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A Study on the Properties of Alosa (*Alosa caspia*) By-Products Protein Hydrolysates Using Commercial Enzymes

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Abstract: In current study, protein hydrolysates were produced using microbial proteases of Alcalase, Protamex and Flavourzyme from by-products (head, skin and viscera) of Alosa (*Alosa caspia*), a major *Clupeonella* species in the Caspian Sea. The results indicated that the protein hydrolysate from Alcalase, had the highest protein content (78.91%), protein recovery (80.42%) and degree of hydrolysis (21.06%). No significant differences in lipid were observed among the enzymes (P>0.05). The results of amino acids composition showed that all hydrolysates were almost similar in amino acids composition. In addition, the protein efficiency ratio showed that all hydrolysates had high nutritional value. The chemical score results indicated that all hydrolysates could fulfill human amino acid requirements. While, in comparison with common carp requirements, the amino acids of methionine, lysine, phenyl alanine and threonine were the limiting amino acids in hydrolysates. According to the results of this study, Alcalase is the most affective enzyme. The by-products of the Alosa hydrolysate has high nutritional value, which can be used as a protein source in fish feed.

Key words: Alosa · Enzymatic hydrolysis · Commercial enzymes · Fish protein · Nutritional value

INTRODUCTION

There are vast amounts of protein rich byproduct materials from seafood industry discarded without any attempt of recovery [1]. Global fish production has almost stagnated and presently stands at 132 million tons [2]. It is estimated that 25 % of the global fish production is regarded as waste and is discarded [3]. Hydrolysis processes have been developed to convert fish by-products and under-utilized fish into the more saleable and acceptable forms [4, 5]. Enzymatic hydrolysis of fish by-products and under-utilized fish resulted in hydrolysates with better organoleptic, functional and nutritional properties that can be used as food ingredients [4, 6]. Commercial enzymes are used for protein hydrolysates production and these enzymes are preferred in endogenous enzymes since the hydrolysis and the properties of resulting products (i.e. the peptide chain length of the hydrolysates) can be controlled and reproducible [7-9]. Enzymatic hydrolysis is influenced by several factors such as pH, hydrolysis time, enzyme to substrate level and temperature that collectively influence the enzyme activity there with structure the process more controllable [8, 10, 11]. Different enzymes have been used for fish protein hydrolysates production, some of these enzymes are from plant source such as Bromelain and Papain [3, 12, 13], or from animal origin such as chymothrypsine or trypsin [14, 15] and pepsin [10] and or from microbial origin such as Alcalase, Flavourzyme, Protamex, Neutrase [15].

Different commercial enzymes have been studied on different fish substrates such as sardine [5, 16], capelin [17], cuttlefish [16], round scad [17], Atlantic cod [18], catla [19], Persian sturgeon [20, 21], beluga sturgeon [15] and yellowfin tuna [22]. The results of these studies show wide variation and appear to indicate that there is an optimal enzyme for each type of substrate and processing method. According to previous studies, lipid extraction is enhanced by tissues disruption with the different steps of hydrolysis processes and part of the resulting oil can be

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recovered after centrifugation of the hydrolysates [23, 24]. In addition to enhancing oil recovery, hydrolysis produces peptidic fractions that are of nutritional and biological interest [8, 25].

Alosa (*Alosa caspia*) is a pelagic fish in the Caspian Sea in Iran. The purpose of the present study was to study the effect of enzymatic hydrolysis time and different enzymes on the properties of protein hydrolysates from Alosa by-products. Protein content, lipid content, protein recovery, degree of hydrolysate and amino acid composition have been analyzed and discussed.

MATERIALS AND METHODS

Materials: Alosa (*Alosa caspia*) was caught in the south coast of the Caspian Sea in Babolsar, Mazandaran, Iran. The fish were placed on ice and transported to the laboratory in 1h and then by-products (head, skin and viscera) were removed and minced in a Moulinex-blender and kept in plastic bags at -20°C until further studies for 10 days. Prior to the hydrolysis process, the minced by-products were thawed overnight in a refrigerator at 4±1°C.

Enzymes: Alcalase 2.4L FG (2.4 AU/g) is a bacterial endoproteinase from a strain of *Bacillus licheniformis*. Protamex (1.5 AU/g) is a bacterial endoproteinase from a strain *Bacillus subtilis*. Flavourzyme 500 L (1.5 AU/g) is an exopeptidase enzyme from microorganism *Aspergillus oryzae.* They were obtained from Novozymes (Bagsvaerd, Denmark).

Preparation of Fish Protein Hydrolysate: In order to inactivate the endogenous enzymes, the minced samples were heated at 85°C for 20 min [15, 20, 21, 22]. The cooked samples were mixed with distilled water 1:2 (w: v) and homogenized by a homogenizer for about 2 min. The pH of the mixture was adjusted to the optimum activity of Alcalase, pH 8.5, Protamex and Flavourzyme, pH 7, by adding 0.2 N NaOH. Each enzyme was added to sample at enzyme/protein substrate ratio of 1%. All reactions were performed in 250 ml glass vessels, in a shaker water bath at 55°C [15, 19-22]. After 60 minutes each sample heated by 95°C for 20 min to inactivate the commercial enzymes [26]. All hydrolysates were then cooled on ice to room temperature and centrifuged at 6700 rpm at 4°C for 20 min in a Z36 HK (Labnet, Germany) centrifuge, to collect the supernatant as fish protein hydrolysates source.

Chemical Composition: Total crude protein and the total lipid content of samples were determined by the method of AOAC [27]. Protein in the Alosa by-products hydrolysates was measured by the Biuret method in the supernatant following centrifugation [28], using bovine serum albumin as a standard protein. The absorbance was read at 540 nm in a UV/vis spectrophotometer (PG Instrument, T80, England).

Protein recovery (PR) was calculated according to Ovissipour *et al*. [20].

PR (%) = [Total protein in supernatant (%)/Total protein in sample (%)] × 100

Degree of Hydrolysis: The degree of hydrolysis was evaluated as the proportion (%) of α-amino nitrogen with respect to the total N in the sample [29].

Amino Acid Composition: The sample preparation was carried out by hydrolysis with 6 M Hcl at 110°C for 24 hours and derivatized by using phenyl isothiocyanate prior to HPLC analysis. Amino acid compositions were analyzed by the method of Heinrikson and Meredith [30] (Knauer, Berlin, Germany) with silica-base bonded with C_{18} reverse phase 5µm column (3 × 150 mm; Knauer, Berlin, Germany) at 40°C.

Chemical Score: The chemical score of the Alosa by-products protein hydrolysates was computed according to Bhaskar *et al*. [19] and Ovissipour *et al*. [15, 20, 22] considering essential amino acid (EAA) profile in a standard protein as described by FAO/WHO [31]. In this formula the chemical score was calculated using the following relation:

Chemical score = EAA in test protein (g 100 g^{-1})/EAA in standard protein (g 100 g^{-1})

Calculation of Protein Efficiency Ratio Value: Protein efficiency ratio values (PER) for Alosa by-products hydrolysates were calculated according to the equations developed by Alsmeyer *et al*. [32] and Lee *et al*. [33], as modified by Shahidi *et al*. [3]. These equations are given in Table 5.

Statistical Analysis: One-way analyses of variances (ANOVA) were used and mean comparison was performed by Duncan's new multiple range test. Statistical analysis was conducted using the SPSS statistical software, (release 16.0) for Windows (SPSS Inc. Chicago, IL).

RESULTS AND DISCUSSION

Proximate Composition: Proximate composition of raw material and Alosa by-products protein hydrolysate are shown in Table (1). There were significant differences in protein content among the enzymes (P<0.05). The Highest protein content was observed in fish protein hydrolysates (FPH) produced by Alcalase (78.91%) and the lowest protein content was observed in FPH produced by Flavourzyme (72.18%). The protein content in FPH has been reported by others within the range of 63.4% to 92.0% [3, 15, 19, 22, 34, 35, 36]. Ovissipour *et al*. [20] reported that the protein content of protein hydrolysates from Persian sturgeon (*Acipenser persicus*) using Alcalase was 65.82%.

The lipid content in the raw material was 8.26%, but most of the lipid was separated out during enzymatic hydrolysis followed by centrifugation. However, the lipid content of all hydrolysates were greatly reduced when compared to that in the raw material (Table 1) and no significant differences in lipid were observed among the hydrolysates produced by different enzymes (P>0.05). Reduction of lipid content has been reported in the protein hydrolysate from capelin [3], Persian sturgeon [20], beluga sturgeon [15], yellowfin Tuna [22] and grass carp [35]. Decreasing of lipids content in the fish protein hydrolysates might significantly contribute to lipid oxidation, which may enhance the storage stability of the products [3, 8, 34, 35].

Protein Recovery: The results of protein recovery obtained by different enzymes during 60 min are presented in Table (2). There were significant differences in protein recovery among the enzymes (P<0.05). In all hydrolysates protein recovery ranged from 42% to 81% and has been increased with increasing incubation time. The results show that the highest and lowest protein recovery have been observed in Alcalase (80.42%) and Flavourzyme (47.66%), respectively (P<0.05). The same results have been reported by Ovissipour *et al*. [21]. Their findings showed that the highest protein recovery of hydrolysate is related to Alcalase in enzymatic hydrolysis of Persian sturgeon (*Acipenser persicus*) visceral protein.

Degree of Hydrolysis: Figure 1 shows the progressing of hydroliysis of Alosa by-products using different enzymes (Alcalase, Protamex, Flavourzyme) during 60 min. DH (in all hydrolysates) increased with increasing incubation time. In all hydrolysate at a high rate during the initial 15 min and then slowed down thereafter (Fig. 1), which indicated that maximum cleavage of peptides occurred within 15 min of hydrolysis. The same results were observed by others [20, 22, 26, 35, 37].

Table 1: Proximate composition of Alosa raw and hydrolysate by-products[a,b]

Material	Protein	Fat
Raw material	13.53±0.45	8.37±0.75
Alcalase	78.91±1.25[a]	0.97±0.09[a]
Protamex	75.81±1.65[ab]	1.15±0.26[a]
Flavourzyme	72.16±2.57[b]	1.02±0.11[a]

[a]Values represent means ± SE (n = 3)

[b]Values in the same columns with different superscripts have significantly difference (α = 0.05)

Table 2: Protein recovery of Alosa by-products hydrolysates at different reaction time and enzymes[a,b]

	Enzymes		
Hydrolysis time (min)	Alcalase	Protamex	Flavourzyme
15	66.12±0.92[c]	50.12±1.92[b]	42.58±0.79[c]
30	72.42±1.48[b]	52.27±2.01[ab]	45.38±0.92[b]
60	80.42±2.11[a]	56.58±1.16[a]	47.66±0.79[a]

[a]Values represent means ± SE (n = 3)

[b]Values in the same columns with different superscripts have significantly difference (α = 0.05)

Table 3: The amino acid composition of Alosa by-products protein hydrolysates (g 100 g^{-1} protein) (60 min)

Amino acid (g 100 g^{-1})	Alcalase	Protamex	Flavourzyme
Histidine [a]	13.48	12.11	11.78
Isoleucine [a]	4.14	3.35	2.62
Leucine[a] 5.69	5.40	5.17	
Lysine[a] 4.70	4.00	3.75	
Methionine[a]	2.53	2.51	2.09
Phenyl alanine[a]	2.57	2.51	3.02
Tyrosine 1.61	1.78	1.91	
Threonine[a]	3.85	3.58	2.76
Arginine[a] 8.87	8.15	8.46	
Valine[a] 6.59	6.19	5.92	
Aspartic acid	8.36	7.02	7.26
Glycine 5.04	4.92	5.04	
Proline 5.65	5.35	5.32	
Serine 4.84	4.82	4.67	
Hydroxy praline	3.38	2.88	2.42
Cystein 2.10	1.42	0.89	
Glutamic acid	11.73	10.30	9.68
Total amino acid	95.51	86.21	83.17
Essential amino acid/non-essential amino acid	1.22	1.24	1.21
Essential amino acid/total amino acid	54.99	55.38	54.78

[a]Essential amino acids

[b]Histidine + Alanine

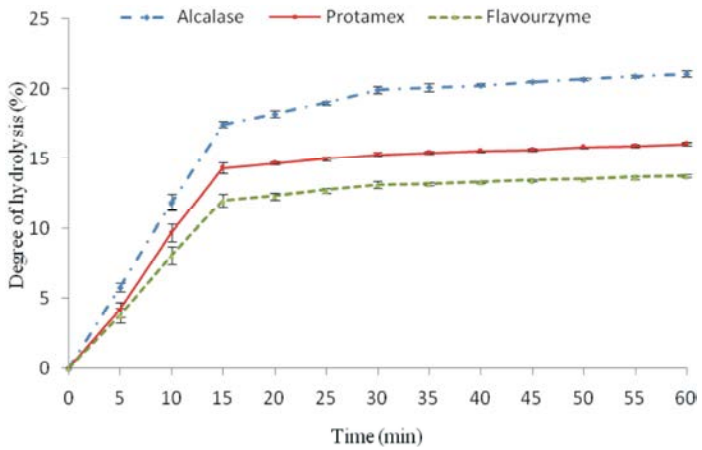

Fig. 1: Hydrolysis plot of Alosa by-products using different enzymes at different time

Table 4: Chemical score, in comparison with FAO/WHO reference protein

Amino acid (g 100 g^{-1})	Reference protein 1[a]	Alcalase	Protamex	Flavourzyme	Reference protein 2[b]	Alcalase	Protamex	Flavourzyme
Histidine	1.6	8.43	7.57	7.36	2.1	6.24	5.77	5.61
Isoleucine	1.3	3.19	2.58	2.02	2.5	1.66	1.34	1.05
Leucine	1.9	3.00	2.84	2.72	3.3	1.73	1.64	1.57
Lysine	1.6	2.94	2.50	2.34	5.7	0.82	0.70	0.66
Methionine	1.7	1.49	1.48	1.23	3.1	0.82	0.81	0.68
Phenyl alanine	-	-	-	-	6.5	0.40	0.39	0.47
Threonine	0.9	4.28	3.98	3.07	3.9	0.99	0.92	0.71
Arginine	-	-	-	-	1.31	6.77	6.22	6.46
Valine	1.3	5.14	4.76	4.55	3.6	1.86	1.72	1.64

[a]Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1990)

[b]Essential amino acid requirements of common carp according to NRC (1993)

Table 5: Calculated protein efficiency ratio (PER) values of Alosa by-products protein hydrolysate

		PER of FPH		
Equation number	Equation	Alcalase	Protamex	Flavourzyme
1	-0.684 + 0.456 [Leu] -0.047 [Pro]	1.53	1.41	1.31
2	-0.468 + 0.454 [Leu]-0.104 [Tyr]	1.95	1.80	1.68
3	-1.816 + 0.435 [Met] + 0.780 [Leu] + 0.211 [His]-0.944 [Tyr]	5.01	4.32	3.76
4	0.08084 [A] -0.1094	2.95	2.68	2.53
5	0.06320 [B]- 0.1539	3.06	2.81	2.70

A = Thr + Val + Met + Ile + Leu + Phe + Lys

B = A+ His + Arg + Tyr

The highest DH was related to Alcalase hydrolysate and the lowest was observed in Flavourzyme. The same results were observed by others [13, 20, 26, 35, 38]. Ovissipour *et al*. [21] used six different enzymes to hydrolyze the Persian sturgeon (*A. persicus*) viscera. Their results showed that the highest and the lowest DH were related to Alcalase and Trypsin, respectively. In addition, they reported that with increasing hydrolysis time, the rate of hydrolysis will be decreased. In current study, significant differences were observed with respect to the DH among the enzymes ($P<0.05$). The DH obtained with Alcalase, Protamex and Flavourzyme were 21.06, 16.08 and 13.76%, respectively.

Amino Acid Composition: The amino acid composition of Alosa by-products protein hydrolysate is given in Table 3. The Alosa by-products protein hydrolysate had an essential amino acid/non-essential amino acid ratio of

1.22, 1.24 and 1.19 for Alcalase, Protamex and Flavourzyme, respectively and had an essential amino acid/total amino acid ratio of 54.99, 55.38 and 54.78 for Alcalase, Protamex and Flavourzyme, respectively (Table 3). According to FAO/WHO [30] for adults, essential amino acid/non-essential amino acid ratio should not be lower than 0.6 and essential amino acid/total amino acid ratio should not be lower than 40%. The highest total amino acid is related to Alcalase (95.51%) and the lowest total amino acid is related to Flavourzyme (83.17%).

Shamloo *et al*. [39] used three different enzymes (Alcalase, Protamex and Flavourzyme) to hydrolyze the red tilapia (*Oreochromis niloticus*) protein. Their results showed that the highest total amino acid is related to Alcalase (81.22%) and the lowest total amino acid is related to Protamex (77.97%).

The Chemical score has been used to evaluate the nutritive value of a protein (Table 4). This parameter contrasts levels of essential amino acids between the test and standard proteins. In this study, the chemical scores computed are based on the reference protein of FAO/WHO [31] for adults and amino acid requirements of juvenile common carp, as listed by NRC [40].

Based on the Alosa by-products protein hydrolysate amino a cid composition and the FAO/WHO [31] and NRC [40] standards, the chemical score results showed that all hydrolysates could fulfill human amino acid requirements. While, in comparison with common carp requirements, the amino acids of methionine, lysine and phenylalanine and threonine were the limiting amino acids in all hydrolysates. Considering this results, in spite of minor deficiencies in certain essential amino acids, fish protein hydrolysate does not lose its nutritional value so can be considered as an ingredient in balanced fish diets [25, 19, 20, 22, 41].

The PER values of the hydrolysates for Alcalase and Protamex and Flavourzyme were 1.53- 5.01, 1.41- 4.32, 1.31- 3.76 respectively (Table 5). PER values of 2.9- 3.14 for shark hydrolysate, 1.97- 2.58 for Cod hydrolysate and 2.85-5.38 for yellowfin Tuna hydrolysate were reported by Diniz and Martin [8] Ovissipour *et al*. [22] and Šližyte *et al*. [41] respectively.

CONCLUSION

In this study the effect of enzymatic hydrolysis time and different enzymes on the properties of protein hydrolysates from Alosa (*A. caspia*) by-products (head, skin and viscera) were investigated. The results showed that hydrolysates which were produced by Alcalase with the longest time (60 min) had the highest DH, protein recovery and protein content. Considering of amino acid compositions, that all hydrolysates can be used for applications in aquaculture/ animal feeds and has a good potential to be an effective nitrogen source in microbial growth media.

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