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

Mahmoudreza Ovissipour[1*], Majid Taghiof[2], Ali Motamedzadegan[3], Barbara Rasco[4], Abbas Esmaeili Molla[5]

[1]Department of Fisheries, Gorgan Agricultural Sciences and Natural Resources University, Iran; Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway
[2]Department of Chemistry, Islamic Azad University of Tonekabon, Tonekabon, Iran.
[3]Department of Food Science, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.
[4]Department of Food Science and Human Nutrition, Washington State University, Washington,USA.
[5]Shahid Rajaee Sturgeon Fish Farm, Sari, Iran.

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Abstract

Fish protein hydrolysate (FPH) was produced from the viscera of beluga sturgeon (*Huso huso*). Using response surface methodology (RSM) and a factorial design to minimize enzyme use and to model the degree of hydrolysis (r^2 = 0.94), the hydrolysis conditions (temperature, time and enzyme activity) were optimized. The optimum conditions were: 50°C, 120 min, protease (Alcalase® 2.4 L) activity on 34 AU/kg protein. The hydrolysates of beluga visceral protein have a relatively high protein (66.43%) and low lipid (1.34%) content. The chemical score of the hydrolysate indicated that the amino acid profile of beluga sturgeon hydrolysate fulfills human adult nutritional requirements, with phenylalanine being the first limiting and predominant amino acid in the hydrolysate.

Keywords: Fish protein hydrolysate, Sturgeon visceral protein, Alcalase, Process optimization

Introduction

According to FAO, the amount of seafood caught worldwide in 2006 was more than 100 million tons (FAO 2006). Large amounts of protein-rich by-products from the seafood industries are discarded or processed into fish meal (Ovissipour et al. 2009). The increasing demand for protein on a global scale turns the focus on under-utilized protein sources (Liaset et al. 2000), and novel processing methods are needed to meet the demand for both human and animal feed use. Proteins from byproducts of fish processing are subjected to enzymatic modification to improve their quality and functional characteristics. Biochemical production of fish protein hydrolysates may be carried out by employing an autolytic process utilizing endogenous enzyme, an accelerated controllable method using exogenous enzymes (Shahidi et al. 1995), or a combination of endogeneous and added enzymes (Kristinsson and Rasco 2000a). The use of fish protein hydrolysates for maintaining the growth of different microorganisms (Gildberg et al. 1989; Safari et al. 2009) or of food and feed ingredients (Kristinsson and Rasco 2000a) has been investigated.

* Corresponding author. Email: mrovissi@yahoo.com. Tel: +98-151-3210330; Fax: +98-151-3240768.

A number of different enzymes have been used for hydrolysis of fish proteins (*e.g.* Papain, Alcalase®, Protamex®, Flavourzyme®, Neutrase®) (Aspmo et al. 2005). These enzymes are from plant source such as papain (Hoyle and Merritt 1994; Shahidi et al. 1995) or from animal origin such as pepsin (Viera et al. 1995) and chymothrypsine or trypsin (Simpson et al. 1998). Enzymes of microbial origin (Alcalase®, Protamex®, Flavourzyme®, Neutrase®) have been also applied. Compared to animal or plant derived enzymes, microbial enzymes offer several advantages, including a wide variety of available catalytic activities, and higher pH and stability of temperature (Diniz and Martin 1997). Alcalase® an alkaline bacterial protease from *Bacillus licheniformis*, has proven to be one of the best enzymes used for producing fish protein hydrolysates (Hoyle and Merritt 1994; Shahidi et al. 1995; Kristinsson and Rasco 2000a, b; Aspmo et al. 2005). Fish protein hydrolysates produced by Alcalase® tend to have less bitter components compared to those made with Papain (Hoyle and Merritt 1994). The cost of the enzyme may influence the economy and commercial viability of the process (Shahidi et al. 1995), and Alcalase® may exhibit a lower cost per unit of enzyme activity compared to other enzymes that could be utilized (Kristinsson and Rasco 2000b). From an economical point of view, the amount of enzyme used should be optimized to prevent enzyme waste and manage its costs.

Fish viscera, one of the most important byproducts from any commercial fisheries, are a rich source of protein and polyunsaturated lipids but with low storage stability if not frozen or otherwise preserved (Raa et al. 1983). Annually, approximately 330 tons of sturgeon is caught of the south coast of the Caspian Sea (IFO 2006). Almost 20-25% of the weight of the sturgeon is viscera, which is produced as byproduct of sturgeon caviar and meat processing industries. The sturgeon wastes are discarded, except for swim bladder and notochord which are used for glue production and as soup ingredient, respectively. Sturgeon viscera could be a valuable protein source for animal feed and human food (Ovissipour et al. 2009). The objective of this study was to optimize reaction conditions (*i.e.* temperature, time and enzyme activity) in order to obtain an optimal degree of hydrolysis of visceral proteins from beluga sturgeon *Huso huso* viscera using commercial enzymes.

Materials and methods

Materials and enzyme

The viscera from sexually immature beluga *Huso huso* were provided by the Iranian Fisheries Organization, Mazandaran, Iran. The fish viscera were kept at 20°C until use (almost 10 days). Alcalase® 2.4 L FG is a bacterial endoprotease produced by *Bacillus licheniformis* (Novozymes, Tehran, Iran). It was stored at 4°C until use for the hydrolysis experiment. All chemical reagents were of analytical grade.

Preparation of fish protein hydrolysate

The preparation of the hydrolysates of the beluga sturgeon viscera was carried out according to our previous study (Ovissipour et al. 2009). First, the fish viscera were minced twice using an industrial mixer at medium speed at approximately 10°C (Jaltajhiz, Tehran, Iran, 5 mm plate size), thawed overnight in a refrigerator at 4°C, then cooked at 85°C in a water bath (W614-B, Fater Rizpardaz, Tehran, Iran) for 20min to inactivate endogenous enzymes (Ovissipour et al. 2009). The cooked viscera and a sodium phosphate buffer 1:2 (w:v) were mixed and homogenized in a Moulinex® blender for about 2min. The pH of the mixture was adjusted as recommended by the Alcalase® producer company at pH 8.5 by adding 0.2 N NaOH. All reactions were performed in 250 ml glass vessels containing 50 g of substrate, in a shaking incubator (Ivymen Systems, Comecta, Spain) with constant agitation, at 200 rpm. After sampling each, the reactions were terminated by heating the solution to 95°C for 20min (Ovissipour et al. 2009), assuring inactivation of the enzyme. The hydrolysates were then cooled on ice until reaching an ambient temperature, centrifuged at 8000g at 10°C for 20min in Hermle Labrotechnik GmbH z206a centrifuge (Germany), the supernatant was collected and the oil phase removed and discarded. The Alcalase® was added to the substrate based on Anson Unit per kg crude protein. Hence, Alcalase® was added in the range of 17 to 51 AU/kg of crude protein.

Proximate composition of hydrolysates

The moisture content was determined by placing approximately 2g of the sample into a pre-weighed aluminum dish. Samples were then dried in an oven at 105 ± 1°C overnight or until a constant weight was reached (AOAC 2005). The total crude protein (N × 6.25) in raw materials was determined using the Kjeldahl method (AOAC 2005). The total lipid in the sample was determined by Soxhlet extraction (AOAC 2005). The ash content was estimated by charring a pre-dried sample in a crucible at 600°C until a white ash was formed (AOAC 2005). The protein in the supernatant was measured by the Biuret method following centrifugation (Layne 1957), using bovine serum albumin as standard. Absorbance was measured at 540 nm in a UV/vis spectrophotometer (Jenway, 6305, UV/vis). The protein recovery was calculated as the amount of protein present in the hydrolysate relative to the initial amount of protein in the reaction mixture (Ovissipour et al. 2009).

Optimization experiments

The hydrolysis conditions were optimized using response surface methodology (RSM) with a completely randomized factorial design. The different factors and the levels at which they were employed, according to preliminary experiments (unpublished data), are presented in Table 1. Three different independent variables, temperature (X_1, °C), time (X_2, minute) and enzyme activity (X_3, AU/kg protein) were employed at five levels (-α, -1, 0 and +1, +α).

The experimental design consists of eight factorial points, six axial points and four replicates at central point (Table 2). The degree of hydrolysis was selected as the response for the combination of the independent variables given in Table 1.

The experimental runs were randomized to minimize the effect of the unexpected variability in the observed response. The behavior of the system was explained by the following equation (1):

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

Where y is the dependent variable (degree of hydrolysis in real value), $ß_0$ is the constant, and $ß_i$, $ß_{ii}$ 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, respectively. The model evaluated the effect of each independent variable to a response (Cao et al. 2008).

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

Factor	Levels				
	-α*	-1	0	+1	+α
Temperature (°C) (X_1)	35	38	45	53	55
Time (min) (X_2)	60	80	120	160	180
Enzyme activity (AU/kg protein) (X_3)	17	22	34	46	51

Notes:
* α= 1.414

Table 2. Experimental design for modeling DH, using RSM

Run No. #	Coded levels of variable			
	X1*	X2	X3	DH (%)
1	1	1	1	30.87
2	1	1	-1	27.71
3	1	-1	1	17.95
4	1	-1	-1	16.90
5	-1	1	1	15.78
6	-1	1	-1	12.65
7	-1	-1	1	12.22
8	-1	-1	-1	11.28
9	0	1.414	0	23.91
10	0	-1.414	0	18.81
11	0	0	1.414	25.23
12	0	0	-1.414	15.08
13	1.414	0	0	26.42
14	-1.414	0	0	15.51
15	0	0	0	24.24
16	0	0	0	24.93
17	0	0	0	23.79
18	0	0	0	25.16

Notes:
*$X1$: temperature, $X2$: time, $X3$: enzyme activity

Degree of hydrolysis

Degree of hydrolysis was estimated according to Hoyle and Merritt (1994), as described previously (Ovissipour et al. 2009). Each run after the specified hydrolysis was terminated by the addition of 20% trichloroacetic acid

(TCA) following centrifugation to collect the peptide-containing supernatant. Then the degree of hydrolysis was computed as follows:

%DH = (10% TCA soluble N in the sample/total N in the sample) × 100

Amino acid composition

The sample preparation was conducted by hydrolysis with 6 M HCl at 110°C for 12h, and derivatization using phenyl isothiocyanate prior to HPLC analysis. The total amino acids was analyzed by the PicoTag® method (Waters Corporation, Milford, MA), using a PicoTag® column (3.9 × 150 mm; Waters) at a flow rate of 1 ml min^{-1} with UV detection. Breez® software was used for data analysis (Ovissipour et al. 2009).

Calculating the chemical score

The chemical score of the protein hydrolysates was computed according to Ovissipour et al. (2009), relative to the essential amino acid (EAA) profile in a standard protein as described by FAO/WHO (1990). In brief, the chemical score was calculated using the following equation:

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

Statistical analysis

The optimization experiments were carried out by the response surface method (RSM) by generating the factorial design (3 factors, 3 levels, and single block) using the experimental design model of the Statistical Analysis System: SAS software release 7 (SAS Institute, Cary, NC, USA) (Little et al. 1991; Nilsang et al. 2005) and MATLAB software release 13.0 (MathWorks Inc., Natick, MA, USA). The significance was determined at a 95% probability level.

Results and discussion

Proximate Composition

The chemical composition of the beluga viscera and the fish protein hydrolysates are shown in Table 3. The fresh beluga sturgeon viscera had a protein content of 13.67% with a relatively high crude fat content (14.34%). The protein content of the hydrolysate was 66.43%, which is within the range of other published studies on FPH of 63.4 to 90.8% protein (Bhaskar et al. 2008; Kristinsson and Rasco 2000b; Onodenalore and Shahidi 1996; Shahidi et al. 1995; Nilsang et al. 2005; Ovissipour et al. 2009). The crude lipid recovered from the beluga sturgeon hydrolysate reached 1.34%, which is similar to the results found by other researchers, since the lipid is decanted or otherwise removed from the hydrolysate (Kristinsson and Rasco 2000a, b; Nilsang et al. 2005; Shahidi et al. 1995).

Table 3. Proximate composition (%) of raw material and fish protein hydrolysate (FPH)[a]

	Protein	Fat	Moisture	Ash	Protein Recovery range (%)
Fresh viscera	13.67 ± 1.4	14.34 ± 0.43	63.51 ± 3.7	6.45 ± 3.21	-
FPH[b]	66.43 ± 3.62	1.34 ± 0.23	7.48 ± 2.7	25.31 ± 2.86	45.2 - 73.7

Notes:

[a] All values are means of triplicate determinations.

[b] Different soluble materials including phosphate buffer.

Ovissipour et al. (2009) reported that the lipid content of Persian sturgeon viscera hydrolysates after 205min and at 100 AU/kg crude protein was 0.18%. The lipid content in FPH was greatly reduced when compared to the raw material, because lipids are usually removed along with the insoluble protein fraction by centrifugal separation (Kristinsson and Rasco 2000b; Nilsang et al. 2005; Ovissipour et al. 2009). Decreasing of lipids content in the protein hydrolysate contributes significantly to the stability of the material towards lipid oxidation (Diniz and Martin 1997; Kristinsson and Rasco 2000b; Nilsang et al. 2005; Shahidi et al. 1995). The lipid fraction is used separately as a feed ingredient or in other commercial applications.

The protein recovery ranged from 45.2 to 73.7%. Kristinsson and Rasco (2000a) reported 40.6 to 79.9% nitrogen recovery, corresponding to a hydrolysate with 5 and 10% DH, respectively. Many researchers indicated that soluble protein recovery would increase by using a longer hydrolysis time and an elevated reaction temperature (Kristinsson and Rasco 2000a, b; Ovissipour et al. 2009).

Optimization of hydrolysis parameters for DH

The degree of hydrolysis has been modeled by keeping significant factors ($P < 0.05$) in the following equation (2):

$$y = 24.7 + 4.74x_1 + 2.98x_2 + 1.88x_3 - 2.05x_1^2 + 2.35x_1x_2 - 1.85x_2^2 - 2.45\ x_3^2 \quad (2)$$

The observed values for DH at different combinations of the independent variables are presented in Table 2. According to the ANOVA (Table 4), in addition to linear and quadratic terms ($P < 0.01$), one cross-product term ($X_1.X_2$) was significant ($P < 0.05$). Statistical analysis indicated that, within each term, all three hydrolysis factors of temperature, time and enzyme activity had a strong and significant influence on DH ($P < 0.05$).

The DH coefficient in this study ($r^2 = 0.94$) was satisfactory, with a low predicted experimental error (Table 4). High correlations of experimental results with those predicted by RSM models for proteolytic reactions have been reported by several researchers. Bhaskar et al. (2008) reported similar results for DH in hydrolysate preparations from catla viscera (*Catla catla*) using an alkaline protease.

The optimum conditions (temperature, time, and enzyme activity) were predicted using response surface graphs for DH (Fig. 1). Fig. 1a shows the effect of time and temperature on DH at pH of 8.5. Quadratic effect of temperature and a linear effect of time are apparent. The results indicated that the DH increases up to 30% with increasing temperature (to a maximum of 50°C) and time of hydrolysis up to 120 minutes. Hydrolysis at a higher temperature (up to 50°C) and a longer time than 120min results in a higher DH value. Fig. 1b shows the effect of time and enzyme activity on DH. Quadratic effect of time and enzyme activity on the DH can be noticed. The DH increased with time, but stayed almost constant after 120min of hydrolysis. Also, the highest DH is observed at the enzyme activity of 34 AU/kg protein. At higher concentrations the DH decreased. The effect of temperature and enzyme activity on the response (DH) is shown in Fig.1c. A quadratic effect of temperature and enzyme concentration is shown. At temperatures higher than 50°C the DH remained constant.

Table 4. ANOVA for DH as affected by temperature, time and enzyme activity, during optimization experiments using Alcalase®

Source	df[1]	Sum of square	Mean square	*F*-ratio
Regression				
Linear	3	420.01	—	34.06**
Quadratic	3	109.38	—	8.87**
Crossproduct	3	46.49	—	3.77*
Total	9	575.88	—	15.57**
Residual				
Lack of fit	5	31.69	6.33	16.00
Pure error	3	1.18	0.39	—
Total error	8	32.88	4.11	—
$r^2 = 0.94$				
Factors				
Temperature (°C)	4	347.89	86.97	21.16**
Time (minute)	4	181.19	45.29	11.02**
EA (AU/kg crude protein)	4	93.23	23.30	5.67**

Notes:
[1] Degree of freedom.
** Significant at 1% level* Significant at 5% level.

These results suggest that a response surface model can be used to predict optimal hydrolysis conditions. The stationary point (maximum) of the fitted model was found by using the first derivatives of the function in equation (3):

$$\begin{aligned} &4.74 - 4.1x_1 + 2.35x_2 = 0 \\ &2.98 + 2.35x_1 - 3.7x_2 = 0 \\ &1.88 - 4.9\ x_3 = 0 \end{aligned} \quad (3)$$

In this study, it was 50 °C for hydrolyzing temperature, 120min for hydrolyzing time and 34 AU/kg crude protein for enzyme activity. Bhaskar et al. (2008) found that the optimum conditions for hydrolyzing visceral waste proteins from the Indian carp (*Catla catla*), in order to attain 50% DH, were 135 minutes, 55°C, and Alcalase® enzyme concentration of 11 AU/liter protein extract at the pH of 8.5. Benjakul and Morrisey (1997) evaluated different combinations of reaction conditions for hydrolyzing protease waste materials recovered from

processing Pacific whiting (*Merluccius productus*), but to lower levels of hydrolysis. A high degree of hydrolysis may reduce the bitterness (Adler-Nissen 1984), and it has been reported that Alcalase® tends to produce less bitter hydrolysates compared to other proteases (Hoyle and Merritt, 1994; Benjakul and Morrisey 1997). Further, it is well known, that the peptide chain length and DH depends upon the extent of hydrolysis, conditions of hydrolysis, enzyme concentration and type of the substrate proteins (Kristinsson and Rasco 2000a). Hence, the optimum conditions for hydrolyzing different substrates will be different and will vary depending upon the substrate used, particularly with the content and reactivity of any endogeneous proteases present.

Amino acid composition

The amino acid composition of beluga visceral protein hydrolysates (n=2) and chemical scores are presented in Table 5. The chemical score provides an estimate of the nutritive value of a protein. This parameter is used to compare levels of essential amino acids between the test and standard proteins. In the current study, the chemical scores computed are based on the reference protein of FAO/WHO (1990) for adults and on amino acid requirements of juvenile common carp, as listed by NRC (1993). The amino acid composition in this study and a comparison with the reference proteins indicate that the amino acid profiles of the beluga sturgeon viscera hydrolysates were generally higher in essential amino acids compared to the suggested amino acid pattern recommended by FAO/WHO for adult humans. Similar results were observed by Ovissipour et al. (2009) for Persian sturgeon viscera hydrolysates. For common carp *Cyprinus carpio* the chemical score of the beluga viscera protein hydrolysate shows that phenylalanine is the first limiting amino acid, and that other amino acids are present at levels exceeding the requirements of juvenile common carp (NRC 1993), except for threonine and histidine which are in adequate but somewhat lower quantities (Table 5). Furthermore, for many fish species including carp, growth rates are produced by diets with large amounts of free amino acids which are inferior to diets of similar amino acid composition in which the nitrogen component is in the form of protein (Walton et al. 1986; Dabrowski and Guderley 2002). Hydrolysates that have an intermediate chain length and limited amounts of free amino acids would be valuable as ingredients in formulated and nutritionally balanced fish diets (Pigott and Tucker 2002). These results agree with our previous study on Persian sturgeon hydrolysates (Ovissipour et al. 2009).

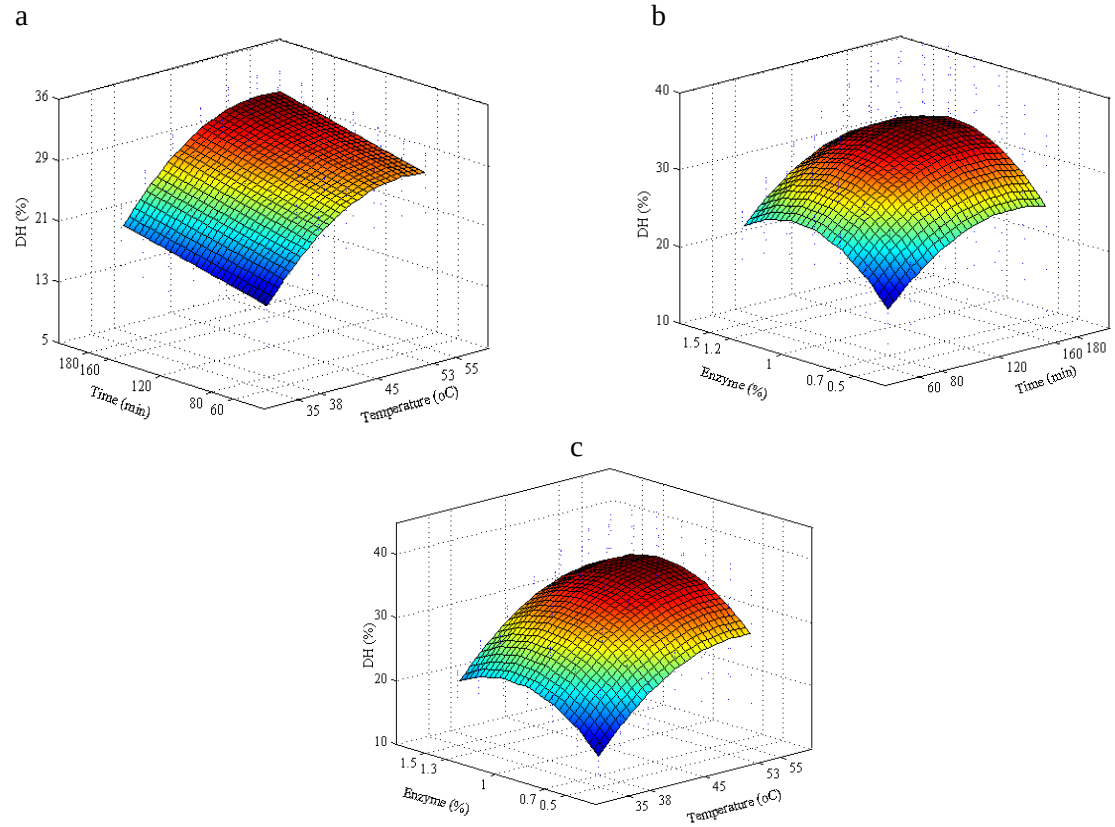

Fig. 1. Response surfaces for DH as a function of different hydrolyzing conditions: (a) time and temperature, (b) time and enzyme activity (c) temperature and enzyme activity.

Table 5. The amino acid composition of beluga sturgeon visceral protein hydrolysate (g/100g) and chemical score in comparison with FAO/WHO reference protein

Amino acid	Quantity (g 100 g^{-1})			Chemical score	
	Protein hydrolysate	Reference Protein 1[a]	Reference Protein 2[b]	RP1	RP2
Histidine	1.98	1.6	2.1	1.23	0.94
Isoleucine	4.23	1.3	2.5	3.25	1.69
Leucine	8.56	1.9	3.3	4.5	1.9
Lysine	7.43	1.6	5.7	4.64	1.3
Methionine[c]	8.87	1.7	3.1	5.21	1.7
Phenyl alanine	3.34	_	6.5	_	0.51
Tyrosine	3.06	_	_	_	_
Threonine	3.77	0.9	3.9	4.18	0.9
Tryptophan	_	_	_	_	_
Arginine	6.89	_	1.31	_	_
Valine	4.62	1.3	3.6	3.55	1.3
Aspartic acid	9.02	_	_	_	_
Glysine	6.56	_	_	_	_
Alanine	6.3	_	_	_	_
Proline	2.76	_	_	_	_
Serine	3.78	_	_	_	_
Glutamic acid	13.43	_	_	_	_

Notes:

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

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

[c] Methionine + cysteine.

Conclusion

The hydrolysis of beluga sturgeon *Huso huso* visceral waste protein using Alcalase® resulted in more than 30% DH. The DH is influenced significantly by enzyme activity, reaction time and temperature. Response surface methodology used for optimizing the condition of hydrolysis resulted in temperature of 50°C, time of 120 min and enzyme activity of 34 AU/kg protein. The sturgeon viscera hydrolysate has a relatively high protein (66.43%) and low lipid content (1.34%), and based on its amino acid composition, has a good potential for applications in aquaculture, as ingredient in animal feeds and as peptone to be an effective nitrogen source in microbial growth media.

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