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Fish protein hydrolysates production from yellowfin tuna *Thunnus albacares* head using Alcalase and Protamex

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Abstract

Protein hydrolysates were prepared from ground yellowfin tuna head, a by-product from tuna canning industry, using 1.5% Alcalase (PHA) and Protamex (PHP) at a natural pH for 4 and 24 h with a water/material ratio of 1:1 (w/v). The higher degree of hydrolysis (DH), nitrogen recovery and protein content of both hydrolysates increased when the longer hydrolysis time was used ($P < 0.05$). In addition, DH and protein content of PHA were higher than those of PHP ($P < 0.05$). The amino acids composition of both PHA and PHP could fulfill human amino acid requirements. With respect to common carp requirements, lysine, methionine and phenylalanine were the limiting amino acids in both protein hydrolysates. However, both hydrolysates had high content of flavoring and essential amino acids. Protein efficiency ratio (PER) results revealed that both hydrolysates had the similar PER. Therefore, protein hydrolysates from yellowfin tuna were found to have high nutritional value and can be used in human and animal diets.

Keywords: Tuna by-products, Hydrolysis, Nutritional value, PER, Alcalase, Protamex, Protein hydrolysate

Introduction

Annually, more than 100 million tons of fish are harvested, of which 29.5% is converted into fish meal (FAO 2006). However, more than 50% of remaining fish tissue is converted to non-edible by-product material (Kristinsson and Rasco 2000a). Recognition of the limited biological resources and increasing environmental pollution have emphasized the need for better and more value-added utilization of under-utilized fish and by-products from the fishing industries (Geurard et al. 2002). Food industries in many locations are no longer permitted to discard their by-products directly to the aquatic environments.

Hence, alternative methods to use by-products must be developed. Using enzymes to hydrolyze proteins is a process, which could improve the physiochemical, functional, sensory and nutritional properties of the intact

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proteins (Šližytė et al. 2005). Hydrolyzing protein can also improve intestinal absorption (Kristinsson and Rasco 2000a). In addition, many studies have revealed that peptone from enzymatic hydrolysis of fish by-products is an appropriate nitrogen source for bacterial growth (Gildberg et al. 1989; Safari et al. 2009; Ovissipour et al. 2009d).

The most common commercial proteases, especially for the hydrolysis of fish protein, are both from plant sources such as papain (Hoyle and Merritt 1994; Shahidi et al. 1995) or from animal origin such as pepsin (Viera et al. 1995), trypsin and chymotrypsin (Simpson et al. 1998; Ovissipour et al. 2009b). Enzymes from microbial origin have also been applied for hydrolysis of fish proteins (Shahidi et al. 1991, 1995; Diniz and Martin 1997; Kristinsson and Rasco 2000b; Aspmo et al. 2005; Batista et al. 2009; Bhaskar et al. 2008; Ovissipour et al. 2009a,b,c,d; Šližytė et al. 2005, 2009). In comparison to animal- or plant- derived enzymes, microbial enzymes offer several advantages including a wide variety of available catalytic activities, greater pH and temperature stabilities (Diniz and Martin 1997). The nutritive value of a protein depends on its capacity to satisfy the needs for nitrogen and the essential amino acids. A widely used method to evaluate protein quality is the protein efficiency ratio (PER) test, which is an expensive and time consuming method (Šližytė et al. 2005). Many researchers have used the equations given by Alsmeyer et al. (1974) and Lee et al. (1978) based on the amino acids composition of proteins to estimate PER (Shahidi et al. 1991, 1995; Diniz and Martin 1997; Šližytė et al. 2005; Ovissipour et al. 2009a). The work of Shahidi et al. (1995) on capelin confirmed that amino acid profiles of protein hydrolysates are generally similar to the raw material except for sensitive amino acids such as methionine and tryptophan. In addition, hydrolysates from cod and sturgeon by-products have been found to have appropriate PER for using in human diets (Šližytė et al. 2005; Ovissipour et al. 2009a).

Extensive hydrolysis of proteins has reduced immunological reactions which is important for hyper allergic infants (Mahmoud 1994). In addition, peptides with lower molecular weight can be easily absorbed in the gut, and can be used for both human and animal diets (Kristinsson and Rasco 2000a; Šližytė et al. 2005).

Degree of hydrolysis (DH), which is defined as the percentage of peptide bonds cleaved, is one of the basic parameters describing the properties of protein hydrolysates (Šližytė et al. 2005). DH is an important factor, which could influence molecular weight, the amount and composition of free amino acids, functional and nutritional properties of hydrolysates.

Tuna canning processing generates solid by-products, constituting 50- 70% of the original raw material. Preparation of tuna by-product hydrolysate would provide the promising means for better utilization of the by-products, traditionally used for pet-food (Guerard et al. 2002). In addition, yellowfin tuna (*Thunnus albacares*) is one of the most important pelagic species in Iran with an annual catch of 41,000 metric tones (Iranian Fisheries Organization) (IFO 2009).

The purpose of this study was to determine the effects of Alcalase and Protamex on DH, nitrogen recovery and the nutritional value of protein hydrolysate from yellowfin tuna head.

Materials and methods

Raw materials

Yellowfin tuna (Thunnus albacares) were caught off the coast of Southern Iran, Persian Gulf, and immediately frozen on board at -20 ºC. The fish were delivered to processing plant (Darya-Khorak Co. Babolsar, Iran) within two weeks. The heads were taken from the frozen fish using an electrical saw, and immediately transferred to the laboratory at Tarbiat Modares University (Noor, Iran). Upon arrival, the tuna heads were minced using an industrial mincer at medium speed (5 mm plate size), pooled and divided into 1 kg portion. Each portion was placed in a plastic container. They were frozen again at -20 ºC within 2 days before analyses and preparation of protein hydrolysate.

Enzyme

Alcalase 2.4 L FG and Protamex are bacterial endopeptidases from strains of Bacillus licheniformis and B. subtilis, as liquid and powder, respectively, provided by Novozymes (Bagsvaerd, Denmark) and stored at 4 ºC until use. All chemical reagents used for the experiments were of analytical grade.

Preparation of fish protein hydrolysate

Minced tuna head were thawed using a refrigerator, followed by cooking at 85 ºC for 20 min in a water bath (W614-B, Fater Rizpardaz, Tehran, Iran) to inactivate the endogenous enzymes (Guerard et al. 2002; Ovissipour et

al. 2009a,b,c; Safari et al. 2009). Prepared sample was mixed with distilled water at a ratio of 1:1 (w:v) and homogenized in a Moulinex® blender for about 2 min at ambient temperature. Alcalase and Protamex were added to the substrate at the levels of 1.5% (v/w) and 1.5% (w/w), respectively, based on the protein content of raw material. All reactions were performed in 250 ml glass Erlenmeyer flask vessels with 50 g of raw material with continuous shaking using a shaking incubator (GTSL20, Jaltajhiz, Tehran, Iran) at a speed of 200 rpm at 55 ºC for 4 and 24 h. No pH control was performed during hydrolysis to avoid salt generated in the hydrolysate and to decrease the cost of process. At the hydrolysis time designated, the reaction was terminated by heating the mixture at 95 ºC for 15 min to inactive the enzymes (Guerard et al. 2002 Ovissipour et al. 2009a,b,c; Safari et al. 2009).

The hydrolysates were then cooled on ice to ambient temperature and centrifuged at 8000 g for 20 min at 10 °C using a Hettich D-7200 centrifuge (Tuttlingen, Germany). The supernatants, containing the fish protein hydrolysates, were then freeze-dried using Operon, FDU-7012 (Gyeonggi-do, South Korea). To monitor the rate of hydrolysis of protein by both enzymes, DH was also determined during the first 4 h.

Chemical composition

Moisture content was determined following AOAC (2002) by placing approximately 2 g of sample into a pre-weighted aluminum dish. Samples were dried in an oven at 105 °C until constant weight was obtained. The total crude protein (N × 6.25) in raw materials and freeze-dried hydrolysates was determined using the Kjeldahl method (AOAC 2002). Total lipid in samples was determined by Soxhlet apparatus (AOAC 2002). Ash content was determined by charring a pre-dried sample in a crucible at 600 °C until constant weight of white ash was obtained (AOAC 2002).

In order to determine the degree of hydrolysis and nitrogen recovery, protein in the soluble fish hydrolysate was measured by the Biuret method (Layne 1957), using bovine serum albumin as a standard protein. Absorbance was measured at 540 nm in a UV/vis spectrophotometer (Jenway, 6305, Staffordshire, UK).

Nitrogen Recovery

Nitrogen recovery was calculated according to Liaset et al. (2002) as follows:

Total nitrogen in hydrolysate / total nitrogen in the minced tuna head × 100.

Degree of hydrolysis

Degree of hydrolysis was estimated according to Hoyle and Merritt (1994). To the supernatant, one volume of 20% trichloroacetic acid (TCA) was added to precipitate the intact protein, followed by centrifugation at 8000 g at 10 °C for 20 min to collect the 10% TCA-soluble materials. The degree of hydrolysis (DH) was computed according to the following equation:

%DH= 100 × (10% TCA-soluble N_2 in the sample/total N_2 in minced tuna head).

Amino acid composition

Samples were hydrolyzed with 6N HCl at 110 °C for 24 h. Derivatisation has been conducted using o-phthaldialdehyde (OPA) prior to HPLC analysis. The total amino acids were analyzed by the Knauer (Germany) HPLC set using C18 column at the flow rate of 1 ml/min with a fluorescence detector (RF-530, Knauer, Germany).

Calculating the chemical score

The chemical score of the protein hydrolysates based on their amino acids composition was computed according to Bhaskar et al. (2008). The ratio of essential amino acids in test proteins (EAA) to requirement patterns as described by FAO/WHO (1990) for adult humans and NRC (1993) for carp was calculated. In brief, the chemical score was calculated using the following equation:

Chemical score = EAA in test protein (g 100/g)/EAA requirement pattern (g 100/g).

Protein efficiency ratio (PER)

PER of tuna head hydrolysates were calculated according to the equations developed by Alsmeyer et al. (1974) and Lee et al. (1978). These equations are presented in Table 3.

Essential Amino Acid Index (EAAI) and Biological Value (BV)

EAAI and BV were computed according to the methods of Oser (1951; 1959), respectively. The following equation was used for BV determination.

BV = 1.09 (EAA Index) – 11.7.

Statistical analysis

The data obtained were subjected to one-way analysis of variance using SPSS statistical software, release 12.0 (SPSS Inc., Chicago, IL, USA). Duncan's new multiple range test was performed to determine the significant differences at 5% level.

Results and discussion

Proximate compositions

Proximate compositions of raw material and liquid protein hydrolysates are shown in Table 1. The protein contents of liquid PHA and PHP with hydrolysis time of 4 h were 76.8 and 72.32%, respectively, while protein contents of 80.2 and 75.4%, respectively, were obtained with the hydrolysis time of 24 h. The protein content of both hydrolysates with the hydrolysis time of 4 and 24 h were within the range reported by others (Shahidi et al. 1995; Kristinsson and Rasco 2000a,b; Bhaskar et al. 2008; Ovissipour et al. 2009a,b,c). Thus the protein content of both protein hydrolysates increased significantly with increasing hydrolysis time. At the same hydrolysis time, the differences in protein content between PHA and PHP were observed ($P < 0.05$). A higher content of soluble peptides was presented in PHA ($P < 0.05$). This result suggested that Alcalase exhibited the higher hydrolytic activity toward proteins in yellowfin tuna head, in comparison with protamex. As a consequence, a greater content of soluble peptides or low molecular weight proteins was presented in PHA. Ovissipour et al. (2009b) found that PHA from Persian sturgeon viscera has higher protein content (73.3%), compared to PHP (71.7%).

Table 1. Chemical composition and nitrogen recovery of protein hydrolysates from Alcalase and Protamex

Parameter (%)[1,2,3]	Tuna head	4h		24h	
		PHA	PHP	PHA	PHP
Protein	20 ± 1.3	76.8 ± 1.32b	72.32 ± 1.72bc	80.2 ± 1.5a	75.4 ± 2.3bc
Fat	5.11 ± 0.45	1.32 ± 0.23b	1.88 ± 0.24a	0.23 ± 0.13c	0.41 ± 0.22c
Moisture	35.7 ± 1.7	10.48 ± 1.42a	12.2 ± 2.6a	7.5 ± 2.3b	6.5 ± 2.8b
Ash	2.1 ± 0.6	9.4 ± 1.74b	11.3 ± 1.4b	13.3 ± 2.4ab	15.8 ± 3.3a
Nitrogen recovery	_	26.9 ± 2.3b	24.3 ± 1.4b	40 ± 0.62a	37.7 ± 1.3a

[1] Values represent percentage of dry matter.
[2] Values represented means ± SE (n=3).
[3] Values in the same row with different superscripts are significantly different (α=0.05).

The lipid content in the raw material was 5%. After hydrolysis, the mixture was centrifuged and most of the lipid was separated as evidenced by the low lipid content in resulting hydrolysate. Lipid contents of PHA and PHP were 1.32 and 1.88% with hydrolysis time of 4 h and were 0.23 and 0.41% after 24 h of hydrolysis. The lowest lipid content was found in PHA with hydrolysis time of 24 h ($P < 0.05$). A separation process following hydrolysis to remove lipids and insoluble materials rendered fish protein hydrolysate with total lipid content less than 0.5% (Shahidi et al. 1995; Kristinsson and Rasco 2000a,b; Ovissipour et al. 2009a). Decreasing lipid content in the hydrolysates might increase stability of the material toward lipid oxidation and might also enhance products stability (Diniz and Martin 1997; Shahidi et al. 1995; Kristinsson and Rasco 2000b). The results of this study revealed that PHA had significantly less lipid content than did PHP ($P < 0.05$). It might be due to the higher proteolytic activity of

Alcalase, compared to Protamex. Kristinsson and Rasco (2000b) assumed that lipids in FPH were centrifuged out with insoluble protein fractions. Many researchers also pointed that Alcalase is the most favored commercial enzyme because of its high proteolytic activity (Shahidi et al. 1995; Kristinsson and Rasco 2000a,b; Aspmo et al. 2005).

Nitrogen recovery, as an index of nitrogen solubilization and hydrolysis yield, increased with increasing hydrolysis time ($P < 0.05$). At the same hydrolysis time, no difference in nitrogen recovery between PHA and PHP was observed ($P > 0.05$). Liaset et al. (2002) reported that nitrogen recovery increased with increasing time.

Degree of hydrolysis

Figure 1 shows the progression of enzymatic hydrolysis of yellowfin tuna head by-products using different enzymes (Alcalase and Protamex) during 4 h. DH increased with increasing hydrolysis time. A rapid increase in DH was observed within the first 2 h, followed by a lower increase in DH. At the same hydrolysis time, PHA possessed the higher DH, compared with PHP ($P < 0.05$). This typical hydrolysis curve has been reported for FPH (Diniz and Martin 1996; Benjakul and Morrissey 1997; Guerard et al. 2002; Ovissipour et al. 2009a,b,c). Guerard et al. (2002) speculated that a reduction in DH increasing rate with increasing hydrolysis time might be due to the limited enzyme activity by the formation of reaction products at high DH. Furthermore, the decrease in hydrolysis rate might also be due to a decrease in the substrate concentration, enzyme inhibition and enzyme deactivation. After 24 h of hydrolysis, PHA and PHP had the DH of 34 and 19%, respectively. Similar results were observed by Batista et al. (2009) who reported higher DH for sardine (*Sardina pilchardus*) hydrolysates prepared using Alcalase in comparison with Protamex.

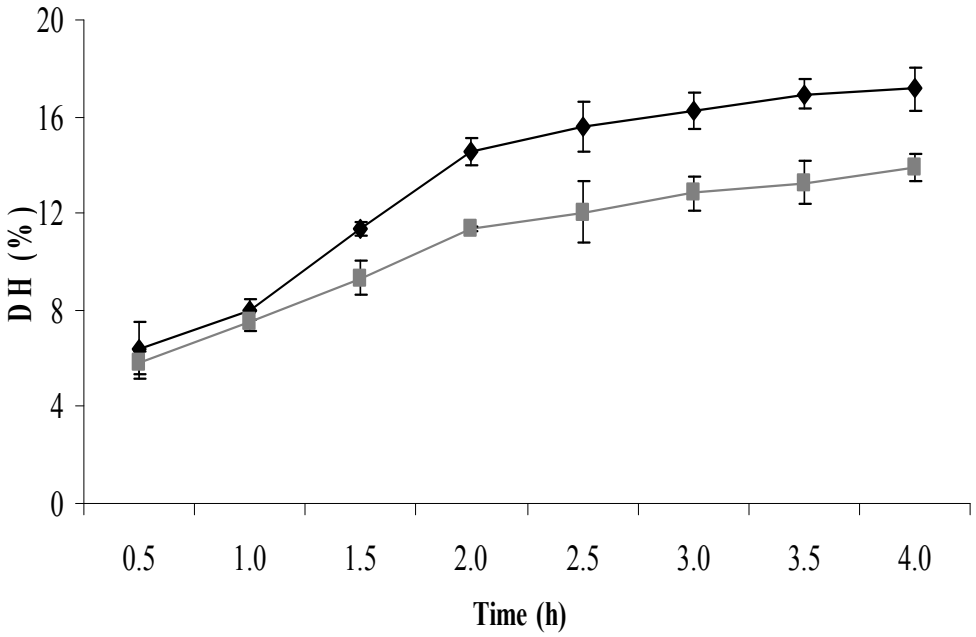

Fig. 1. Hydrolysis curves for tuna by-products protein hydrolysates with Alcalase ♦ and Protamex ■

Amino acid composition

The amino acids composition and chemical score of protein hydrolysates, both PHA and PHP, from yellowfin tuna head are given in Table 2. Chemical score provides an estimate of the nutritive value of a protein by comparing the levels of essential amino acids between the test and standard proteins. In the present study, 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).

The amino acid composition of the current study and comparison with reference proteins revealed that the amino acid profiles of both PHA and PHP were generally higher in essential amino acids, compared with the suggested requirements patterns by FAO/WHO (1990) for adult humans. Similar results were reported by Ovissipour et al. (2009 a,c) for Persian and beluga sturgeons visceral hydrolysates.

The results of chemical scores based on common carp requirements showed that both PHA and PHP had lysine, phenylalanine and methionine as the limiting amino acids, while other amino acids were present at the adequate quantities (Table 2). This result agreed with other findings (Bhaskar et al. 2008; Ovissipour et al. 2009a,c). Bhaskar et al. (2008) reported that phenylalanine and methionine in Catla Alcalase-hydrolysates were the limiting amino acids for common carp.

Based on amino acid composition, both PHA and PHP were shown to be high quality food and feed ingredients. Firstly, the essential amino acids were 51.0 and 59.18% of all amino acids, and the ratio of essential amino acid (EAA) to non-essential amino acids (NEAA) were 1.04 and 1.43 for PHA and PHP, respectively.

Table 2. Amino acid composition and chemical score of protein hydrolysates from yellowfin tuna head prepared using Alcalase and Protamex for 24 h

Amino acid	Amount (g/100g) in Fish protein hydrolysates		Reference protein pattern		PHA		PHP	
	PHA	PHP	1	2	RP1	RP2	RP1	RP2
Histidine[a]	5.25	5.54	1.6	2.1	3.28	2.5	3.46	2.63
Isoleucine[a]	5.54	4.82	1.3	2.5	4.26	2.21	3.7	1.92
Leucine[a]	5.37	5.24	1.9	3.3	2.82	1.62	2.75	1.58
Lysine[a]	2.37	1.86	1.6	5.7	1.48	0.41	1.16	0.32
Methionine[a]	2.66	2.12	1.7	3.1	1.56	0.85	1.24	0.68
Phenyl alanine[a]	3.29	2.3	_	6.5	_	0.50	_	0.35
Tyrosine	2.54	1.67	_	_	_	_	_	_
Threonine[a]	4.48	4.34	0.9	3.9	4.97	1.14	4.8	1.11
Tryptophan[a]	_	_	_	_	_	_	_	_
Arginine[a]	6.83	5.78	_	1.31	_	5.21	_	4.41
Valine[a]	7.14	6.13	1.3	3.6	5.49	1.98	4.71	1.7
Aspartic acid[b]	9.85	9.42	_	_	_	_	_	_
Glysine[b]	6.47	5.37	_	_	_	_	_	_
Alanine[b]	3.06	2.84	_	_	_	_	_	_
Serine	5.87	3.56	_	_	_	_	_	_
Glutamic acid[b]	13.36	3.43	_	_	_	_	_	_
∑AA	84.08	64.42	_	_	_	_	_	_
∑EAA	42.93	38.13	_	_	_	_	_	_
∑FAA	32.74	21.06	_	_	_	_	_	_
∑EAA/∑AA	0.5105	0.5918	_	_	_	_	_	_
∑FAA/∑AA	0.39	0.32	_	_	_	_	_	_
∑EAA/∑NEAA	1.04	1.43	_	_	_	_	_	_

RP1: Chemical score calculated with FAO/WHO (1990) reference protein as the base. RP2: Chemical score calculated with amino acid requirements as per NRC (1993).

[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).

[a] Essential amino acids.

[b] Flavor amino acids.

AA: Amino acid, EAA: Essential amino acids, FAA: Flavor amino acid, NEAA: Non-Essential amino acid.

Both values exceeded the reference values of 40% and 0.6 for human, which are recommended by World Health Organization (WHO)/ Food and Agriculture Organization (FAO) (FAO/WHO 1990). Secondly, the hydrolysates had a high content of the flavor enhancers, glutamic acid, aspartic acid, glycine and alanine (39 and 32% of the total amino acids for PHA and PHP, respectively). Thirdly, the arginine content was high in both hydrolysates. Arginine is one of the most important amino acids participating in protein synthesis and other physiological functions such as detoxification and energy conversion (Cao et al. 2008) and plays an important role in cardiovascular disease treatment (Niittynen et al. 1999). The same results were reported by Cao et al. (2008) who found that protein hydrolysates from shrimp could fulfill human requirements in terms of amino acids and shrimp hydrolysates were rich in arginine.

PER values were 1.7-2.71 for PHA and 1.7-2.82 for PHP (Table 3). These results indicated that PER was not influenced by type of enzyme used for hydrolysis. The results of current study were in agreement with Šližytė et al. (2005) who reported 1.3-2.53 as PER for cod visceral hydrolysates using Flavourzyme and 1.25-2.5 for that using Neutrase.

Table 3. Prediction equation for some of the nutritional indices of protein hydrolysate from yellowfin tuna head head prepared using Alcalase and Protamex for 24 h

PER Equations[a]	PHA	PHP
-0.468 + 0.454 [Leu] -0.104 [Tyr]	1.705	1.73
-1.816 + 0.435 [Met] + 0.780 [Leu] + 0.211 [His] -0.944 [Tyr]	2.39	2.82
0.08084 [X_7] -0.1094	2.38	2.05
0.06320 [X_{10}] -0.1539	2.71	2.36
EAAI[b]	119	106
BV[c]	118	104

[a] X_7 = Thr + Val + Met + Ile + Leu + Phe + Lys
$X_{10} = X_7$ + His + Arg + Tyr
[b] Essential Amino Acids Index
[c] Predicted Biological Value

The results of EAAI and BV are given in Table 3. The results indicated that both nutritional indices were higher for PHA, compared with PHP. Sindayikengera and Xia (2006) showed that whey protein hydrolysates prepared using Protamex had higher nutritional indices in comparison with whey protein.

Conclusion

Yellowfin tuna is one of the most important pelagic species throughout the world for canned products. This may cause vast amounts of by-products having high protein and lipid content. Enzymatic hydrolysis of fish by-product tends to provide fish protein hydrolysates (FPH) with high nutritional values. Both Alcalase and Protamex were effective to produce hydrolysates with appropriate amino acids composition and PER values. Both hydrolysates could fulfill human amino acids requirements and had high potential as protein ingredient for fish feed production.

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