is known, mass of nitrogen can be determined, and by using protein mass (N x 6.25)[9], mass of protein can be known.

$$V \text{ of (N) } H_2SO_4 = V \text{ of (N) } NH_3 \quad (2)$$

$$M = n/V \quad (3)$$

Where N is Normality, n is number of moles, and V is volume (L). Finally, calculation was then made to obtain the protein based on the equation below[9]:

$$\%P = N \times 6.25 \quad (4)$$

F. Isolation, Purification and Characterization of Bioactive Peptide

i. Centrifugation

The samples was centrifuged at 4100 rpm, 4 ℃ for 40 minutes[10]. 3 layers were formed at the end of the centrifugation process. The first layer is fat (solid form), the second is supernatant, and the last layer is suspended solids. The first and third layer was removed using a pipette. By using dropper then, the peptide hydrolysate (supernatant) was then taken out using a dropper and placed it into a clean and empty beaker.

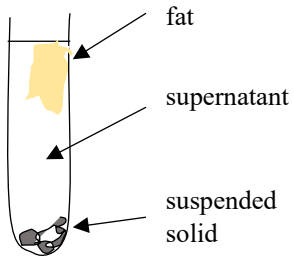

Figure 1. Sample after centrifugation.

ii. Filtration

The separated hydrolysate was then filtered using P5 filter paper. The pore size of this P5 paper is 4 micron. This process was repeated for 2 times or until a clear solution was obtained.

iii. Fourier Transform Infrared (FTIR) Spectroscopy

The peptides were then analyzed using FTIR in order to identify the functional groups present in the samples (fish, shrimp, okara) in the region of 4000-400 cm-1. The results were compared with standard reading of functional group (fish,shrimp,okara) in order to determine the existence of the functional group in the samples.

iv. UV-Vis Spectrometer

The reading of absorbance was determined by using a spectrophotometer at a wavelength of 280 nm. The blend is diluted to get an accurate range of the instrument. The blend used in this analysis is a mixture of buffer and distilled water. 10 ml of Potassium Dihydrogen Phosphate (KH_2PO_4) were mixed with 10ml of Di-Potassium Hydrogen Phosphate (K_2HPO_4) to get the mixture of a buffer. The mixture buffer then were blend with distilled water in a ratio (1:10) of buffer mixture to distilled water. The results of the absorbance value then were recorded.

III. RESULT AND DISCUSSION

1. Degree of Hydrolysis (DH)

i. Degree of hydrolysis for Fish, Okara and Shrimp

Table I. Effect of Hydrolysis time on Degree of Hydrolysis (DH) of Fish, Okara and Shrimp

Hydrolysis Time (h)	Enzyme Concentration (%)	DH (%) of Fish	DH (%) of Okara	DH (%) of Shrimp
2	2.0	16.20	38.82	4.46
3	2.0	20.17	44.86	5.35
4	2.0	24.41	48.89	8.02
5	2.0	24.64	49.46	7.13

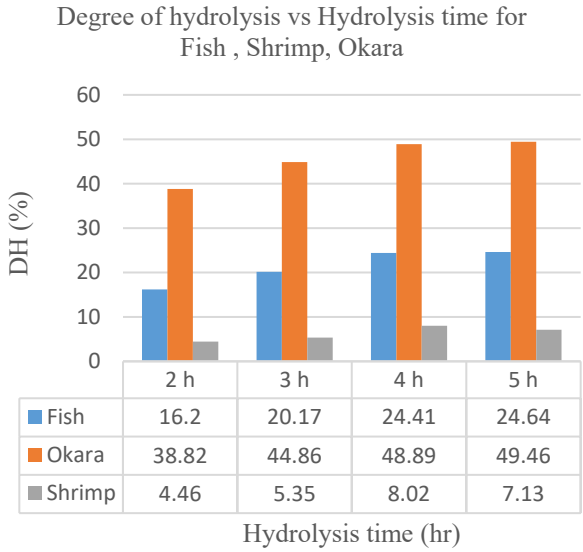

Figure 2. Graph of Degree of Hydrolysis (%) vs Hydrolysis time of Fish, Okara and Shrimp

Table I and figure 2 provides the data for the degree of hydrolysis of fish, okara and shrimp at a variable of hydrolysis time. Figure 1 show the DH is increased with the increment of hydrolysis time for fish, okara and shrimp. The increased in hydrolysis time from 2 hour to 4 hours increased the protein yield from 16.20 % to 24.41 % for fish, 38.82% to 48.89 % for okara and 4.46% to 8.02 % for shrimp. The optimum rate of

DH for fish and okara is at 5 hours of hydrolysis time. Whereas, the optimum condition of shrimp in extracting peptide is at 4 hours of hydrolysis time.

It can be seen clearly that in the first 2 hours of hydrolysis time, the reaction occurs at a rapid rate and it started to slow down after 4 hours of hydrolysis time for the overall reaction. This is due to decreasing substrate concentration and saturated reaction site by enzyme molecules[7]. As the time of hydrolysis time proceeds, the amount of enzyme to bind with the active site of the substrate is decreased as the concentration of substrate decreased.

The value of DH is expected to reach a steady state after 5 hours of hydrolysis time where there is no reaction take place anymore. Based on previous research on hydrolysis study done by V.v Ramakrishnan [7], it has been reported that the variable time of hydrolysis ranging from 1 to 5 hours having a similar pattern and trends of hydrolysis rate. It has been observed that there is no reaction occur after 4 hours of the hydrolysis process.

Moreover, if the reaction proceeds after the 4 hours of hydrolysis process, it did not cause any insignificant increment in the protein yield. In order to reduce the operating cost of protein extraction, shorter time with a higher increment in protein yield should be used and considered in a reaction. Therefore, the optimum condition in extracting the peptide is at 4 hours of hydrolysis time for fish, okara and shrimp.

2. *Fourier Transform Infrared (FTIR) Spectroscopy*

i. *Ftir of Fish, Okara and Shrimp*

The graph consist of the sample before centrifuge, unfiltered solid, supernatant filter 1 until 3 times of hydrolysate sample at optimum condition of DH. The wavelength exist for fish waste, okara and shrimp waste represented as Fw, Okara and Sw in table II.

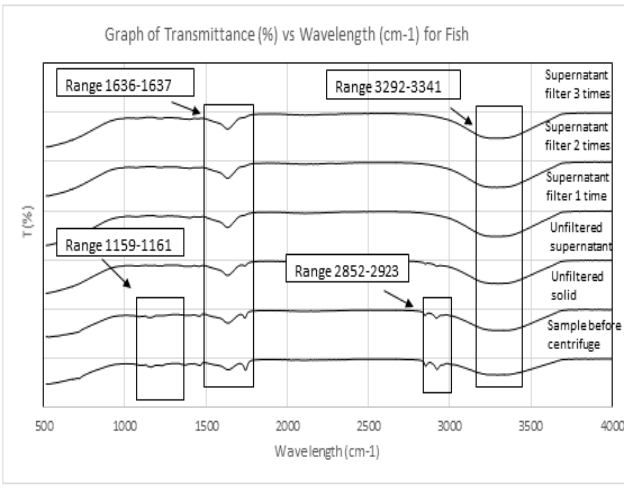

Figure 3. FTIR analysis of fish sample with the optimum degree of hydrolysis (DH)

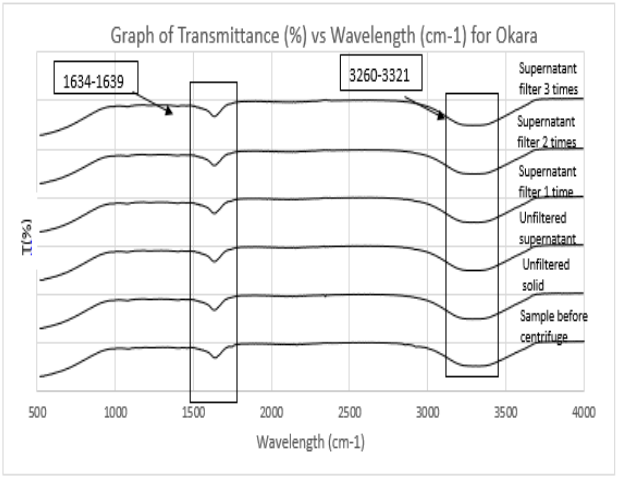

Figure 4. FTIR analysis of okara sample with the optimum degree of hydrolysis (DH)

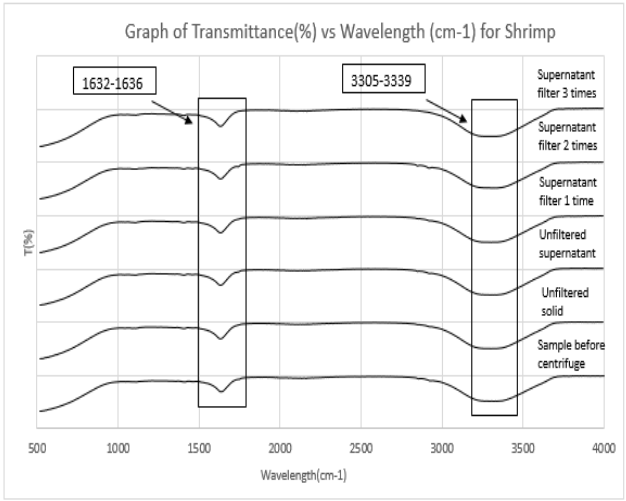

Figure 5. FTIR analysis of shrimp sample with the optimum degree of hydrolysis (DH)

Table II. The functional group of protein presence in defined wavelength

Wavelength (cm-1) based on journal	Wavelength (cm-1) based on result	Functional group	Reference
3293 (Fw)	3292-3341	O-H axial deformation	[11]
2852-2924 (Fw)	2852-2923	C-H axial deformation	[12]
1655 (Fw)	1636-1637	unsaturated acyl group (–C=C–)	[13]

1158-1159 (Fw)	1159-1161	C-N axial deformation	[12]
3304 (Okara)	3260-3321	O-H group	[14]
1627-1722 (Okara)	1634-1639	C=O stretching (amide I)	[15]
3418-3390 (Sw)	3305-3339	O-H groups	[16]
1590-1650 (Sw)	1632-1636	N-H bending	[17]

Figure 3, 4 and 5 shows analysis of fish, okara and shrimp sample with the optimum degree of hydrolysis (DH). The analysis consists of the sample before centrifuge (unfiltered), unfiltered solid, supernatant filter 1 until 3 times. The sample before centrifuge is the sample that comes directly after the hydrolysis reaction without undergoing any centrifugation and filtration process. All of these samples were undergo a hydrolysis reaction.

The reason of variation of samples is to evaluate and identify which samples have the highest number of functional group contents. From table II, it can be seen that the functional group exist for the overall sample of fish consist of functional group of unsaturated acyl group (–C=C–) and O-H axial deformation is detected at (1636 to 1637) wavelength cm-1 and (3292 to 3341) wavelength cm-1. Moreover, there are additional functional group exist for a sample before centrifuge and unfiltered solid sample where the C-N axial deformation detected at (1159 to 1161) wavelength cm-1 and C-H axial deformation at (2852 to 2923) wavelength cm-1.

For ftir analysis for okara and shrimp, there were only two functional groups exist detected. The first functional group detected is at (1634 to 1639) wavelength cm-1 which mainly are C=O stretching (amide I), the second group obtained is at (3260 to 3321) wavelength cm-1 which is the O-H group for okara. Whereas for shrimp analysis, the first functional group detected is at (1632 to 1636) wavelength cm-1 which mainly are N-H bending of secondary amide and the second group obtained is at (3305 to 3339) wavelength cm-1 which is the O-H group. There is no additional functional group exists in the overall sample for okara and shrimp.

From overall analysis of ftir shows that different types of food sample (fish, shrimp, okara) exhibit a different functional group in a specific range of wavelength.

3. UV-Vis Spectrometer

i. Uv-Vis of Fish, Okara and Shrimp

Table III. UV-Vis analysis of fish, okara, shrimp sample with the optimum degree of hydrolysis (DH)

Sample	Absorbance Value of Fish	Absorbance Value of Okara	Absorbance Value of Shrimp
Before centrifuge	0.9810	0.8595	0.5899
Supernatant	0.3315	0.6963	0.3016
Supernatant filter 1 time	0.3057	0.2193	0.1481
Supernatant filter 2 times	0.2679	0.1596	0.1617
Supernatant filter 3 times	0.2626	0.1410	0.1444

Table III provide the data for UV-Vis analysis of fish, okara, shrimp sample with the optimum degree of hydrolysis (DH). The analysis consists of a sample before centrifuge (unfiltered), supernatant unfiltered, and supernatant filter 1 until 3 times. The sample before centrifuge is the sample that comes directly after the hydrolysis reaction without undergo any centrifugation and filtration process. Then, another 4 samples left in the table were produced after the protein hydrolysate undergoing a centrifugation process where it produces a layer of solution.

From table III, the highest absorbance value for the overall sample was at a before centrifuge sample of fish, okara and shrimp in which the absorbance value is 0.9810, 0.8595 and 0.5899 respectively. This is because there is no protein in a sample being filtrated or removed, therefore, in this sample, the concentration of protein presence is at an optimum concentration for fish, okara and shrimp. Moreover, the absorbance value is decreased as the number of filtration for supernatant is increased. As the number of filtration increased, it removes a certain protein content in a sample having a pore size smaller than 4 microns. The filtration paper used in the filtration process is 4 micron, P5 paper.

When comparing the result from DH and UV-Vis analysis, it shows that an optimum degree of hydrolysis (DH) of fish which is 24.41 %, the absorbance value that indicates the concentration of protein exist in the sample is at an optimum concentration which is 0.9810. For okara and shrimp, the optimum DH which at 48.89 % and 8.02 % resulting in the absorbance value of 0.8595 and 0.5899 respectively.

Thus, it shows that the optimum concentration of protein content is at a sample directly before undergo any filtration process (before centrifuge sample). Therefore, the filtration process should be done only one time in order to reduce the amount of protein being removed in this experiment.

CONCLUSION

In conclusion, the optimum condition in extracting the peptide is at 4 hours of hydrolysis time for fish, okara and shrimp. In order to reduce the operating cost of protein extraction, shorter time with a higher increment in protein yield should be used and considered in a reaction. The highest degree of hydrolysis (DH) for fish, shrimp and okara are at 2% enzyme concentration, 60 ℃ after 4 h of hydrolysis. By converting the food by-product into a value added product (protein hydrolysate) it can help to preserve the environment and reduce the accumulation of food waste.

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