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RESEARCH PAPER

Protein Quality of Hydrolyzed Dark Muscle Protein of Skipjack Tuna (*Katsuwonus pelamis*)

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Abstract

Protein quality of protein hydrolysates prepared from dark flesh skipjack tuna, hydrolyzed using 2.04 % Alcalase® (FPH-A) and 3 % Protamex® (FPH-P) were evaluated and compared with commercial fish protein hydrolysate (FPH-C). The protein quality was determined using the analysis of molecular weight, amino acid profile, amino acid score/chemical score, protein digestibility correction amino acid score, essential amino acid index, protein efficiency ratio, biological value and its in-vitro digestibility. The results conclude that the FPH-C possessed wider distribution of molecular weight ranged from 237 Da to 11770 Da (mean 3231), in which the largest proportion were at <1000 Da (20%) and >5000 Da (20%). FPH-P possessed narrower distribution range (1210-7246 Da) which 50% of the protein was at 2000-3000 Da. On the other hand, FPH-A's was at range of 730-6440 Da dominated with low molecular weight protein (40% of 1000-2000 Da). Skipjack tuna hydrolysate prepared using Alcalase® showed higher aminoacids concentration, chemical score of protein, protein efficiency ratio (PER), biological value and protein digestibility correction amino acid score (PDCAAS) compared to hydrolysate repared using protamex and commercially sold hydrolysate tested.

Keywords:

Introduction

Fish is known to be a source of protein rich in micro and macro minerals (calcium, phosphorus, fluorine and iodine), essential amino acids (lysine, methionine, cystein, threonine, and tryptophan) (Sikorski, 1994), fats, fat soluble vitamins and unsaturated fatty acid (Fernandez & Venkatrammann, 1993; Ismail, 2005; Usydus *et al*., 2009). Skipjack tuna (*Katsuwonus pelamis*) is a medium-sized fish of the tuna family. It is a cosmopolitan pelagic fish found in tropical and warm-temperate waters and it is a very important species for fisheries. The global production of skipjack tuna has reached to 59.1 % of total production (4.5 million tons per year) (FAO, 2010). Large amounts of protein-rich fish processing by-products are discarded as waste. In the canning process left solid wastes such as fish viscera, dark flesh, head, gills, bone, and skin can be as high as 70 % of the original material. Sultanbawa and Aksnes (2006) reported that the processing discards from tuna canning industry are estimated at 450000 tons annually. Therefore they recommended that the tuna industry must look at avenues to add value to tuna processing discard (Herpandi, *et al*., 2011).

In tuna, dark flesh is a band of dark tissue that lies beneath the skin throughout the body and located near the backbone. The high lipid content, less stable proteins, high concentration of heme proteins, lower pH values and higher concentration of sarcoplasmic proteins of dark flesh have been suggested to contribute difficulties in its fishery industry (Elena, *et al*., 2011). The characteristics of dark flesh, that make it not acceptable for fishery industry, are the strong dark color and the highly susceptibility to lipid oxidation speeding up its deterioration (Nishioka, *et al*., 2007). By-products from the canning industry produced protein-rich especially dark flesh. Normally its was processed into other products, such as fish meal and fertilizer. However, these by-products can be utilized as functional ingredients in food system. In order to increase the utilization of these fishery waste products, hydrolysis of the fish proteins with proteolytic enzymes should be considered to convert the fish into fish protein hydrolysate. Enzymatic hydrolysis of protein is an efficient way to recover potential bioactive peptides. Fish protein hydrolysate has been shown to have potential for nutritional applications (Wergedahl *et al*., 2004). Proteolytic enzymes from plants and microorganisms are most

suitable for preparing fish protein hydrolysates (Chalamaiah, *et al*., 2010). Alcalase and Protamex have been found to be the best enzymes for preparation of skipjack tuna fish protein hydrolysates (Herpandi, *et al*., 2012).

The quality of the protein can be determined in relation to the composition of standard protein, which is recognized as the most relevant for the assessment of the protein quality in the nutrition of all populations (Usydus *et al*., 2009). The evaluation of protein quality is carried out on the basis of the amounts of limiting amino acids. There are 9 essential amino acids, 6 conditionally essential amino acids and 5 are nonessential amino acids in humans (McGuire and Beerman, 2007).

The objectives of this study were to investigate the protein quality of skipjack tuna hydrolyzed with Alcalase® and Protamex®. To assess the nutritional quality of fish proteins in the product tested, the digestibility and composition of the protein were determined. Therefore, those are beneficial to improve protein quality in food and important as supplement.

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 the laboratory of Food Technology Programme, in the School of Industrial Technology, Universiti Sains Malaysia in cold storage truck and was stored at -20^{o}C until further use. Prior to the hydrolysis, one packet from each sample block was thawed for 12 hours in refrigerator at 4°C. The following enzymes were obtained from Novo Nordisk (Denmark): Industrial endo and exopeptidase mixtures, 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 use. All chemical reagents used for experiments are of analytical grade.

Production of Protein Hydrolysate

The thawed dark flesh of tuna was minced in a blixer (Robot Couple, France) followed by heating at 85°C for 20 minute in a water bath (Daihan Scientific, Korea) to deactivate the endogenous enzymes (Guerard *et al.*, 2001) and facilitate the removal of fat present in the material. The heat treated raw material was then allowed to cool and proceeded with centrifugation at 3500 rpm at 4°C for 20 min (Union 5KR centrifuge, Hanil Science Industry, South Korea) for oil separation. The separated oil was then removed and the protein rich solid was used for the subsequent experiment. The protein rich solid (sample) was mixed with sodium phosphate buffer 1:2 (w/v) and homogenized at 10000 rpm (IKA T25 digital Ultra Turrax, Germany) for about 2 min at ambient temperature. The condition of FPH production was based on response surface methodology (RSM) optimized condition from previous study (Herpandi *et al*., 2013). The condition for Alcalase® 2.4L was 65.41°C, pH 8.87, and 2.04% Alcalase® 2.4L for 5.73 h whereas for Protamex® was 58°C, pH 6.57, and 3% Protamex® for 4 h. After each treatment, the reaction was terminated by heating the solution in water bath (JP Selecta, Spain) (Alcalase® 2.4L FG optimum at 85°C for 10 min, and Protamex® at 85°C for 10 min), assuring the inactivation of the enzyme. The hydrolysate was 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. The supernatant was frozen-dried and stored in screw-cap inert bottle for hydrolysate protein quality, digestibility analyses.

Molecular Weight

The molecular weight distribution profile of FPH was determined by gel permeation chromatography (GPC) using a high-performance liquid chromatography system (Waters 1525, Mildford, USA) in a row of three GPC ultrahydrogel column (ultrahydrogel linear, ultrahydrogel 500, and ultrahydrogel 120) by using Waters 2414 Refractive Index Detector and Waters 1525 Binary HPLC Pump. A 0.2% sample solution in 0.1 M sodium nitrate was filterred with Whatman 0.45 µm PTFE before injection to the column. The temperature was set at 40^{o}C and then a 20 µl of sample was injected to the column with 45 min run time and flow rate at 1 ml/min. Six series of Shodex standard (P-28, Lot number: 80502, Showadenco, Japan) consist of 78.8 x 10^4, 40.4 x 10^4, 21.2 x 10^4, 11.2 x 10^4, 4.73 x 10^4, 0.59 x 10^4 Å were used as standard molecular weight.

Amino Acid Profile

Amino acid profile was determined following the official method of AOAC (2000). Amino acid analysis was done in two steps; first step was the hydrolysis of protein to generate the amino acids, whereas the second step was the analysis of amino acids using high performance liquid chromatography. Protein hydrolysis was carried out using 3 different methods which were acid hydrolysis using 6M HCl (acid hydrolysis) for generating amino acids other than methionine, cysteine and tryptophan; hydrolysis using performic acid (performic hydrolysis) to generate sulfur-based amino acids such as cysteine and methionine; and alkaline hydrolysis using 4.3N LiOH for generating amino acid tryptophan (alkaline

hydrolysis).

The amino acid derivatives were produced from acid, performic and alkaline hydrolysis methods. Fluor (*6-aminoquinolyl-N-hydroxysuccinimidyl carbamate*) reagent kit containing acetonitrile, borate buffer and AccQ Fluor powder. Approximately 10 µL of sample/standard was mixed with 70 µL of borate buffer in a 1.5 ml Eppendorf tube. After that, 20 µL of AccQ fluor reagent was added and vortexed vigorously. The sample was then rested for 1 minute for the formation of amino acid derivative. Approximately 10 µL of amino acid derivative was then injected into a high performance liquid chromatography (HPLC) equipped with *Waters AccQ.Tag Amino Acid Analysis Column* (3.9 mm x 150 mm) operated at a column temperature of 36˚C. Internal standard of L-alpha-amino-n-butiric acid (AABA) was used as an internal standard during the analysis. The detection of amino acid was done using a fluorescence detector. Mobile phase utilized were *AccQ.Eluent A* and *AccQ.Eluent B* or 60% acetonitrile with a flow rate of 1 ml/min controlled using Waters 410 HPLC pump.

Amino Acid Score or Chemical Score

The chemical score of the protein hydrolysates was computed based on their amino acids composition according to Ovissipour *et al*. (2011). Chemical score is the ratio of essential amino acids (EAA) in the test proteins compared to the EAA for egg as described by FAO/WHO (1990). In brief, the chemical score was calculated using the following equation:

$$Chemical\ score = \frac{EAA\ in\ test\ protein\ (g/100g)}{EAA\ requirement\ pattern\ (g/100g)}$$

Protein Digestibility Correction Amino Acid Score

Protein digestibility-corrected amino acid scores (PDCAAS) of the samples were calculated by multiplying the lowest amino acid ratio (mg of an essential amino acid in 1.0 g test protein/mg of the same amino acid in 1.0 g reference pattern suggested by FAO/WHO (1990) for 10-12 year old children for the nine essential amino acids plus tyrosine, cystine, and histidine) by the *in vitro* protein digestibility. The PDCAAS scores were expressed in percentage terms (El and Kavas, 1996).

Essential Amino Acid Index

Essential amino acid index (EAAI) was calculated based on the procedure of Oser (1951). The ratio value was taken from essential amino acid in the test protein relative to their respective amounts in whole egg protein calculated by FAO/WHO (1990).

Protein Efficiency Ratio

Protein efficiency ratio (PER) value of protein hydrolysates were calculated using equations developed by Alsmeyer *et al*. (1974) and Lee *et al*.(1978):

$$PER^a = -0.468 + 0.45[LEU] - 0.105\,[TYR]$$

$$PER^b = -1.816 + 0.435[MET] + 0.780[LEU] + 0.21[HIS] - 0.944[TYR]$$

$$PER^c = 0.08084[\sum AA_7] - 0.1094$$

where $\sum AA_7$ = threonine + valine + methionine + isoleucine + leucine + phenylalanine + lysine

Biological Value

Biological value (BV) was computed according to methods of Oser (1951) and Oser (1959). The following equation was used for biological value determination:

$$BV = 1.09\ (EAA\ Index) - 11.7$$

***In vitro* Digestibility**

In vitro digestibility was determined with an enzymatic *in vitro* technique following method of Babji and Lechumanan (1989).

(i) Preparation of sodium caseinate control and FPH samples

Each sample of protein hydrolysate and caseinate control weighing 6.25 mg protein/ml correspondingly was placed into glass vials. Glass-distilled water (10 ml) was pippetted into each vial and then the contents of all the vials were mixed thoroughly by vortexing. In order to allow for hydration of protein, the samples and a solution were stored for at least 1 hour at 5 ºC. Protein samples and the caseinate control were then adjusted to pH 8.00±0.03 at 37 ºC.

(ii) Preparation of three-enzyme solution

This solution was prepared by mixing 16.55 mg of Trypsin (13700 units/g), 22.18 mg of Chymotrypsin (83.9 units/g), and 5.10 mg Peptidase (102 units/g) and dissolving them in 10 ml of glass-distilled water. Next, the pH of three-enzyme solution was adjusted to around 7.97 and then 0.05 M of NaOH was added dropwise until the pH reached 8.00±0.03 for no more than 2 minutes. The vials were immediately placed in an ice bath.

(iii) Protease solution

Accurately, 11.21 mg of protease (5 units/mg) were dissolved completely in 10 ml of glass-redistilled water. The pH of protease solution was then adjusted to pH 8.00±0.03 in the same manner as described for the three-enzyme solution.

(iv) Determination

The pH of sodium caseinate control was readjusted to 8.00±0.003 in the 37°C reaction vessel. When the pH had stabilized at 8.00±0.003, 1.0 ml of three-enzyme solution was added and a stopwatch was concurrently started. At exactly 10 minutes after

the addition, 1.0 ml of the protease solution was added. The vial was then immediately placed in the 55 °C water bath. At exactly 19 minutes, the vial was transferred from the water bath to the reaction vessel. And finally, the pH value was recorded at exactly 20 minutes. Samples of protein hydrolysate were measured in the same way as described for the control.

(v) Calculation

In vitro digestibility of the protein sample was calculated using the following equation:

$$\%\ Digestibility = 234.84 - 22.56X$$

where, X is the pH at 20 minutes.

Statistical Analysis

The data obtained were subjected to one-way analysis of variance using SPSS statistical software, versions 17.0 (SPSS Inc., Chicago, IL, USA). Duncan's new multiple range test was performed to determine the significant differences at 5% level.

Results and Discussions

The protein quality derived from skipjack protein hydrolysted is determined by molecular weight, amino acid composition, chemical score and essential amino acid index, predicting efficiency ratio and biological value, In vitro digestibility and protein digestibility corrected amino acid score. These parameters are important for further hydrolysate protein applications in food and supplement. Furthermore, it can fulfill human requirements in protein.

Molecular Weight

The mean molecular weight of the FPH-C (3231 Da) was followed by FPH-P was (2335 Da) and FPH-A (2170 Da) (Table 1). The size of a molecule, along with other information, can be clue to its identity. The results showed that FPH-P gave higher minimum size molecular weight compare to FPH-A. The FPH-C sample has the lowest minimum size molecular weight (237 Da) compared to FPH-P (1210 Da) and FPH-A (730 Da). However, FPH-C, also has the highest maximum size molecular weight (11770 Da) compared to the others showing a higher range in the molecular weight of its hydrolysates.

The molecular weight distribution of Skipjack dark flesh protein hydrolysate is presented in Table 2. From the results, FPH-P had highest molecular weight distribution (50%) in the range of 2000-3000 Da, followed by molecular weight of 1000-2000 Da (25%). Contrary with FPH-A, which the domination of molecular weight distribution was in the range of 1000-2000 Da (40%), followed by molecular weight of 2000-3000 Da (25%). FPH-C however had molecular weight which was quite distributed from 1000 Da to >5000 Da.

The quality of protein hydrolysate was influenced by enzyme specificity and hydrolysis conditions (Borseau et al., 2009). Protamex® and

Table 1. Molecular weight of skipjack dark flesh protein hydrolysate produced using Protamex®, Alcalase® 2.4L FG and commercial fish protein hydrolysate commercial

Protein Hydrolysate	Molecular weight (Daltons)		
	Min	Max	Mean
FPH-P	1210	7246	2335
FPH-A	730	6440	2170
FPH-C	237	11770	3231

FPH-P; skipjack dark flesh protein hydrolysate produced using Protamex®
FPH-A; skipjack dark flesh protein hydrolysate produced using Alcalase® 2.4L FG
FPH-C; commercial fish protein hydrolysate

Table 2. Molecular weight distribution of skipjack dark flesh protein hydrolysate produced using Protamex®, Alcalase® 2.4L FG and commercial fish protein hydrolysate commercial

Molecular weight (Daltons)	Molecular weight distribution (%)		
	FPH-P	FPH-A	FPH-C
>5000	3	4	20
4000-5000	6	5	10
3000-4000	16	11	14
2000-3000	50	25	17
1000-2000	25	40	19
<1000	0	15	20

FPH-P; skipjack dark flesh protein hydrolysate produced using Protamex®
FPH-A; skipjack dark flesh protein hydrolysate produced using Alcalase® 2.4L FG
FPH-C; commercial fish protein hydrolysate

Alcalase® are both endopeptidase enzymes which have an ability to degrade major protein in alpha-peptide bonds. Compared to exopeptidase that has ability to generate more free-amino acid from the degradation of oligopeptide, endoprotease tend to produce bigger size of peptides (Chapot-Chartier, 2004; Dudley and Steele, 2004). However, the hydrolysis using Protamex® and Alcalase® could also produce low molecular weight protein if carried out on longer time of hydrolysis. Ovissipour *et al*. (2011) previously mentioned that the hydrolysis of up to 120 minutes using Protamex® could generate low molecular weight of peptide at range of 60-7320 Da. Similarly, Alcalase® was also reported to be able to generate low molecular weight at longer time of hydrolysis at range of 200-1000 Da at 120 min of hydrolysis time (Foh *et al*., 2010). According to Peña-Ramos *et al*. (2004) the chromatographic determination of Alcalase®-hydrolyzed whey protein revealed numerous oligopeptides with molecular weight estimated to <1000 Da and between 1000 and 2500 Da. Alcalase® hydrolysated whey protein has >80 % a small peptides with of molecular weight <2000 Da after 5 h of hydrolysis (Doucet *et al*., 2003).

Hale and Bauersfeld (1978) reported the effects of various processing conditions and commercially available proteolytic enzymes on yield and composition of water-soluble fish protein hydrolysate. Pihlanto (2006) reported that the relationship between the peptides and their activities is not known in detail but dependent on various characteristics like, amino acid, molecular weight, hydrophobicity, charge and acid-basic character.

Amino Acid Compositions

Amino acid compositions of FPH are listed in Table 3. The fish protein hydrolysate contained 18 amino acids, nine of which are essential amino acids. The most abundant amino acids composition of FPH prepared by using Protamex® (FPH-P) was lysine (6.70 ± 0.10 g/ 100g) followed by leucine (4.71 ± 0.07 g/ 100g), histidine (3.93 ± 0.02 g/ 100g), threonine (3.17 ± 0.02 g/ 100g), valine (3.10 ± 0.05 g/100g), isoleucine (2.76±0.05 g/ 100g), phenylalanine (2.22±0.03 g/ 100g), methionine (1.91±0.02 g/ 100g) and tryptophan (1.73±0.19 g/ 100g).

The FPH-A showed similar trend like FPH-P, except the concentration of valine was higher than threonine and the concentration of tryptophan was

Table 3. Amino acid compositions of skipjack dark flesh protein hydrolysate produced using Protamex®, Alcalase® 2.4L FG and commercial fish protein hydrolysate commercial

No.	Amino acid (g/100 g)		FPH-P	FPH-A	FPH-C	Egg Ref. Pattern (FAO, 1965)
1	Histidine[a]	His	3.93±0.02	4.42±0.15	ND	2.4
2	Isoleusine[a]	Ile	2.76±0.05	3.21±0.10	2.55±0.05	6.6
3	Leucine[a]	Leu	4.71±0.07	5.69±0.15	4.32±0.09	9.1
4	Lysine[a]	Lys	6.70±0.10	7.34±0.17	4.11±0.04	6.6
5	Methionine[a]	Met	1.91±0.02	2.29±0.05	2.22±0.05	5.5
6	Phenylalanine[a]	Phe	2.22±0.03	2.71±0.08	2.27±0.04	10.1
7	Threonine[a]	Thr	3.17±0.02	3.63±0.09	2.65±0.05	5.0
8	Tryptophan[a]	Trp	1.73±0.19	2.56±0.11	1.37±0.04	1.8
9	Valine[a]	Val	3.10±0.05	3.65±0.11	3.18±0.07	7.4
10	Alanine	Ala	4.30±0.03	4.94±0.07	5.70±0.18	
11	Arginine	Arg	4.77±0.10	5.17±0.39	3.28±0.04	
12	Aspartic acid	Asp	6.77±0.01	7.59±0.13	5.01±0.13	
13	Cysteine	Cys	0.42±0.02	0.63±0.06	0.34±0.02	
14	Glutamic acid	Glu	10.29±0.05	11.29±0.08	10.08±0.22	
15	Glysine	Gly	3.10±0.02	3.49±0.06	12.81±0.46	
16	Proline	Pro	2.31±0.05	2.59±0.08	5.28±0.18	
17	Serine	Ser	2.67±0.01	3.12±0.12	3.36±0.09	
18	Tyrosine	Tyr	2.57±0.06	3.09±0.03	0.97±0.14	
	∑AA		67.41±0.36	77.42±0.63	69.47±2.24	
	∑NEAA		37.19	41.92	46.81	
	∑EAA		30.22	35.50	22.66	54.5
	∑FAA		24.45	27.32	33.59	
	∑EAA/∑AA		0.45	0.46	0.33	
	∑FAA/∑AA		0.36	0.35	0.48	
	∑EAA/∑NEAA		0.81	0.85	0.48	

Notes:
[a]Essential amino acid
AA: Amino acid, EAA: Essential amino acids, FAA: Flavor amino acid, NEAA: Non-Essential amino acid
FPH-P; skipjack dark flesh protein hydrolysate produced using Protamex®
FPH-A; skipjack dark flesh protein hydrolysate produced using Alcalase® 2.4L FG
FPH-C; commercial fish protein hydrolysate

higher than methionine. From the result, it shows that histidine was not detected in FPH-C. Table 3 showed that the content of nine essential amino acids in the fish protein hydrolysate were comparable with that of the amino acid reference pattern established by the FAO (1965). The composition of histidine, lysine and tryptophan were found to be higher in FPH-A compared to egg reference pattern established by the FAO.

Essential amino acids content of our sample was 30.22 g/100 g FPH-P and 35.50 g/100 g FPH-A, that is almost 44.83 and 45.86 % of total amino acids, respectively. These values were much higher compared to FPH-C which has only 32.62% (22.66 g/100 g) of its total amino acids as essential amino acids. Moreover, compared to FPH-C, the FPH produced (FPH A and FPH P) had higher proportion of ratio of essential amino acid (EAA) to non-essential amino acids (NEAA). FPH-A exhibited ∑EAA/∑NEAA, at 0.85 slightly higher than that of FPH-P (0.81), whereas FPH-C had a much lower ratio of 0.48. This finding suggests that resulted sample, FPH-P and FPH-A are suitable for human consumption since it exceeded the requirement of World Health Organization and Food and Agriculture Organization (FAO/WHO, 1990) that stated the minimum EAA content is 40% of total amino acid and at ratio more than 0.6.

Even though glutamic acid, aspartic acid, glycine, and alanine are non-essential amino acid, their high concentrations in the samples (36.27% in FPH-P and 35.29% in FPH-A) could be advantageous since they are responsible to the development of flavour by acting as flavour enhancers (Ovissipour *et al*., 2010). Moreover, the resulted FPH-A was also richest in arginine compared to FPH-P and FPH-C. Cao *et al*. (2008) and Niitynen *et al*. (1999) previously mentioned that even though it is non-EAA, arginine is one of the most important amino acids that plays an important role in human body. Arginine participates in protein synthesis and physiological functions (detoxification and energy conversion), and it has been found that arginine is advantageous to be utilized in cardiovascular disease treatment.

Chemical Score and Essential Amino Acid Index, Predicting Efficiency Ratio and Biological Value

Chemical score provides an estimate of the nutritive value of a protein by comparing the levels of essential amino acids between samples and standard proteins (Ovissipour *et al*., 2010). Chemical scores were determined based on the reference protein of FAO/WHO (1990) for adults and amino acid requirements of juvenile common carp as presented by NRC (1993) as shown in Table 4. The amino acid composition of the present study and comparison with reference proteins revealed that the amino acid profile of both FPH-P and FPH-A were generally higher in histidine, isoleucine, leucine, lysine, threonine, tryptophan and valine compared with the suggested requirements patterns by FAO/WHO (1990) for adult humans.

The amino acid score in FPH-P and FPH-A compared to protein reference pattern established by FAO/ WHO for adults were methionine + cysteine with chemical score 1.12 and 1.35, respectively. Meanwhile, the amino acids score in FPH-C was phenylalanine + tyrosine which were 1.19 (Table 4). It means that the amino acids profile of the FPH-P, FPH-A and FPH-C were generally higher in essential amino acids compared to the suggested amino acid pattern recommended by FAO/ WHO for adult humans. Similar result was observed by Ovissipour *et al*. (2009a) about beluga sturgeon viscera hydrolysates that fulfilled amino acid pattern recommended by FAO/ WHO for adult humans.

However, the chemical score of phenylalanine + tyrosine was found to be lower in FPH-P and FPH-A (0.31 and 0.37, respectively) compared to reference protein pattern established by NRC (1993). The chemical score in FPH-C was 0.31 for phenylalanine

Table 4. Chemical score of skipjack dark flesh protein hydrolysate produced using Protamex®, Alcalase® 2.4L FG and commercial fish protein hydrolysate commercial

EAA (g/100 gram)		Refference Protein Pattern[1]	Refference Protein Pattern[2]	Chemical Score					
				FPH-P[1]	FPH-A[1]	FPH-C[1]	FPH-P[2]	FPH-A[2]	FPH-C[2]
Histidine	His	1.6	1.7	2.46	2.76	-	2.31	2.60	-
Isoleusine	Ile	1.3	4.2	2.12	2.47	1.96	0.66	0.77	0.61
Leucine	Leu	1.9	7	2.48	3.00	2.28	0.67	0.81	0.62
Lysine	Lys	1.6	5.1	4.19	4.59	2.57	1.31	1.44	0.81
Methionine + Cysteine	Met+Cys	1.7	2.6	*1.12*	*1.35*	1.30	0.74	0.88	0.85
Phenylalanine + Tyrosine	Phe+Tyr	1.9	7.3	1.17	1.43	*1.19*	*0.31*	*0.37*	*0.31*
Threonine	Thr	0.9	3.5	3.52	4.03	2.94	0.91	1.04	0.76
Tryptophan	Trp	0.5	1.1	3.45	5.13	2.73	1.57	2.33	1.24
Valine	Val	1.3	4.8	2.39	2.81	2.45	0.65	0.76	0.66

[1]Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1990).
[2]Essential amino acid requirements of common carp according to NRC (1993).
FPH-P; skipjack dark flesh protein hydrolysate produced using Protamex®
FPH-A; skipjack dark flesh protein hydrolysate produced using Alcalase® 2.4L FG
FPH-C; commercial fish protein hydrolysate

+ tyrosine. Similar finding was observed by Ovissipour *et al*. (2009a). The authors reported that beluga sturgeon viscera hydrolysates had chemical score which was phenylalanine + tyrosine (0.51) as a comparison to reference protein pattern established by NRC (1993). This result agreed with other findings reported by Bhaskar *et al*. (2008) and Ovissipour *et al*. (2009a,b). Phenylalanine and methionine in Catla alcalase-hydrolysates were found to be limiting amino acids for common carp (Bhaskar *et al*., 2008). Similar results were reported by Ovissipour *et al*. (2010) who also found that methionine was the limiting amino acid in hydrolysates isolated from yellowfin tuna head. Limiting amino acid became important for further purposes, such as food fortification. Limiting amino acid as the lowest concentration is the major factor that in order to fulfill body requirement, it is needed to be added by other source of food.

The essential amino acid index is shown in Table 5. Results indicated that amino acid index were higher for FPH-A compared to FPH-P. The essential amino acids index in FPH-P and FPH-A were greater compared to FPH-C. Similar results were also reported by Ovissipour *et al*. (2010) that essential amino acids index of yellowfin tuna protein hydrolysate using Alcalase® was higher compared with Protamex® hydrolysate (74.26 to 69.39). The lower essential amino acids index for FPH-C is in accordance with the lower essential amino acid score (Table 4 and 5) for Phenylalanine + Threonine. Lysine showed the highest amino acid index of fish protein hydrolysate in accordance with the higher essential amino acid score. These results suggest that FPH-A had higher nutritional indices compared to FPH-P. This finding is in agreement with the report of Ovissipour *et al*. (2010) which found similar result from yellowfin tuna head hydrolysate prepared using Protamex® and Alcalase®.

From the result, it showed that FPH-A has higher amino acids index, biological value and predicting PER compared to FPH-P (Table 5 and 6). PER values were 1.09-2.11 for FPH-P and 1.64-2.45

Table 5. Essential amino acid index of skipjack dark flesh protein hydrolysate produced using Protamex®, Alcalase® 2.4L FG and commercial fish protein hydrolysate commercial

Essential Amino Acid		Egg Ratio 100			Log Egg Ratio		
		FPH-P	FPH-A	FPH-C	FPH-P	FPH-A	FPH-C
Histidine	His	234.00	245.54	-	2.00	2.00	
Isoleusine	Ile	59.64	64.98	48.40	1.78	1.81	1.68
Leucine	Leu	73.90	83.49	59.51	1.87	1.92	1.77
Lysine	Lys	144.92	148.49	78.03	2.00	2.00	1.89
Methionine + Cysteine	Met+Cys	49.62	55.57	50.51	1.70	1.74	1.70
Phenylalanine + Threonine	Phe+Thr	31.46	35.79	28.13	1.50	1.55	1.45
Threonine	Thr	90.51	96.79	66.28	1.96	1.99	1.82
Tryptophan	Trp	136.94	189.96	95.12	2.00	2.00	1.98
Valine	Val	59.92	65.76	53.92	1.78	1.82	1.73
		Sum of log (Egg Ratios)			16.57	16.84	14.04
		Mean log Egg Ratios			1.84	1.87	1.75
		EAA index			69.39	74.26	56.81

*EAA = Essential Amino Acid
FPH-P; skipjack dark flesh protein hydrolysate produced using Protamex®
FPH-A; skipjack dark flesh protein hydrolysate produced using Alcalase® 2.4L FG
FPH-C; commercial fish protein hydrolysate

Table 6. Predicting equation for some of nutritional of skipjack dark flesh protein hydrolysate produced using Protamex®, Alcalase® 2.4L FG and commercial fish protein hydrolysate commercial

Equation[a]	Predicting PER		
	FPH-P	FPH-A	FPH-C
-0.468 + 0.454[Leu] - 0.104[Tyr]	1.40	1.80	1.39
-1.816 + 0.4435[Met] - 0.780[Leu] + 0.211[His] - 0.944[Tyr]	1.09	1.64	-
0.08084[X_7] - 0.1094	1.88	2.20	-
0.08084[X_{10}] - 0.1539	2.11	2.45	1.61
Biological Value	63.94	69.24	50.22

[a]X_7=Thr+Val+Met+Ile+Leu+Phe+Lys; X_{10}=X7+His+Arg+Tyr
FPH-P; skipjack dark flesh protein hydrolysate produced using Protamex®
FPH-A; skipjack dark flesh protein hydrolysate produced using Alcalase® 2.4L FG
FPH-C; commercial fish protein hydrolysates

for FPH-A (Table 6). The results indicated that PER was influenced by the type of enzyme used for hydrolysis thus effect its degree of hydrolysis. The result was supported by the finding of Ovissipour *et al*. (2010) and Šližytė *et al*. (2005). Šližytė *et al*. (2005) reported that the PER of cod visceral hydrolysates varied between 1.3-2.53 using Flavourzyme® and 1.25-2.5 for that using Neutrase®. Furthermore, Ovissipour *et al*. (2010) reported 1.7-2.71 as PER for yellowfin tuna head hydrolysis produced using Alcalase® and 1.7-2.82 for that using Protamex®.

The calculation on biological values showed that FPH-A exhibited highest biological value (69.24) followed by FPH-P (63.94) and FPH-C (50.22). Since biological value represents the competence of the absorbed amino acids to meet the body's metabolic demand (WHO, 2007), these results showed that FPH prepared using Alcalase® 2.4L FG would exhibit higher nutritional value compared to that of processed with Protamex®.

In vitro Digestibility and Protein Digestibility Corrected Amino Acid Score

Figure 1 shows the *in vitro* digestibility of FPH-P, FPH-A and FPH-C in comparison with casein. The result shows that there is no significantly difference between FPH-P and FPH-A for *in vitro* protein digestibility. In addition, the results confirmed that the *in vitro* protein digestibility of FPH-P (65.00±0.66 %) and FPH-A (66.12±0.58 %) were significantly lower than that of FPH-C ($P<0.05$). The FPH-C (72.42±1.19 %) has lower *in vitro* digestibility compared to casein (87.28±0.51 %, significantly different $P<0.05$)). This result suggests that according to *in vitro* digestibility simulation, FPH-C was easier to be digested compared to FPH-P and FPH-A. However, even though FPH could provide more available amino acid nitrogen during digestion, it might not represent higher nutritional value compared to FPH-A and FPH-P; since it had lower BV which indicated the lower competence of amino acid absorption during human metabolism.

According to Sindayikengera (2006), protein structure plays an important role in the digestibility of FPH. This protein structure was postulated to affect the resistance of FPH to be enzymatic hydrolysed during *in vitro* digestion. On the other hand, protein digestibility could be reduced as a result of complex chemical reactions such as protein-fat interaction or protein-protein interactions taking place when food is treated at high temperatures (El and Kavas, 1996).

Nutritional evaluation of FPH-P, FPH-A and FPH-C was performed by using the protein digestibility corrected amino acid score (PDCAAS) (Table 7). The PDCAAS was calculated on the basis of essential amino acid composition. The results showed that the PDCAAS of FPH-A ranged from 0.28 to 1.54, FPH-P from 0.23 to 1.34 and FPH-C from 0.26 to 0.91. The lowest values which then represent the PDCAAS were found in phenylalanine + tyrosine in FPH-P was 0.23, in FPH-A was 0.28 and in FPH-C was 0.26. Our results were lower compared to the report of Abdul-Hamid *et al*. (2002) which quoted the PDCAAS value of 0.82. Our low values of PDCAAS were mostly affected by the low content of phenylalanine + tyrosine. Previous results reported by Ovissipour *et al*. (2010) also mentioned that the FPH generated from tuna (head and viscera) possessed relatively low content of phenylalanine + tyrosine compared to other fish such as Coho salmon, herring and tilapia as reported by Nakajima *et al*. (2009), Sathivel *et al*. (2003) and Abdul-Hamid *et al*. (2002), respectively.

Conclusions

Both skipjack tuna hydrolysates, and especially FPH-A, can be used in food systems as a natural

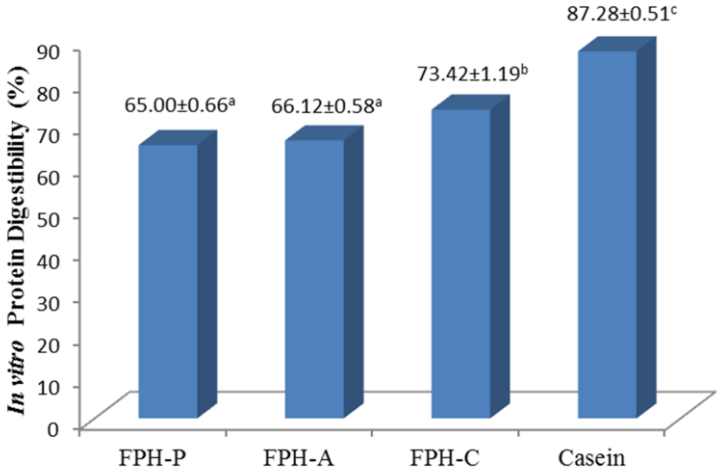

Figure 1. *In vitro* protein digestibility of skipjack dark flesh protein hydrolysate produced using Protamex®, Alcalase® 2.4L FG and commercial fish protein hydrolysate commercial in comparison with casein.

Table 7. Protein digestibility correction amino acid score of skipjack dark flesh protein hydrolysate produced using Protamex®, Alcalase® 2.4L FG and commercial fish protein hydrolysate commercial

EAA (g/100 gram)		PDCAAS		
		FPH-P	FPH-A	FPH-C
Histidine	His	1.34	1.54	-
Isoleusine	Ile	0.64	0.76	0.67
Leucine	Leu	0.46	0.57	0.48
Lysine	Lys	0.75	0.84	0.52
Methionine + Cysteine	Met+Cys	0.50	0.61	0.65
Phenylalanine + Tyrosine	Phe+Thr	0.23	0.28	0.26
Threonine	Thr	0.61	0.71	0.57
Tryptophan	Trp	1.02	1.54	0.91
Valine	Val	0.58	0.69	0.67

PDCAAS, protein digestibility-corrected amino acid scoring
FPH-P; skipjack dark flesh protein hydrolysate produced using Protamex®
FPH-A; skipjack dark flesh protein hydrolysate produced using Alcalase® 2.4L FG
FPH-C; commercial fish protein hydrolysate

additive with good protein quality. The FPH-A showed greater protein quality compared to FPH-P and FPH-C. In general skipjack tuna hydrolysates are considered safe products and they are not restricted use in food. Moreover, both hydrolysates have nine essential amino acids which are important to human body.

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