Production of Fish Protein Hydrolysates by Acid and Enzymatic Hydrolysis

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***Abstract*—In this study, two hydrolysis methods, acid hydrolysis and enzymatic hydrolysis, were proposed to produce fish protein hydrolysates (FPH) from 72% of protein in dry basis of low-valued marine fish. Both methods showed an ability to hydrolyse fish protein to FPH. Acid hydrolysis was performed by 4, 6 and 8M of HCl in high pressure (15 psi) at 121˚C for 90 minutes. Results showed 30-35% degree of hydrolysis (DH) and only 0.1 - 0.4 % of high molecular weight protein left after the reaction. For enzymatic hydrolysis, papain was used to digest the substrate with concentrations of 2, 4 and 6% (w/w) duration of the reaction were 5 10 and 15 hours at 40˚C. Results revealed that DH were increased significantly as the time increased while enzyme concentration has no significant effect on DH in the range of 20 - 24 % and high molecular weight protein in the FPH were 0.18 - 0.33 %. Thus acid hydrolysis is a suitable and economically beneficial method to produce FPH as a protein additive in animal feed industry.**

***Index Terms*—acid hydrolysis, enzymatic hydrolysis, fish protein hydrolysates, papain**

I. Introduction

Annually, large amount of marine fish are caught to use as a raw material in sea food industries leading to approximately 100,000 tons per year of fish byproduct are obtained from se[1]a food process [1] including a lot of small fish that do not match the quality criteria and cannot be used in industrial process. These types of fish waste are usually be either discarded from fishery and aquaculture or sold as low-valued products. These low-valued fish contain valuable proteins and essential amino acids. Therefore, hydrolysis of fish protein would be a proper strategy for economic gain under the consideration of fish processing waste into high value products with the improvement in both quality and quantity. Fish protein hydrolysates (FPH) are products of hydrolysis reaction on peptide bonds in proteins and result in shorter peptides or amino acids which are easy for animal to absorb. Generally, protein can be hydrolyzed by chemical process or protease enzymes. However, FPH obtained from these two methods are different in quality.

Acid hydrolysis is considered as a conventional method for producing protein hydrolysates, strong acid or alkaline are used to cleave peptide bonds under high pressure and temperature. This method consumes less cost and time, nevertheless, hydrolysis reaction by chemical is difficult to control product quality due to its harsh reaction and unspecific peptide bonds cleaving. Therefore this pathway causes racemization of amino acids, reformation of L-form amino acids to D-form amino acids which cannot be utilized by human and animal [2]. Moreover, some essential amino acids such as tryptophan and cysteine will be diminished during the reaction [3]. FPH produced by this process usually be used as an additive in animal feed, culture media and plant fertilizer [4]. They also contain undesirable peptides that cause bitterness and that limit the use of FPH in food application. During hydrolysis reaction, hydrophobic residues expose to a certain length at the end of the polypeptide chain result in bitterness of FPH [5].

Recently, enzymatic hydrolysis of fish protein attracts more attention as a process that produces high quality FPH which could be a valuable raw material for producing bioactive peptides for the treatment of diseases [6]. Even though, this method is more time consuming and production cost is obviously higher, yet less productivity, FPH obtained from this method are more nutritional and offering vast array of application including animal nutrition, food additive, pharmaceutical and cosmetic [7,8,9]. Several commercially available protease enzymes have been used to hydrolyze fish protein, such as Alcalase, Flavourzyme, Papain, Neutrase and Bromilane [10]. Papain had been chosen and reported as a protease enzyme that is widely used to produce protein hydrolysates. Abdulazeez, et al. (2013) reported the use of 1% papain to digest King fish protein at 37˚C for 6 hours. FPH obtained by this method is an ideal choice of protein used in biomedicine and commercial aspects [11]. However, the high cost of enzyme is still one of the obstacles that make its production in industrial scale economically difficult.

This research studied the potential of both acid and enzymatic hydrolysis to produce fish protein hydrolysate

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from low-valued fish in order to use as the supplementation in conventional nourishment feed for enhancing nutrition in aquaculture feed production. Effects of acid and enzyme concentrations to hydrolysis reaction were determined including an effect of duration in enzymatic hydrolysis was examined. Efficiency of FPH production by both methods was also evaluated.

II. Materials and Methods

A. Raw Material

As illustrated in Fig. 1, low-valued marine fish, Ponyfish (*Eubleekeria splendens*), Yellow stripe travally (*Selaroides leptolipis*) and Mackerel (*Decapterus maruadsi*), were purchased from the local fish market. Mixture of the fish in equal proportion was washed and minced for uniformity. The homogenous raw material was packed in plastic bags then stored at -20˚C until use. Raw material was prepared and provided by INTEQC FEED Co.,Ltd., Samut Sakhon, Thailand.

Figure 1. Preparation of FPH (a) low-valued fish as a raw material (b)fish were washed (c) wholly minced fish and (d) grinded material fish

B. Enzyme

Papain derived from *Carica papaya* was purchased from Millipore Corp., USA.

C. Fish Protein Hydrolysates Preparation

For acid hydrolysis, FPH were prepared by thawing raw material fish in room temperature then mixed 2:1 with distilled water, 4, 6 and 8 M of HCl was added to 50 mL fish solution. Acid hydrolysis was performed under high pressure (15 psi) at 121˚C for 90 minutes. Hydrolysis reaction was terminated by adjusting pH value to 5 by 6 M NaOH then filtered to separate some huge pieces of bone.

For enzymatic hydrolysis, commercial papain enzyme was used to digest the raw material, 2%, 4% and 6% (w/w) of papain were added to the raw material, after adjusting pH to 5, hydrolysis reaction was carried out in an incubator shaker at 40˚C, 200 rpm for 5 10 and 15 hours. Enzyme was inactivated at 90˚C for 30 minutes before being filtered.

D. Proximate Analysis

The moisture content was determined by spreading 2 g of sample on an aluminum dish that has been pre-weighted. Dried in an oven at 100˚C for 3 hours then placed in a desiccator until reaching a constant weight. Total weight loss during drying process represents moisture content in the sample [12]. The protein content in raw material was determined by using Kjeldhal method with nitrogen factor equal to 6.25 [13]. The total lipid was determined by Soxhlet extraction [13]. The ash content was analyzed by incineration of 2 g of sample in a furnace at 550˚C for 3 hours or until the white ash was formed [12].

E. Degree of Hydrolysis Analysis

Degree of hydrolysis (DH) is defined as the ratio between number of peptide bonds cleaved during hydrolysis reaction and total peptide bonds in raw material. DH of protein hydrolysates can be determined by measuring the amount of free amino acid according to the method described by Adler-Nissen (1979) [14]. To the protein hydrolysates, phosphate buffer pH 8.2 and trinitrobenzenesulfonic (TNBS) were added. In dark chamber at 50˚C, TNBS and amino group in amino acids formed yellow complex compounds. After adding 0.1 N HCl, absorbance was measured at 340 nm spectrophotometrically. Another method commonly used to determine DH is trichloroacetic (TCA) soluble assay. DH is equal to the percentage of total nitrogen in protein hydrolysates that has been coagulated by 20% trichloroacetic acid (TCA) and centrifuged at 4000 rpm for 30 minutes, in relation to the total nitrogen of the sample. Total nitrogen can be measured by Kjeldahl method [13] and the conversion factor used was 6.25.

F. Protein Analysis

Determination of high molecular weight protein can be done based on Bradford's assay method [15]. Color of Coomassie Brilliant Blue G-250 in Bradford reagent shifts from red to blue when binding with proteins. Absorbance was measured at 595 nm. Proteins content then was calculated using standard curve of bovine serum albumin.

G. Statistical Analysis

Proximate analysis of raw material fish and DH of FPH produced by acid hydrolysis were done in triplicate. DH of FPH produced by enzymatic hydrolysis and protein content were done in duplicate and data were averaged. Standard deviation was calculated. Means of DH and protein were analyzed by statistical analysis of ANOVA and Tukey's range test using SPSS 16.0 program.

III. Results and Discussion

A. Proximate Analysis of Raw Material Fish

As shown in Table I, raw material fish used in this research composed of 79.67% of moisture, 15.69% of protein, 3.51% of ash and 2.56% of lipid base on wet basis. On dry basis, protein lipid and ash were 72.10%, 11.76% and 16.13%, respectively. Moisture content of raw material fish was 71 - 80% which was within the

range of a published study on various types of marine fish fleshes [16]. Based on the results, the protein content of raw material fish was found 15 - 16% which appears to be slightly lower than that of marine fish flesh average protein [16] due to the wholly minced fish composed of frames, bones and visceral.

TABLE I: PROXIMATE COMPOSITION OF RAW MATERIAL FISH

Proximate composition	% wet basis	% dry basis
Moisture	79.67	-
Protein	15.69	72.10
Lipid	2.56	11.76
Ash	3.51	16.13

B. Moisture and Ash Content

Ash and moisture content in FPH produced by acid hydrolysis method were determined and showed in Fig. 2. Moisture content of FPH hydrolyzed by 4, 6 and 8 M HCl were 92.66%, 76.33% and 85.00%, respectively. Our results is in agreement of the previously published as Abdulazeez et al. (2013) reported that moisture content of protein hydrolysates of minced skin of Narrow-barred Spanish mackerel (*Scomberomorus commersoon*) was 76.8%. Our result revealed that FPH produced by acid hydrolysis had high moisture content. High moisture content was found on this experiment because of added water to raw material fish during FPH preparation. For industrial aspect, high moisture content would increase the production cost since the production process will require more energy for water evaporation.

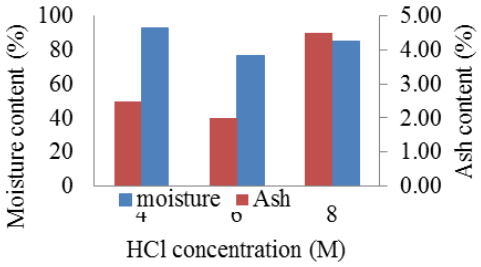

Figure 2. Moisture and ash content of FPH obtained by acid hydrolysis using different acid concentrations at 121˚C for 90 minutes.

When treated fish raw material with increasing acid concentrations 4, 6 and 8 M HCl, the ash contents yielded 2.50, 2.00 and 4.50, respectively [11]. Ash content indicated inorganic matter in FPH which showed highest value of 4.5% when digested with high HCl concentration while lower acid concentrations had 2.5% and 2.0%. Once we consider in animal feed industry, ash content could inform the quality of the feed, it means that the feed may contain some impurities such as sand or bones. Besides inorganic matter, ash content detected in this research might include NaCl generated during the adjustment of pH value by adding NaOH or some leftover fish bones.

C. Degree of Hydrolysis

When producing hydrolyzed protein, it is important to measure DH defined as the percentage of peptide bonds cleaved [14] and indicated the efficiency of protein hydrolysis reaction. DH obtained from acid hydrolysis with 4, 6 and 8M HCl was measured by TCA soluble assay and the results were shown in Fig. 3.

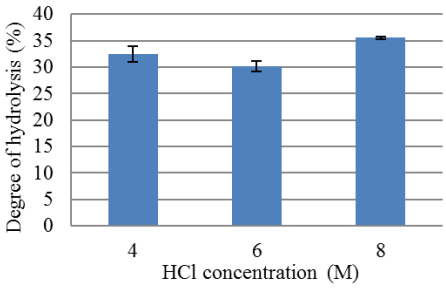

Figure 3. DH obtained by acid hydrolysis using different acid concentrations at 121˚C for 90 minutes.

The highest DH value of 35.56% was detected with 8 M HCl and followed by 4M (32.50 %) and 6M (30.21%), respectively. Nevertheless, there was no significant differences between DH of 4 and 6M ($p = 0.218$). According to the study of Shahidi, et al. (1995), fish proteins were completely hydrolyzed with 6M HCl at 110˚C for 20 - 24 hours [17]. For acid hydrolysis, an increasing reaction time can raise the production cost and completely hydrolyzed fish protein hydrolysates have low nutritional quality due to the loss of some essential amino acids and more derivatives from the racemization may occur.

For enzymatic hydrolysis, papain was used as the proteolytic enzyme to breakdown minced fish protein resulting in both soluble and insoluble fraction. In industrial point of view, the soluble fraction contains the hydrolyzed proteins that can be converted and incorporated with other main components in animal feed. In this research, enzymatic reactions were performed by 2%, 4% and 6% of papain concentrations at 40˚C for 5, 10 and 15 hours. DH was determined according to the method of Adler-Nissen (1979) and the results were then shown in Fig. 4.

As expected that DH was increased with increasing reaction times ($p<0.0001$). The 15 hours of reaction time gave DH in the range of 20 - 24 % yet there were no significant differences in DH among the enzyme concentrations. Our result is confirmed with the published of Abdulazeez, et al. (2013) who studied papain digestion of FPH and reported that hydrolysis reaction by 1% concentration at 37˚C for 6 hours gave 22.2% of DH [11].

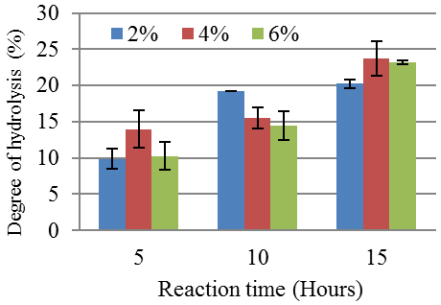

Figure 4. DH obtained by enzymatic hydrolysis using various enzyme concentrations at 40˚C for 5, 10 and 15 hours

When consider the reaction time, acid hydrolysis gave more preferable higher DH than that of enzymatic hydrolysis. When hydrolyzing with enzyme such as papain, the reaction depends on temperature and its optimal temperature is reported at 70˚C [18]. Therefore, increasing reaction temperature might increase the ability to hydrolyze protein within limited range.

D. High Molecular Weight Protein

After hydrolysis reaction, large protein molecules were digested to smaller peptides and amino acids. According to the principle of Bradford assay, color of Comassie Brilliant Blue G-25 will switch from red to blue when binding with proteins and the absorbance can be measured at 595 nm [15]. In this research, Bradford assay was used to determine high molecular weight proteins that left over in FPH. Proteins that can be detected by this method are only proteins that have molecular weight more than 3000 Dalton, smaller proteins, peptides and amino acids cannot be detected [19].

The results shown in Fig. 5 and 6 suggested that FPH hydrolyzed by HCl had high molecular weight protein in the range of 0.10 -0.14%. The highest value at 0.14% when treated with 4M HCl concentration and protein was 0.12% and 0.10% when hydrolyzed with 6 and 8M, respectively but there were no significant difference among HCl concentrations (p=0.353).

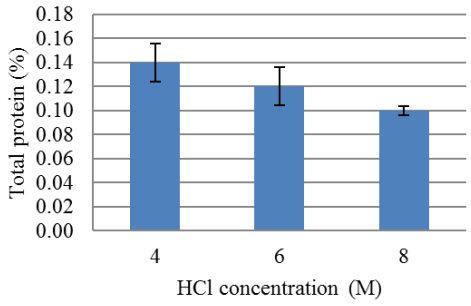

Figure 5. High molecular weight protein in FPH obtained by acid hydrolysis using different acid concentrations at 121˚C for 90 minutes

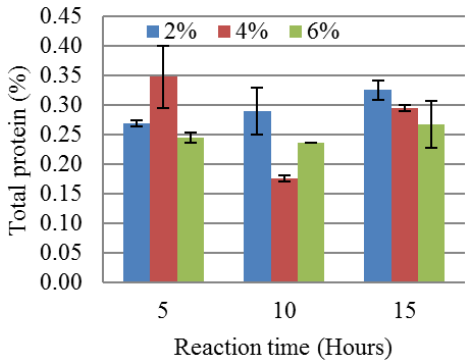

Figure 6. High molecular weight protein in FPH obtained by various enzyme concentrations at 40˚C for 5, 10 and 15 hours

For enzymatic hydrolysis, there are no significant differences in leftover protein among all enzyme concentrations and reaction times. Anyway, protein detected in enzymatic FPH were 0.18 - 0.33 % which were slightly higher than those of acid hydrolysis. It can be concluded that most of the proteins had already been hydrolyzed to peptides or amino acids that were smaller than 3000 Dalton under these hydrolysis conditions.

IV. Conclusion

The results of this research clearly revealed that low-valued marine fish had a potential to be utilized as protein source for producing protein hydrolysates. DH and Bradford assay results indicated that the raw material fish was rapidly hydrolyzed to small peptides by both acid and enzymatic hydrolysis. Papain can hydrolyze fish protein with increasing DH as reaction time increases but there is no influence by enzyme concentration. Acid hydrolysis seems to be more efficient and economically appropriate method for FPH production for animal feed application. It can hydrolyze protein in shorter period of time when compared with enzymatic hydrolysis due to a higher DH obtained. Instead of discard some low-valued fish, the value added FPH from this alternative source of protein can be produced. Nevertheless, optimization for the hydrolysis condition using response surface methodology and further characterization should be evaluated.

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