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Degree of hydrolysis and free tryptophan content of Skipjack Tuna *(Katsuwonus pelamis)* protein hydrolysates produced with different type of industrial proteases

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Abstract: Protein-rich by-products from the canning industry, especially dark flesh of skipjack, have limited uses due to several factors such as darken color, susceptibility to oxidation and off flavour. Protein hydrolysates from skipjack dark flesh was produced with different type of industrial proteases (Alcalase®2.4L FG, Protamex®, Neutrase®1.5MG and Flavourzyme®500MG) for 60, 120, 180 and 240 min with level of proteases used of 0.5, 1, 1.5 and 2% per weight of raw material. The degree of hydrolysis and free tryptophan content of hydrolysate were investigated. The results shows longer time with higher concentration of enzyme has increased the degree of hydrolysis. Alcalase®2.4L FG had the highest degree of hydrolysis among all proteases followed by Protamex®, Flavourzyme®500MG and Neutrase® 1.5MG. All enzymes increase free tryptophan content linearly with the increament of protease enzyme level. The longer the hydrolysis time, the higher the content of free tryptophan produced.

Keywords: Skipjack dark flesh, protein hydrolysate, degree of hydrolysis, free tryptophan, protease

Introduction

Tuna (*Thunus* spp) and tuna-like species are economically very important and significant source of food. The global production of tuna has reached 4.5 million tons per year, and skipjack tuna accounts for 59.1% of total production (FAO, 2010). Tuna is generally processed as a raw meat and marketed as loins/steaks or as a canned food. In the canning process, approximately one-third of the whole fish is available for value addition activity. Guerard et al. (2002) reported that fish canning industries left solid wastes such as fish viscera, gills, flesh dark/dark muscle, head, bone, and skin. These wastes can be as high as 70% of the original material. Sultanbawa and Aksnes (2006) reported that processing discards from tuna canning industry are estimated at 450000 tons annually. In addition, they recommended that the tuna industry must, therefore, look at avenues to add value to tuna processing discards.

Protein-rich by-products from the canning industry, especially dark flesh of the fish, have limited uses due to their darken colour, susceptible to oxidation and prone to off flavour. Due to these factors, they are normally discarded or processed into other market-value products, such as fish meal and fertilizer. However, these by-products contain proteins that can be utilized as functional ingredients in food systems. Hydrolysis process is one of the methods that has been developed to convert fish by-products and under-utilized fish into a more marketable and acceptable form such as fish protein hydrolysates that can be widely used in food systems (Kristinsson and Rasco, 2000).

By using the commercial enzymes to produce hydrolysate from low value marine species can be a feasible technology. It makes vast underutilized resources into useful food ingredients for direct human consumption. Enzymes used to hydrolyze fish protein have at least one common characteristic: it must be food-grade and if they are of microbial origin, the producing organism has to be non-pathogenic (Pedersen, 1994). Generaly, the variety of food-grade proteolytic enzymes is very wide and offers enzymologists a good opportunity in producing various types of fish by-product hydrolysates. Enzymes of microbial origin have been applied to the hydrolysis of fish proteins. In comparison to animal- or plant derived enzymes, microbial enzymes have other several advantages including a wide variety

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of available catalytic activities and greater pH and temperature stabilities (Diniz and Martin, 1997). From a technical and economical point of view, microbial such as alcalase that operates at alkaline pH has been reported to be the most efficient agent in hydrolysis of fish proteins (Dufosse *et al.*, 2001). Other enzyme preparations have also shown excellent potential for hydrolyzing fish protein to produce highly functional fish protein hydrolysates including Protamex (Choi *et al.,* 2009), Flavourzyme, Corolase (Kristinsson and Rasco, 2000), Umamizyme (Guerard *et al*., 2002) and Kojizyme (Nilsang *et al*., 2005).

There are several reports about enzyme application from the hydrolysis of by-product of fish processing and from the under-utilized fish species. In Malaysia presently, the research work on hydrolysis of fish protein by-product is scanty. Various previous studies were more focused on the under-utilized fish species such as Black tilapia (Hamid *et al*., 2002) and Threadfin bream only (Norma *et al.*, 2004).

Fish protein hydrolysates, obtained by controlled enzymatic hydrolysis, are among the best protein hydrolysates in terms of it nutritional properties, a well balanced amino acid composition and highly digestible (Kristinsson and Rasco, 2000). Unfortunately, they are underutilized byhuman due to their bitterness and fishy flavours. Bitterness taste is a major problem affecting the sensory acceptability of protein hydrolysates. One of amino acids that is commonly express bitter taste is tryptophan (Pedersen, 1994). Thus, the aim of this study was to observe the effect of using different types of enzymes, times of hydrolysis and enzyme concentrations on the flesh dark tuna hydrolysate in term of the degree of hydrolysis and the content of free tryptophan.

Materials and Methods

Raw material and chemicals

Frozen blocks of Skipjack tuna (Katsuwonus pelamis) by-product (dark flesh parts) were obtained from PT. Medan Tropical Canning & Frozen Industries (Medan, Indonesia). This frozen material was transported to a laboratory of Food Technology Division, in the School of Industrial Technology, Universiti Sains Malaysia in cold storage truck at -20°C until further used. Prior to the hydrolysis, one packet from each sample block were thawed overnight in refrigerator at 4°C. The following enzymes were obtained from Novo Nordisk (Denmark): Industrial endo and exopeptidase mixtures, Flavourzyme® 500MG, Neutrase® 1.5MG, Protamex® and Alcalase® 2.4LFG. These enzymes comply with the recommended purity specifications for food-grade enzymes recommended by Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC). These enzymes were stored at 4°C until further used. Meanwhile, Tryptophan standard 5-Fluoro-L-tryptophan Biochemical ≥98.0% (HPLC grade) was purchased from Sigma company. All chemical reagents used for experiments are of analytical grade.

Production of protein hydrolysate

The thawed dark flesh tuna were minced in a blixer (Robot Couple, France) followed by heating at 85°C for 20 minute in wise bath (Daihan Scientific, Korea) to inactivate the endogenous enzymes (Guerard et al., 2001) and facilitate the removal of fat presented in the material. The heat treated raw material was then allowed to cool and proceeded with centrifugation protocol at 3500 rpm for 20 min at 4°C (Union 5KR centrifuge, Hanil Science Industry, Korea) for oil separation. The separated oil was then removed and the protein rich solid was used for the next experiments. The protein rich solid (sample) were mixed with sodium phosphate buffer 1:2 (w/v) and homogenized (IKA T25 digital Ultra Turrax, Germany) for about 2 min at ambient temperature. The pH of the mixture was adjusted to optimum activity for each enzyme (Alcalase® 2.4L FG at pH 8, Neutrase® 1.5MG at pH 7, Protamex® and Falvourzyme® 500MG at pH 7.5), by adding 2 N NaOH. The different concentrations (0.5, 1.0, 1.5, and 2% w/w of the raw material) were added into protein rich solid sample. All reactions were performed in 250 ml glass vessels, in a shaking incubator (LM-570R Orbital Shaker Incubator) with constant agitation (200 rpm) at temperatures of enzymatic hydrolysis were adapted as the manufacturer recommends for each enzyme (Alcalase® 2.4LFG optimum at 55°C, Flavourzyme® 500MG and Protamex® at 50°C, Neutrase® 1.5MG at 45°C). Hydrolysis is prolonged for 4 hours and sampling is carried out hourly. After each treatment, the reaction was terminated by heating the solution in water bath (JP Selecta, Spain) at (Alcalase® 2.4L FG optimum at 85°C for 10 min, Flavourzyme®500MG at 90°C for 5 min, Protamex® at 85°C for 10 min and Neutrase® 1.5MG at 80°C for 10 min), assuring the inactivation of the enzyme. The hydrolysate were then cooled on ice to room temperature and centrifuged at 10000 rpm at 4°C for 20 min in a Kubota 6500 (Japan) centrifuge, to collect the supernatant.

Degree of hydrolysis

The degree of hydrolysis was estimated according to the method established by Hoyle and Merritt (1994).

To the supernatant, one volume of 20% trichloroacetic acid (TCA) was added, followed by centrifugation at 10000 rpm at 4°C for 10 min to collect the 10% TCA-soluble materials. Total nitrogen in the 10% TCA soluble material and the substrate was estimated by Kjeldahl method using Kjeltec protein analyzer. Tl

$$\%DH = 100\ X\ (\frac{10\%TCA - solublenitrogeninthesample}{totalnitrogeninthesample})$$

Free tryptophan content

Preparation standard and working solution

Standard stock solutions of tryptophan (404 mg/mL) were prepared in acidic water (ultra-pure water adjusted to pH 6.3 with 0.1M hydrochloric acid). The solutions were then stored away from light at 4°C. Working solutions for tryptophan were prepared from these solutions and diluted with acidic water. Then, five ascending concentration levels were prepared. In order to get persistent result, the stock solutions were freshly prepared every 2 weeks, and the working solutions were prepared on the same day of analyses. An aliquot (20µL) of each standard working solution was subjected to HPLC analysis. For quantification, peak areas were correlated with the concentrations according to the calibration curve.

Instrumentation and chromatographic conditions

A Waters HPLC system (Waters Corporation, USA) equipped with a Waters Alliance 2690 Separation Module, a Waters fluorescence detector and a Waters Millennium 32 workstation version 3.2 was used. The separation was performed with a Hypersil ODS C18 column (250×4.6 mm, 5 µm) (Thermo Scientific, Waltham, MA, USA) fitted with a Hypersil ODS guard column. The HPLC conditions were as follows: the mobile phase consisted of the mixture of methanol: 40mM sodium acetate buffer (adjusted to pH 4.5 with acetic acid; 20:80, v/v) filtered through a 0.22mm membrane and degassed; a flow rate of 1.00 mL/min; column temperature was 26°C. The fluorescence was recorded at the optimal wavelength for tryptophan (λex = 280 nm and λem = 348 nm) for another 15 min. These conditions were based on preliminary trials from method described by Sanchez-Machado (2008) for isocratic liquid chromatography, with minor modifications.

Extraction of free tryptophan

Free tryptophan was extracted from the freeze-dried samples with acidic water. Two hundred milligrams of finely ground sample was placed in a volumetric flask and diluted to 25mL with acidic water to obtain a concentration of 10 mg/mL. The samples were then sonicated for 2 min for complete dissolution process (Sanchez-Machado *et al.,* 2008).

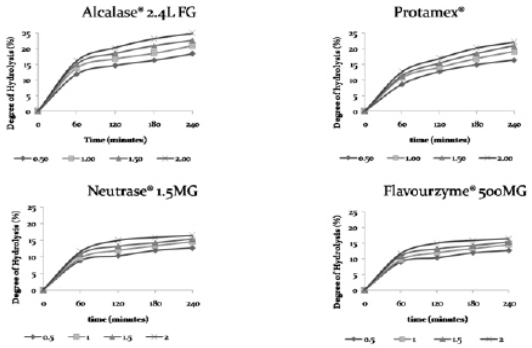

Figure1. Degree of hydrolysis of skipjack tuna dark flesh hydrolysates

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test. Pearson's correlation method was performed to analyse correlation between several data. Analysis was performed using SPSS software (SPSS 16.0 for Windows, SPSS Inc, Chicago, IL, USA).

Results and Discussion

Enzymatic hydrolysis of protein is one of the technique to improve the functionalities of protein. The important properties of protein hydrolysates were determined by the degree of hydrolysis and the degree of bitterness attribute. These in turn are dependent on the nature of the protein and the specificity of the enzyme used, as well as on the hydrolysis conditions, particular pH and temperature. Degradation of protein renders it more soluble. Other functional properties, such as emulsifying, foaming, viscosity, gelatinization and water absorption capacity are also affected by the hydrolysis. The choice of enzyme for given application depends on the substrate and the desired properties of the final hydrolysates. The hydrolitic curves obtained with Alcalase® 2.4L FG, Neutrase® 1.5MG, Protamex® and Flavourzyme® 500MG at different initial enzyme concentrations are given in Figure1.

Degree of hydrolysis of flesh dark of skipjack treated with four enzymes (Alcalase® 2.4L FG, Protamex®, Neutrase® 1.5MG and Flavourzyme® 500MG) increased linearly with the increment of incubation time and enzyme concentration. The result was in agreement with previous studies. Normah *et al.* (2004) found that DH increased as incubation time and enzyme-substrate ratio increased on the hydolysis of threadfin bream by alcalase. Guerard *et*

al. (2001) and Guerard *et al*. (2002) reported the same conclusion for yellowfin tuna hydrolysis by alcalase and umamizyme, respectively.

Meanwhile, Klompong *et al*. (2007) observed that when the incubation time is longer and the enzyme concentration used is higher (alcalase and flavourzyme), the degree of hydrolysis of yellow stripe trevally will be increased. The study of Mukhin et al. (2001) demonstrated that the increased incubation time and enzyme concentration will increase the DH of Iceland scallop processing waste using a proteinases complex obtained from king crab hepatopancrease. At the same temperature rate, the increased enzymatic hydrolysis time resulted in increased DH of a Persian sturgeon viscera hydrolysate by alcalase.

Each type of enzyme used to hydrolyze dark flesh of skipjack gave a significant different effect on the degree of hydrolysis for each concentration and incubation time. The highest degree of hydrolysis was enzyme Alcalase® 2.4L FG enzyme, followed by enzyme Protamex®, Flavourzyme® 500MG and Neutrase® 1.5MG, respectively. Alcalase® 2.4L FG showed the highest efficiency compared to the other three for the hydrolysis of the dark flesh. This result was typical with those previously reported for the enzymatic hydrolysis of cod muscle by alcalase, protamex and neutrase (Ravallec-Pleet *et al*., 2001), small croaker by protamex and flavourzyme (Choi *et al*., 2009) and Silver carp by alcalase and flavourzyme (Dong *et al*., 2008).

Tryptophan is an amino acid that contributes a bitter taste. The amounts of this amino acid are changed during enzymatic hydrolysis (Nilsang *et al*., 2005). Neutrase and Flavourzyme hydrolysis exhibited a continuously increasing of tryptophan. This also occurred on Protamex hydrolysis. But it decreased at incubation time of 240 minute (Figure 2). Whereas, hydrolysis using alcalase gave the highest concentration of free tryptophan at 60 minute of incubation time, but then its decreased nearly constant until at final incubation of 240 minute. All enzymes performed higher content of free tryptophan with the increased of enzyme concentration. Comparison of all types of enzymes showed that Neutrase gave the lowest amount of free tryptophan followed by Protamex®, Alcalase® 2.4L FG, and Flavourzyme® 500MG, respectively.

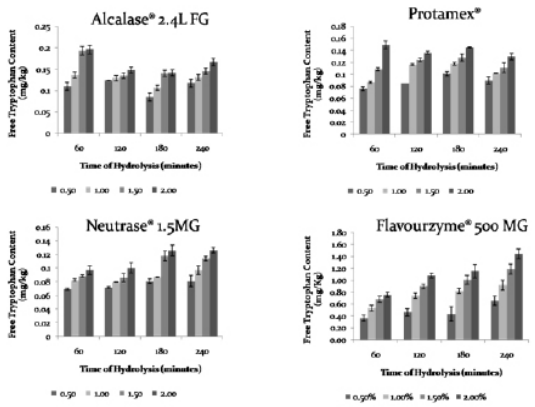

Figure 2. Free tryptophan content of skipjack tuna dark flesh hydrolysates

Currently, the study related to free tryptophan content during fish protein hydrolysis is still limited. So far, researcher that used the content of free tryptophan as the parameter of optimization of enzymatic hydrolysis was Nilsang *et al*. (2005), who conducted the study on fish soluble concentrate, a by-product from canned fish industry, by using Flavourzyme and Kojizyme.

Conclusion

Protein from the waste of processed skipjack tuna was successfully hydrolyzed with various commercial enzymes (Alcalase®2.4L FG, Protamex®, Flavourzyme®500MG and Neutrase® 1.5MG). The present study suggested that degree of hydrolysis increased linearly with time and concentration of enzyme. Alcalase®2.4L FG gave the highest degree of hydrolysis among all proteases followed by Protamex®, Flavourzyme®500MG and Neutrase® 1.5MG. Each type of enzyme gave different effect on each parameter. Protease enzyme level significantly affected the amount of free tryptophan for all type of enzyme. Overall, the longer the hydrolysis time, the higher the content of free tryptophan, except on hydrolysis by alcalase which gave nearly constant value.

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