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The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera

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ABSTRACT

Protein hydrolysate was prepared from the viscera of Persian sturgeon (*Acipenser persicus*), a major sturgeon species in the Caspian Sea. Hydrolysis was performed at three different temperatures (35, 45 and 55 °C), pH 8.5, using commercially available Alcalase® and an enzyme to substrate ratio of 0.1 AU/g viscera protein over a 205 min incubation period. Protein and lipid content of the hydrolysate were 65.82%, and 0.18%, respectively. Protein recovery and degree of hydrolysis ranged from 34.97% to 61.96% and 13.32% to 46.13%, respectively. The highest degree of hydrolysis was observed at 55 °C after 205 min ($p < 0.05$). The amino acid score of the hydrolysates was similar to that of the FAO/WHO reference protein. It is revealed that Persian sturgeon visceral protein hydrolysate amino acid fulfils adult human requirements. Based on National Research Council guidelines, phenylalanine is the first limiting amino acid in the hydrolysate. However, the hydrolysate has the potential for application as an ingredient in formulated diets.

1. Introduction

Much of the protein-rich byproducts from seafood processing plants are discarded without any attempt at recovery. At the same time many processors are no longer allowed to discard their offal directly into the sea, resulting in a very high cost of refining the material before it is discarded. To meet the need of the seafood processing industry, an alternative for discarding these byproducts should be developed (Kristinsson & Rasco, 2000a).

Every year over 132 million tons of fish are harvested, of which 29.5% is converted into fish meal (FAO, 2006). Possibly more than 50% of the remaining fish tissue is considered to be non-edible waste material. With a dramatically increasing world population and a world catch of fish of more than 100 million tons per year, there is obviously an increased need to utilise our sea resources with more intelligence and foresight (Kristinsson & Rasco, 2000a). By applying enzyme technology for protein recovery in fish processing, it may be possible to produce a broad spectrum of food ingredients or industrial products for a wide range of applications. This would utilise both fisheries byproducts, secondary raw materials and, in addition, underutilised species that would otherwise be discarded. Fish viscera, one of the most important byproducts, are a rich source of protein and polyunsaturated lipids but with low storage stability if not frozen or otherwise preserved (Raa, Gildberg, & Strom, 1983).

Enzymatic modification of proteins using selected proteolytic enzyme preparations to cleave specific peptide bonds is widely used in the food industry (Mullally, O'Callaghan, FitzGerald, Donnelly, & Dalton, 1994). The most common commercial proteases reported used for the hydrolysis of fish protein are from plant sources such as papain (Hoyle & Merritt, 1994; Shahidi, Han, & Syniwiecki, 1995) or from animal origin, such as pepsin (Viera, Martin, Sampaiao, Omar, & Gonsalves, 1995), chymotrypsin and trypsin (Simpson, Nayeri, Yaylayan, & Ashie, 1998). Enzymes of microbial origin have been also applied to the hydrolysis of fish proteins. In comparison to animal- or plant-derived enzymes, microbial enzymes offer several advantages, including a wide variety of available catalytic activities, and greater pH and temperature stabilities (Diniz & Martin, 1997). Generally, Alcalase® 2.4 L-assisted reactions have been repeatedly favoured for fish hydrolysis, due to the high degree of hydrolysis that can be achieved in a relatively short time under moderate pH conditions, compared to neutral or acidic enzymes (Aspmo, Horn, & Eijsink, 2005; Benjakul & Morrissey, 1997; Bhaskar, Benila, Radha, & Lalitha, 2008; Hoyle & Merritt, 1994; Kristinsson & Rasco, 2000a, 2000b; Shahidi et al., 1995).

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Hydrolysing protein can also improve intestinal absorption (Kristinsson & Rasco, 2000a), and be used as a source of peptides, such as peptone, for ingredients in microbial growth media (Gildberg, Batista, & Strom, 1989).

The work to date on fish protein hydrolysate (FPH) has been somewhat sporadic, with most research conducted on industrial and animal feed application, and some directed to the potential of using powdered hydrolysates in food formulations, with some fish protein hydrolysates having excellent functional properties (Kristinsson & Rasco, 2000a).

Persian sturgeon, one of the most important sturgeon fishes on the south coast of the Caspian Sea, is caught for both meat and caviar production. Sturgeon processing waste is usually discarded, without any recovery except for the swim bladder. Also the notochord is recovered, and kept as frozen or dried, to use in soups or as a nutritional supplement. Sturgeon viscera is rich in protein, which can be used as animal feed, and its proteins may potentially serve as a human food ingredient. However, there have been no reported attempts studying the effect of enzymatic hydrolysis on sturgeon viscera protein. In this study the effects of time and temperature on the hydrolysis of sturgeon visceral proteins were investigated.

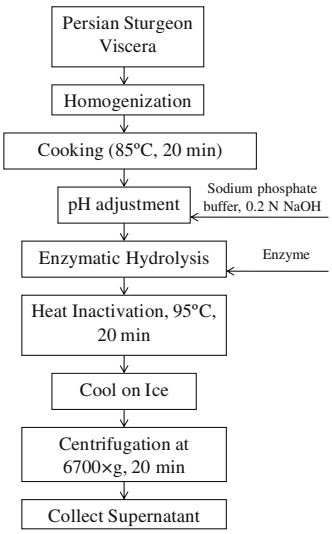

Fig. 1. Enzymatic hydrolysis process flow chart for the preparation of fish protein hydrolysates.

2. Materials and methods

2.1. Materials

Persian sturgeon (*Acipenser persicus*) was caught from the south coast of the Caspian Sea in Sari, Iran. The fish were immediately transferred to the laboratory, where viscera were removed and kept at −20 °C until use. Prior to the hydrolysis process, viscera were thawed overnight in a refrigerator at 4 ± 1 °C.

2.2. Enzymes

Alcalase® (with a declared activity of 2.4 AU/g and a density of 1.18 g/ml) is a bacterial endoproteinase from a strain of *Bacillus licheniformis*. It was provided by the Iranian branch of the Danish company Novozyme, and stored at 4 °C until use. All chemical reagents used for experiments were of analytical grade.

2.3. Preparation of fish protein hydrolysate

The Persian sturgeon protein hydrolysate production scheme is given in Fig. 1. The fish viscera were first minced in a Moulinex® blender, and then heated at 85 °C for 20 min to inactivate endogenous enzymes (Guerard, Guimas, & Binet, 2002). The cooked viscera were mixed with sodium phosphate buffer 1:4 (w:v) and homogenised in a Moulinex® blender for about 2 min at ambient temperature. The pH of the mixture was adjusted to the optimum activity of Alcalase, pH 8.5, by adding 0.2 N NaOH. Alcalase was added to the substrate based on its enzyme activity (0.1 AU/g protein). All reactions were performed in 250 ml glass vessels, in a shaking incubator (Jaltajhiz, Iran) with constant agitation (200 rpm) at three different temperatures: 35, 45 and 55 °C (Bhaskar et al., 2008). After each treatment, the reaction was terminated by heating the solution at 95 °C for 20 min (Guerard et al., 2002), assuring the inactivation of the enzyme. The hydrolysate were then cooled on ice to room temperature and centrifuged at 6700*g* at 10 °C for 20 min in a Hettich D-7200 (Tuttlingen, Germany) centrifuge, to collect the supernatant.

2.4. Chemical composition

Moisture content was determined by placing approximately 2 g of sample into a pre-weighted aluminium dish. Samples were then dried in an oven at 105 °C until a constant weight (AOAC, 2005). The total crude protein ($N \times 6.25$) in raw materials was determined using the Kjeldahl method (AOAC, 2005). Total lipid in sample was determined by Soxhlet extraction (AOAC, 2005). Ash content was estimated by charring in a predried sample in a crucible at 600 °C until a white ash was formed (AOAC, 2005).

Protein in the fish hydrolysates was measured by the Biuret method in the supernatant following centrifugation (Layne, 1957), using bovine serum albumin as a standard protein. Absorbance was measured at 540 nm in a UV/vis spectrophotometer. The Gerber method was applied to measure total lipid content of the hydrolysate (Collares, Gonçalves, & Ferreira, 1997). Protein recovery was calculated as the amount of protein present in the hydrolysate relative to the initial amount of protein present in the reaction mixture.

2.5. Degree of hydrolysis

Degree of hydrolysis was estimated according to the method of Hoyle and Merritt (1994). To the supernatant, one volume of 20% trichloroacetic acid (TCA) was added, followed by centrifugation at 6700*g* at 10 °C for 20 min to collect the 10% TCA-soluble materials. The degree of hydrolysis (DH) was computed as:

$$\%DH = 100 \times (10\%\ \text{TCA} - \text{soluble } N_2 \text{ in the sample/total } N_2 \text{ in the sample})$$

2.6. Amino acid composition

Sample preparation was conducted by hydrolysis with 6 M HCl at 110 °C for 12 h and derivatisation using phenyl isothiocyanate prior to HPLC analysis. The total amino acids and free amino acids were analysed by the Pico Tag method (Waters Corporation, Milford, MA), using a Pico Tag column (3.9 × 150 mm; Waters) at a flow rate of 1 ml min^{-1} with UV detection. Breez® software was applied to data analysis.

2.7. Chemical score

The chemical score of the protein hydrolysate was computed according to Bhaskar et al. (2008), relative to the essential amino acid (EAA) profile in a standard protein as described by FAO/

Table 1
Proximate composition of sturgeon raw material and protein hydrolysates (FPH).[a]

	Protein	Fat	Moisture	Ash
Fish viscera	15.48 ± 0.25	15.68 ± 1.34	39 ± 00	5.76 ± 0.05
FPH	65.82 ± 7.02	0.18 ± 0.4	4.45 ± 0.67	7.67 ± 1.24

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

WHO (1990). In brief, the chemical score was calculated using the following equation:

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

2.8. Calculation of protein efficiency ratio value

Protein efficiency ratio (PER) values for Persian sturgeon hydrolysates were calculated according to the equations developed by Alsmeyer, Cunningham, and Happich (1974) and Lee, Elliot, Rickansrud, and Mugberg (1978), as modified by Shahidi, Naczk, Pegg, and Synowiecki (1991) and Shahidi et al. (1995). These equations are given in Table 4.

2.9. Molecular weight distribution of components in the hydrolysate by SDS-PAGE

SDS-PAGE was performed on all samples using a 4% stacking gel and 15% acrylamide gel, according to Laemmli (1970). Protein concentration of the samples was determined by the Biuret method (Layne, 1957).

The electrophoresis was performed in a water-cooled electrophoresis system. The protein markers used were purchased from Sigma–Aldrich, St. Louis, MO (albumin, bovine 66 kDa; ovalbumin, chicken 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, rabbit 36 kDa; carbonic anhydrase, bovine 29 kDa; trypsinogen, bovine, PMSF-treated 24 kDa; trypsin inhibitor, soybean 20.1 kDa; and α-lactalbumin, bovine 14.2 kDa; aprotinin 8 kDa).

2.10. Statistical analysis

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

3. Results and discussion

3.1. Proximate composition

Proximate composition of raw material and protein hydrolysate are shown in Table 1. The maximum protein content of the hydrolysates was 65.82%, within the range reported by others of 63.4% to 90.8% (Bhaskar et al., 2008; Kristinsson & Rasco, 2000a, 2000b; Onodenalore & Shahidi, 1996; Shahidi et al., 1995).

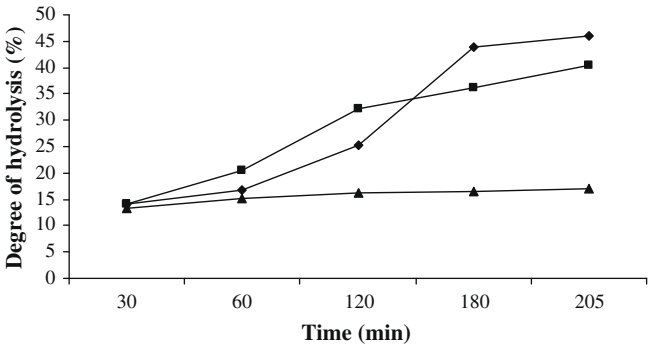

Fig. 2. Hydrolysis curves for Persian sturgeon visceral protein hydrolysate with Alcalase® 2.4 L at temperatures of 35 (▲), 45 (■) and 55 (♦) °C.

The lipid content in the raw material was relatively high and close to 16%, but in this study most of the lipid was separated out during centrifugation to give a final content of 0.18%. Some other researchers who have used a separation process following fermentation to remove lipids and insoluble materials for FPH production (Kristinsson & Rasco, 2000b) reported values under 0.5% for total lipid content (Kristinsson & Rasco, 2000a, 2000b; Shahidi et al., 1995). Decreasing lipid content in the protein hydrolysates might significantly increase stability of the material towards lipid oxidation, which may also enhance the product stability (Diniz & Martin, 1997; Kristinsson & Rasco, 2000b; Shahidi et al., 1995).

The protein recovery ranged from 40% to 62%. The protein recovery increased as the hydrolysis progressed and was higher at the higher reaction temperatures (Table 2). Protein recovery was 35% to 38%, 46% to 61% and 56% to 62% for hydrolysis at 35 °C, 45 °C and 55 °C, respectively. Significant differences in protein recovery were observed at different temperatures ($p < 0.05$).

3.2. The effect of time and temperature on degree of hydrolysis

Fig. 2 shows the progression of hydrolysis of Persian sturgeon viscera using Alcalase® 2.4 L. The DH% increased with increasing in incubation time. Hydrolysis was nearly completed after 205 min at 55 °C. The rate of hydrolysis was slow at 35 °C. The same results observed by others (Kristinsson & Rasco, 2000b), using Atlantic salmon, and (Guerard, Duffose, De La Broise, and Binet, 2001), using yellowfin tuna. Guerard et al. (2002) speculated that a reduction in the reaction rate may be due to the limitation of the enzyme activity by formation of reaction products at high degrees of hydrolysis. However, decrease in hydrolysis rate may also be due to a decrease in the concentration of peptide bonds available for hydrolysis, enzyme inhibition and enzyme deactivation (Guerard et al., 2002).

The results of this study revealed that, the degree of hydrolysis will increase at increasing hydrolysis temperature. Significant differences were observed with respect to temperature ($p < 0.05$). The highest DH%, 46.13, was achieved at 55 °C after 205 min. Shahidi et al. (1995) reported a higher degree of hydrolysis at higher

Table 2
Protein recovery of sturgeon viscera hydrolysates at different reaction times and temperatures.[a,b]

Temperature (°C)	Hydrolysis time (min)				
	30	60	120	180	205
35	34.97 ± 0.56[c]	42.77 ± 1.08[c]	37.07 ± 0.96[c]	37.15 ± 0.57[c]	38.38 ± 0.15[c]
45	45.59 ± 0.7[b]	48.02 ± 0.24[b]	54.53 ± 0.18[b]	48.96 ± 0.47[b]	60.51 ± 0.75[b]
55	56.37 ± 0.26[a]	58.29 ± 0.46[a]	59.14 ± 0.19[a]	60.42 ± 1.13[a]	61.96 ± 0.20[a]

[a] Values represent means ± SE (n = 3).
[b] Values in same columns with different superscripts are significantly different at α = 0.05.

Table 3
The amino acid composition of Persian sturgeon visceral protein hydrolysate (g 100 g^{-1}) (55 °C, 205 min) and chemical score, in comparison with FAO/WHO reference protein.

Amino acid	Quantity (g 100 g^{-1})				Chemical score	
	FAA[a]	FPH	Reference Protein 1[b]	Reference Protein 2[c]	RP-1	RP-2
Histidine	1.65	2.08	1.6	2.1	1.3	0.99
Isoleucine	1.02	3.8	1.3	2.5	2.92	1.52
Leucine	2.35	7.13	1.9	3.3	3.75	2.16
Lysine	1.34	6.8	1.6	5.7	4.25	1.19
Methionine[d]	7.7	10.3	1.7[b]	3.1	6.05	3.32
Phenylalanine	0.78	3.14		6.5		0.48
Tyrosine	0.77	2.34				
Threonine	1.64	3.5	0.9	3.9	3.8	0.89
Tryptophan	_	_				
Arginine	6.78	7.28		1.31		5.6
Valine	2.28	5.79	1.3	3.6	4.45	1.6
Aspartic acid	0.58	8.3				
Glycine	1.43	5.4				
Alanine	2.6	6.3				
Proline	1.23	3.46				
Serine	1.07	4.2				
Glutamic acid	0.65	13.7				

RP1: Chemical score calculated with FAO/WHO reference protein as the base. RP2: Chemical score calculated with amino acid requirements as per NRC (1993).
[a] Free amino acids.
[b] Suggested profile of essential amino acid requirements for adults (FAO/WHO, 1990).
[c] Essential amino acid requirements of common carp according to NRC (1993).
[d] Methionine + cysteine.

temperature. They found that hydrolysis of capelin proteins at 65 °C using Alcalase® results in approximately 22% DH. Bhaskar et al. (2008) also observed higher degrees of hydrolysis at elevated temperatures.

3.3. SDS-PAGE

The electrophoresis pattern of the Persian sturgeon hydrolysate showed a high degree of hydrolysis with Alcalase®, with no peptides size bigger than 10 kDa. The same results were observed by Benjakul and Morrissey (1997) and Kristinsson and Rasco (2000b), with an average chain length being under 10 amino acids long (Dong, Fairgrieve, Skonberg, & Rasco, 1993). The molecular weight distribution and average peptide size is affected by enzyme specificity (Kristinsson & Rasco, 2000b).

3.4. Amino acid Composition

The amino acid composition of Persian sturgeon visceral protein hydrolysate and chemical score are given in Table 3. Chemical score provides an estimate of the nutritive value of a protein. This parameter compares levels of essential amino acids between the test and standard proteins. In the current study, chemical scores computed are based on the reference protein of FAO/WHO (1990) for adults, and amino acid requirements of juvenile common carp, as listed by NRC (1993). The amino acid composition in this study and comparison with reference proteins revealed that the amino acid profiles of the Persian sturgeon viscera hydrolysates were generally higher in essential amino acids, compared with the suggested pattern of requirement by FAO/WHO for adult humans.

The results of the common carp chemical score revealed that phenylalanine is the most limiting amino acid, while all other amino acids (except threonine and histidine that are in almost the same quantity as that of the requirement of juvenile common carp) are present in adequate or excess quantities (Table 3). Based on the Persian sturgeon visceral protein hydrolysate amino acid composition, and the FAO/WHO (1990) and NRC (1993) standards, the hydrolysates fulfil both human and common carp requirements. Further, for many fish species, growth rates produced by diets with large amounts of free amino acids are inferior to diets of similar amino acid composition in which the nitrogen component is protein (Walton, Cowey, Coloso, & Adron, 1986). Based on the results, in spite of minor deficiencies in certain essential amino acids, the protein hydrolysate does not lose its nutritional value so can be considered as an ingredient in balanced fish diets.

The PER values of the hydrolysates were 2.4–6.45 (Table 4). PER values of 2.86–3.24 for cod hydrolysates and 2.61–3.11 for capelin hydrolysates were reported by Shahidi et al. (1991, 1995), respectively.

Table 4
Calculated protein efficiency ratio (PER) values of Persian sturgeon visceral protein hydrolysate.

Equation number	Equation[a]	PER of Persian sturgeon visceral hydrolysate
1	−0.684 + 0.456 [Leu] −0.047 [Pro]	2.405
2	−0.468 + 0.454 [Leu] −0.104 [Tyr]	2.525
3	−1.816 + 0.435 [Met] + 0.780 [Leu] + 0.211 [His] −0.944 [Tyr]	6.45
4	0.08084 [ΣAA7] −0.1094	3.16
5	0.06320 [ΣAA10] −0.1539	3.14

[a] AA_7 = Thr + Val + Met + Ile + Leu + Phe + Lys. $AA_{10} = AA_7$ + His + Arg + Tyr.

4. Conclusion

There is no attempt to produce fish protein hydrolysates in Iran, despite vast fisheries byproducts, with an annual amount of 145,000 tons.

When hydrolysing Persian sturgeon visceral protein using commercial enzyme (Alcalase®), it is noticeable that DH% of FPH is affected by time and temperature. The results suggested that, in order to obtain a high degree of hydrolysis, this process should be performed at higher temperatures and for longer times. The hydrolysates showed good protein recovery up to 61% which increased by increasing DH%. Hydrolysates from Persian sturgeon visceral protein may potentially serve as a good source of desirable peptides and amino acids. The results of this study revealed that there is a good potential in Iran for food and feed grade protein hydrolysates production for various uses.

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