Int Aquat Res (2011) 3: 93-99
ISSN 2008-4935
Available online at www.intelaquares.com
Special Issue of 'Sturgeon Fishes and their Future' Conference, Ukraine (2011)

International Aquatic Research

Optimization of enzymatic hydrolysis of visceral waste proteins of beluga *Huso huso* using Protamex

Abbas Esmaeili Molla[1*], Hrachya G. Hovannisyan[2]

[1]*Shahid Rajaee Sturgeon Fish Farm, P.O.Box: 833, Sari, Iran*
[2]*Yerevan State Medical University, Yerevan, Armenia*

Abstract

Fish protein hydrolysate (FPH) was produced from the viscera of beluga (*Huso huso*). Hydrolysis conditions (enzyme activity, temperature, and time) were optimized using response surface methodology (RSM). Lack-of-fit test revealed a non-significant value for the model, indicating that the regression equation was adequate for predicting the degree of hydrolysis under any combination of the variables ($P < 0.05$). The optimum conditions to reach the highest degree of hydrolysis were: 39.21 °C, 114.2 min, and a protease (Protamex) activity of 27.41 AU/kg protein. The amino acid composition of protein hydrolysates of beluga in optimum conditions showed a high nutritional value of protein hydrolysates from beluga viscera.

Keywords: Fish protein hydrolysates, Beluga visceral protein, Protamex, Optimization, RSM

Introduction

World fish production has almost stagnated and presently stands at 143 mmt (FAO 2008). Fish sources once appeared to be inexhaustible, and by-products arising out of fish processing were looked as worthless materials discarded without an attempt of recovery (Kristinsson and Rasco 2000a).

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 utilize our sea resources with more intelligence and foresight (Kristinsson and Rasco 2000a; Ovissipour et al. 2009a). By applying enzyme technology for protein recovery in fish processing, it may be possible to produce a broad spectrum of food ingredients and improve and upgrade the functional and nutritional properties of protein. This would utilize both fisheries byproducts, secondary raw materials, and in addition, underutilized species that would otherwise be discarded or processed to low price (non-value added products). Fish viscera, one of the most important byproducts, are a rich source of protein and lipids but with low storage stability if not frozen or otherwise preserved.

In the process of hydrolyzation, proteolytic enzymes are used to solubilize the fish protein, resulting in two distinguishable fractions, soluble and insoluble. The insoluble fraction may be used as animal feed (Kristinsson and Rasco 2000a), and the soluble fraction, which contains the hydrolyzed protein, may be converted into a food ingredient, incorporated into food systems, or used as a nitrogen source for bacterial growth. Dehydration of the soluble hydrolysate results in a more stable powder, high in protein content. Such a product is known as fish protein

* Corresponding author. Email: esmaeili45@yahoo.com. Tel: +989113520759.

hydrolysate (FPH). Produced under controlled proteolysis, FPH possesses desirable functional properties, and high nutritional value (Kristinsson and Rasco 2000a). The variables with the most important roles in this complex enzymatic reaction have been reported to be enzyme concentration, protease specificity of the enzyme, pH, temperature of the reaction, the nature of the protein substrate, and the degree of hydrolysis attained (Adler-Nissen 1986).

Beluga visceral protein has been used as a substrate for enzymatic hydrolysis by Alcalase (Ovissipour et al. 2009c). In addition, Protamex assisted reactions have been repeatedly favored 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 the neutral or acidic enzymes (Kristinsson and Rasco 2000a, b; Hoyle and Merritt 1994; Shahidi et al. 1995; Aspmo et al. 2005; Bhaskar et al. 2008; Ovissipour et al. 2009b).

RSM is a useful technique for the investigation of complex processes. It has been successfully applied to optimize seafood processing operations (Shahidi et al. 1995; Diniz and Martin 1997; Bhaskar et al. 2008). RSM defines the effect of the independent variables alone, and in combinations, in the process.

In addition to analyzing the effects of variables, this experimental methodology generates a mathematical model that accurately describes the overall process using a significant estimation. Beluga (*Huso huso*) is one of the most important bentic species in Iran with an annual catch of 2700 metric tons (Iranian Fisheries Organization). The objective of this study was to optimize reaction conditions (i.e. enzyme activity, temperature and time) to obtain optimal degree of hydrolysis from visceral waste proteins of beluga viscera using Protamex.

Materials and methods

Beluga (*Huso huso*) caught in Shahid Rajaee Sturgeon Fish Farm, North of Iran, was immediately frozen on board at -20 ºC. The fish was delivered to the processing plant (Darya-Khorak Co., Babolsar, Iran) within 2 weeks at -20 ºC. The viscera were removed while frozen, using an electric saw, and immediately (1 h) transferred to the laboratory. Once received in the laboratory, fish viscera were minced twice using an industrial mixer at medium speed (5 mm plate size) then pooled, and divided into plastic containers.

All raw materials were frozen again at -20 ºC until analysis. Compositional analyzing experiments were conducted within 2 days after mince freezing. Protamex is a bacterial endoproteinase from a strain of Bacillus subtilis with a proteolytic activity of 1.5 Anson unit/g, with activity temperature ranges of 35 to 70 °C (Novozymes, 2003). It was provided from the Iranian branch of the Danish company Novozymes (Novozymes, Tehran, Iran), and stored at 4 °C until used. All chemical reagents used for the experiment were of analytical grade.

Preparation of fish protein hydrolysate

Preparation of beluga viscera hydrolysates was performed according to our previous study (Ovissipour et al. 2009a, b). Briefly, the fish viscera were first minced twice using an industrial mixer at medium speed (5 mm plate size), then for each run, a 50 g sample was strewn into the 250 ml glass vessel (Erlenmeyer flask) and cooked at 85 °C in a water bath (W614-B, Fater Rizpardaz, Tehran, Iran) for 20 min to inactivate endogenous enzymes (Guerard et al. 2002; Ovissipour et al. 2009a, b). Heat inactivation was performed due to RSM study. In optimization studies, endogenous enzymes are usually deactivated in order to investigate the effect of commercial enzymes. The cooked viscera were mixed with sodium phosphate buffer 1:2 (w:v), and homogenized in a Moulinex® blender for about 2 min. The pH of the mixture was adjusted to the optimum activity of Protamex, pH 8.5 by adding 0.2 N NaOH. Enzyme was added according to the experimental runs (Table 1A, B). All reactions were performed in a shaking incubator (Ivymen System, Comecta, Spain) with constant agitation (200 rpm). After each sampling, reactions were terminated by heating the solution to 95 °C for 15 min (Guerard et al. 2002; Ovissipour et al. 2009a, b), assuring enzyme inactivation. The hydrolysates were cooled on ice and centrifuged at 8000 g at 10 °C for 20 min in Hermle labortechnik GmbH z 206A (Germany) centrifuge, to collect the supernatant. Finally, the soluble phase was spray-dried (inlet air t=170 °C, outlet air t= 80 °C).

Proximate Composition

Moisture content of whole viscera was determined by placing approximately 2 g of sample into a pre-weighted aluminum dish. Samples were then dried in an oven at 105 °C overnight or to constant weight (AOAC 2002). Total crude protein (N × 6.25) in raw materials and FPH was determined using the Kjeldahl method (AOAC 2002). Total

lipid in samples was determined by Soxhlet extraction (AOAC 2002). Ash content was also estimated by charring a pre-dried sample in a crucible at 600 °C until a white ash was formed (AOAC 2002). Protein in the supernatant was measured, following centrifugation, by the Biuret method (Layne 1957). Bovine serum albumin was used as a standard protein to determine the standard curve. Absorbance was measured at 540 nm in a UV/vis spectrophotometer (Jenway, 6305, UK).

Optimization of experiments

RSM with a completely randomized factorial design, was applied to optimize hydrolysis conditions. Different experimental treatments are summarized in Table 1A. Three independent variables namely enzyme activity (X_1, Anson Unit/kg Protein), temperature (X_2, °C), and time (X_3, minute) were employed at five levels (-α, -1, 0, +1, and +α). Experimental planning was based on a preliminary study of enzymatic hydrolysis suggested by enzyme manufacturer (Novozymes, Bagsvaerd, Denmark) and our previous studies (Ovissipour et al. 2009a, b). In addition, we studied two different RSM experiments with different independent variables ranges, which at least current study RSM has been selected (Table 1A). Degree of hydrolysis was measured as a response of the independent variables given in Table 1B. The behavior of the system was explained by the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

Where, Y is the dependent variable (degree of hydrolysis in real value), $ß_0$ is constant, $ß_i$, $ß_{ii}$ and $ß_{ij}$ are coefficients estimated by the model. X_i and X_j are levels of the independent variables which represent the linear, quadratic, and cross-product effects of the X_1, X_2 and X_3 on the response (DH), respectively. The model evaluated the effect of each independent variable to the response.

In this study, we used five different enzyme to substrate ratios based on enzyme activity (Anson Unit) (Table 1A). One Anson unit (AU) is defined as the amount of enzyme that will release 1.0 mEq of tyrosine from urea–denatured hemoglobin per minute at 25 °C at the pH of 7.5 (Aspmo et al. 2005).

Degree of hydrolysis

Degree of hydrolysis was estimated according to Hoyle and Merritt (1994) as described previously by Ovissipour et al. (2009a, b). This method is based on the enzyme deactivation by lowering the pH. Each run after the specified hydrolysis was terminated by the addition of 20% trichloroacetic acid (TCA) followed by centrifugation to collect the 10% TCA soluble material as the supernatant. Then, degree of hydrolysis was computed as:
%DH = (10% TCA soluble N in the sample/total N in the sample) × 100.

Amino acid analysis

Sample preparation was conducted by hydrolysis of protein with 6 N HCl at 110 °C for 24 h. Derivatisation was applied using o-phthaldialdehyde (OPA) prior to HPLC analysis (Antoine et al. 1999). The total amino acids were analyzed by the Knauer (Germany) HPLC set using Spherical type column (Knauer, Germany) at the flow rate of 1 ml/min with fluorescence detector (RF-530, Knauer, Germany).

Statistical analysis

The optimization experiments were carried out through response surface method (RSM) by generating the factorial design (3 factors, 3 levels, and single block) generated using experimental design model of Statistical Analysis System: SAS software release 7 (SAS Institute, Cary, NC, USA) and Design-Expert® software 6.0.6 (MN, USA). Significance was determined at a 95% probability level.

Table 1-A. Independent variables, their coded, and actual levels used in experiment

Factor	Levels				
	-α[1]	-1	0	+1	+α
Temperature (°C) (X_1)	33	38	45.5	53	58
Time (min) (X_2)	53	80	120	160	187
Enzyme activity (AU/kg protein) (X_3)	14	22	34	46	55

[1] α= 1.68

Table 1-B. Experimental design for modeling DH, using RSM

Run No. #	Coded levels of variable		
	X1	X2	X3
1	1	1	1
2	1	1	-1
3	1	-1	1
4	1	-1	-1
5	-1	1	1
6	-1	1	-1
7	-1	-1	1
8	-1	-1	-1
9	0	1.68	0
10	0	-1.68	0
11	0	0	1.68
12	0	0	-1.68
13	1.68	0	0
14	-1.68	0	0
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

X_1: temperature, X_2: time, X_3: enzyme activity

Table 2. ANOVA table of DH affected by enzyme activity, temperature, and time during optimization experiments

Source	Sum of Squares	df[1]	Mean of Squares	F-value	*P*-value
Model	280.99	9	31.22	45.19	< 0.0001
X_1	5.25	1	5.25	7.59	0.0203
X_2	98.28	1	98.28	142.25	< 0.0001
X_3	119.22	1	119.22	172.55	< 0.0001
X_1^2	12.97	1	12.97	18.78	0.0015
X_2^2	19.42	1	19.42	28.11	0.0003
X_3^2	2.32	1	2.32	3.35	0.0971
X_1X_2	5.95	1	5.95	8.61	0.0149
X_1X_3	21.45	1	21.45	31.05	0.0002
X_2X_3	0.66	1	0.66	0.96	0.3510
Residual	6.91	10	0.69	-	-
Lack of fit	5.72	5	1.14	4.79	0.0553
Pure error	1.19	5	0.24	-	-

[1]df: Degree of freedom

Results and discussion

The influence of X1, X2, and X3 on the hydrolysis by Protamex was determined using factorial design as mentioned in the previous section. The best explanatory model equation for the DH value obtained from Protamex hydrolysis coded data is described in this equal:

$$Y=34.22+(0.62\times x_1)+(2.68\times x_2)+(2.95\times x_3)-(0.95\times x_1^2)-(1.16\times x_2^2)-(0.4\times x_3^2)-(0.86x_1x_2)-(1.64\times x_1x_3)-(0.29\times x_2x_3)$$

According to the related ANOVA table (Table 2), the linear, quadritic and cross-product terms were significant ($P < 0.05$) except quadritic term of enzyme activity and cross-product of time and enzyme activity ($P > 0.05$). Statistical analysis also indicated that within each term, all three hydrolysis factors had a strong and significant influence on DH ($P < 0.05$). In fact, Adler-Nissen (1986), investigating the hydrolysis of soy protein by bacterial proteases, pointed out the hydrolyzing conditions markedly influenced the peptide bond cleavage in the protein substrate. The same results were observed by other researchers (Diniz and Martin 1997; Bhaskar et al. 2008; Ovissipour et al. 2010a, 2011).

The results model showed that all linear and quadratic terms contributed to the responses which are in agreement with Diniz and Martin (1997). The adjusted coefficient of determination (r^2) implies that 97% of the behavior variation could be explained by the fitted model. Moreover, a lack of fit test, which indicates the fitness of the model obtained, was not significant, indicating that the model is sufficiently accurate for predicting the degree of hydrolysis for any combination of experimental independent variables.

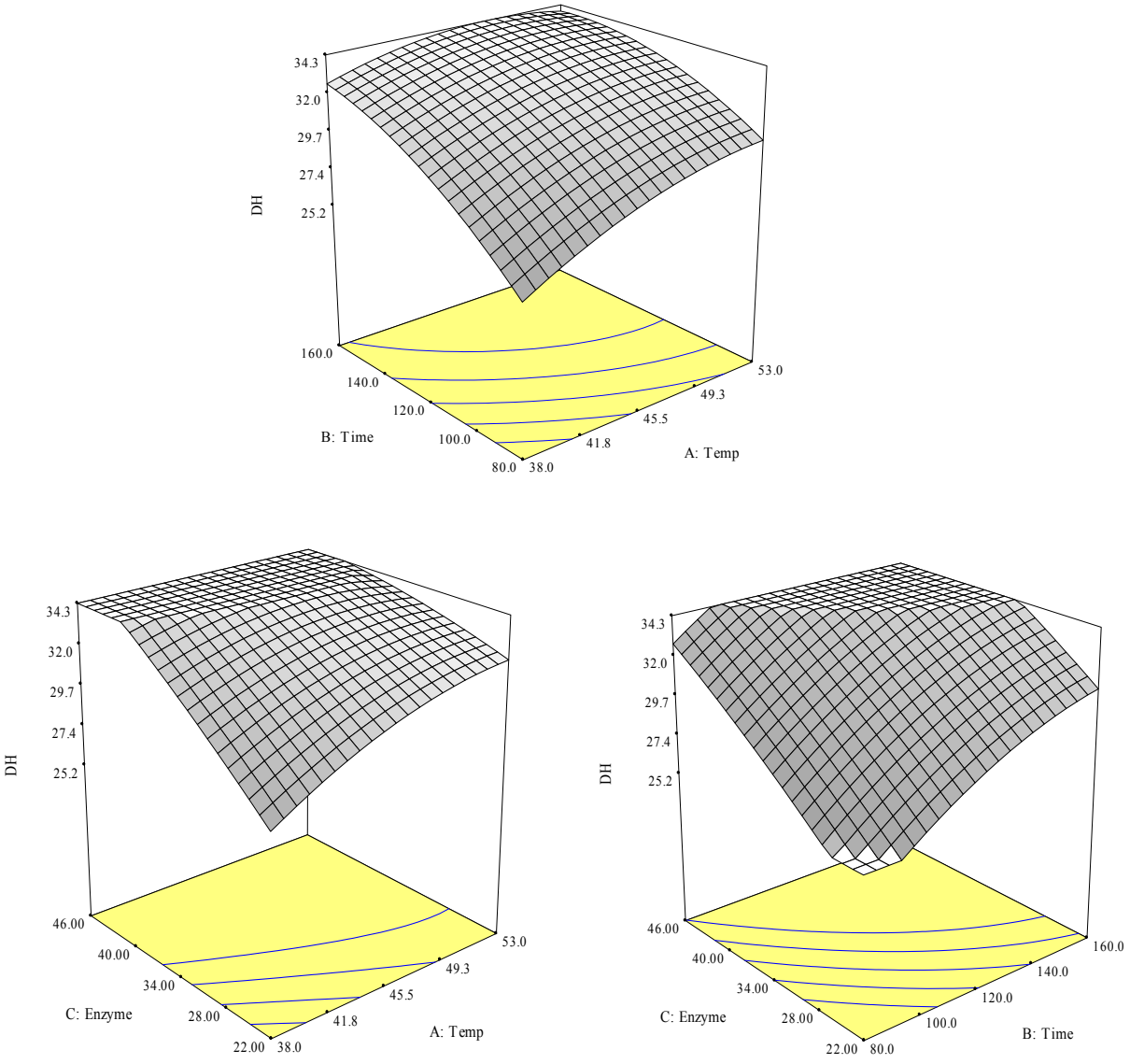

Fig. 1. Response surfaces for the effect of variables on DH as a function of different hydrolyzing condition

The combined contour plots and response surface graphs were generated by the predictive model to predict the critical points and the effectiveness of each factor. The combined effects of each pair of variables indicate that in the hydrolysis of beluga protein, an increase in DH is achieved by increases in temperature, time, and enzyme activity reaction (Fig. 1), up to certain levels; DH slightly decreases beyond those certain criteria. Such a decrease in hydrolysis rate over higher enzyme activity values, temperatures, and time may be due to the reduction in enzyme activity (Guerard et al. 2002; Ovissipour et al. 2009a). Similar dependence between enzyme activity, temperature, and reaction time have been observed for hydrolytic reactions of food proteins using enzymes of microbial origin (Shahidi et al. 1995; Diniz and Martin 1997; Bhaskar et al. 2008).

A Decrease in DH% by increasing time has been reported by many researchers (Guerard et al. 2002; Ovissipour et al. 2009a, b). Guerard et al. (2002) pointed that reduction in DH% by increasing time may be due to the limitation of enzyme activity by formation of reaction products at high degree of hydrolysis, decrease in concentration of peptide bonds available for hydrolysis, enzyme inhabitation, and enzyme deactivation. The optimum conditions to reach the highest degree of hydrolysis were: 39.21 °C, 114.2 min, and a protease (Protamex) activity of 27.41 AU/kg protein.

Amino acids composition of hydrolysate

Amino acids composition of the beluga visceral protein hydrolysates at optimum conditions are presented in Table 3. The amino acids composition revealed that, despite some decreasing in amino acids, protein hydrolysates from beluga is a nutrient source of amino acids.

Table 3. The amino acid composition of beluga sturgeon visceral protein hydrolysate (g/100g)

Amino acid	Quantity
Histidine	1.87
Isoleucine	4.53
Leucine	7.45
Lysine	8.13
Methionine	9.17
Phenyl alanine	2.67
Tyrosine	3.56
Threonine	4.12
Tryptophan	_
Arginine	7.19
Valine	3.52
Aspartic acid	9.45
Glysine	5.86
Alanine	6.53
Proline	3.16
Serine	3.42
Glutamic acid	11.65

Conclusions

Beluga (*H. huso*) is one of the most important commercial species in Iran. Hydrolysis of beluga visceral waste protein using Protamex resulted in DH values of more than 30%. The DH is significantly influenced by enzyme activity, reaction time and temperature. RSM used for optimization of hydrolysis conditions, resulted in a temperature of 39.21 °C, for 114.2 min and on enzyme activity of 27.41 AU/kg protein. The beluga hydrolysate has high potential for applications in aquaculture, and animal feeds. It is also an effective nitrogen source (as peptone) for microbial growth media.

References

Antoine FR, Wei CI, Littell RC, Marshall MR. 1999. HPLC method for analysis of free amino acids in fish using o-Phthaldialdehyde precolumn derivatization. J Agri Food Chem 47: 5100-5107.

AOAC 2002. In: Hortwitz, W. (ed) Official Methods of Analysis of AOAC International, 17th ed. Gaithersburg: AOAC International.

Aspmo SI, Horn SJ, Eijsink VGH. 2005. Enzymatic hydrolysis of Atlantic cod (*Gadus morhua* L.) viscera. Process Biochem 40: 1957-1966.

Bhaskar N, Benila T, Radha C, Lalitha, RG. 2008. Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for preparing protein hydrolysate using a commercial protease. Bioresource Technol 99(2): 335-343.

Diniz AM, Martin AM. 1997. Optimization of nitrogen recovery in the enzymatic hydrolysis of dogfish (*Squalus acanthias*) protein: Composition of the hydrolysates. Int J Food Sci Nutr 48: 191-200.

FAO 2008. Food and Agricultural Organisation of the United Nations. Year book of fishery statistics. Rome, 98, 1&2.

Guerard F, Guimas L, Binet A. 2002. Production of tuna waste hydrolysates by a commercial neutral protease preparation. J Mol Catalysis B: Enzymatic 19-20: 489-498.

Hoyle NT, Merritt JH. 1994. Quality of fish protein hydrolysate from herring (*Clupea harengus*). J Food Sci 59: 76-79.

Kristinsson HG, Rasco BA. 2000a. Fish protein hydrolysates: Production, biochemical and functional properties. Crit Rev Food Sci Nutr 40: 43–81.

Kristinsson HG, Rasco BA. 2000b. Biochemical and functional properties of Atlantic salmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. J Agri Food Chem 48: 657-666.

Layne E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods in Enzymology, 3: p. 450. New York: Academic Press.

Liaset B, Nortvedt R, Lied E, Espe M. 2002. Studies on the nitrogen recovery in enzymatic hydrolysis of Atlantic salmon (*Salmo salar*, L.) frames by Protamex™ protease. Process Biochem 37: 1263-1269.

Novozymes 2003. Protamex. Product data sheet. www.novozymes.com.

Ovissipour M, Abedian AM, Motamedzadegan A, Rasco B, Safari R, Shahiri H. 2009a. The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from the Persian sturgeon (*Acipenser persicus*) viscera. Food Chem 115: 238-242.

Ovissipour M, Safari R, Motamedzadegan A, Shabanpour B. 2009b. Chemical and biochemical hydrolysis of Persian sturgeon (*Acipenser persicus*) visceral protein. Food Bioprocess Technol, DOI 10.1007/ s11947-009-0284-x.

Ovissipour M, Taghiof M, Motamedzadegan A, Rasco B, Esmaeili Mulla A. 2009c. Optimization of enzymatic hydrolysis of visceral waste proteins of beluga sturgeons *Huso huso* using Alcalase. Int Aquat Res 1: 31-38.

Ovissipour M, Safari R, Motamedzadegan A, Rasco B, Pourgholam,R, Mohagheghi E, Esmaeili Mulla A. 2009d. Use of hydrolysates from Yellowfin tuna *Thunnus albacares* fisheries by-products as a nitrogen source for bacteria growth media. Int Aquat Res 1: 73-77.

Ovissipour M, Abedian AM, Motamedzadegan A, Nazari RM. 2010a. Optimization of enzymatic hydrolysis of visceral waste proteins of Yellowfin tuna (*Thunnus albacares*). Food Bioprocess Technol, DOI 10.1007/s11947-010-0357-x.

Ovissipour M, Benjakul S, Safari R, Motamedzadegan A. 2010b. Fish protein hydrolysates production from yellowfin tuna (*Thunnus albacares*) head using Alcalase and Protamex. Int Aquat Res 2: 87-95.

Ovissipour M, Abedian AM, Motamedzadegan A, Nazari RM. 2011. Optimization of protein recovery during hydrolysis of yellowfin tuna (*Thunnus albacares*) visceral proteins. J Aquat Food Prod Technol, In Press.

Shahidi F, Han XQ, Syniwiecki J. 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem 53: 285-293.