



Bioactive properties of marine fish skin gelatin hydrolysate: Optimisation using response surface methodology

VAHIDEH MOKHTARNEZHAD, ALI TAHERI AND ALI MOTAMEDZADEGAN*

Chabahar Maritime University, P. O. Box. 99717- 56499, Chabahar, Sistan and Baluchestan Province, Iran

*Sari Agricultural Sciences and Natural Resources University, Mazandaran Province, Sari

Km 9 Farah Abad Road - 48181 68984, Iran

e-mail: taherienator@gmail.com

ABSTRACT

Adverse effects of free radicals on food oxidation have increasingly been investigated recently. Antioxidants have many health benefits as bioactive materials and are widely used in food industries. The effects of three main factors namely, time (60, 150, 240 min), temperature (40, 52, 65°C) and enzyme to substrate ratio (1, 3, 5%) on fish gelatin hydrolysis were studied in order to obtain optimum antioxidative properties. Response surface methodology (RSM) was used to fit the model and ridge analysis to optimise the selected factors. The percentage of Fe²⁺ chelating activity was considered as the response. R² confirmed that the model fitted well with the experimental data (R² = 0.97). Optimal conditions were found to be at a temperature of 56.55°C, enzyme to substrate ratio of 4.93% and time of 149.23 min. Under optimum conditions, metal chelating activity achieved was 82.33%. The results showed that fish skin gelatin hydrolysate had good antioxidative properties and could be a candidate for *in vivo* studies for industrial applications.

Keywords: Antioxidant, Fish gelatin hydrolysate, Metal chelating activity, Response surface methodology

Introduction

Gelatin is a water soluble protein produced by hydrolysis of animal collagen (Gomez-Guillen *et al.*, 2011) and because of its special functions and properties, is widely used in various industries (Karim and Bhat, 2009). Raw materials used for the extraction of collagen and gelatin are skin, bone, tendons and cartilage of animals (Gómez-Guillen *et al.*, 2011). Usually, gelatin is made from skin and bones of pigs and cows. Pigskin gelatin is not accepted in Islam and Judaism and cow's gelatin is acceptable if produced according to religious requirements (Badii and Howell, 2006). Due to the outbreak of bovine spongiform encephalopathy (BSE) and foot and mouth disease in cows (FMD) as along with the prohibition of collagen produced from the skin and bones of pigs due to religious reasons, it is essential to introduce alternative sources (Duan *et al.*, 2011).

Lipid oxidation during processing and storage of food products harms quality by changing their appearance, flavour, odour, shelf-life and nutritive value. This effect leads to decreased consumer acceptance (Aewsiri *et al.*, 2010; Sarmadi and Ismail, 2010). Also, oxidised foods cause serious illness (Aleman *et al.*, 2011a). Furthermore, uncontrolled generation of free radicals attacks the membrane proteins, fats and DNA, which leads to the development of many chronic diseases, including

cardiovascular diseases, neurological disorders, diabetes, inflammatory diseases, hypertension, aging, stomach ulcers and certain types of cancer (Mendis *et al.*, 2005b; Phanturat *et al.*, 2010). Antioxidants can protect the body from free radicals and lipid peroxidation and delay its complications (Najafian and Babji, 2012).

Synthetic antioxidants like BHT (butylated hydroxytoluene), BHA (butylated hydroxyl anisole), or N-propyl gallate have shown intense antioxidant activity. But their use is significantly reduced because of the potential health risks. Hence, identification of antioxidants from natural sources became a topic of interest (Gimenez *et al.*, 2009). In the past decade, several studies have focused on the antioxidant properties of marine proteins and peptides. According to them, protein hydrolysis of aquatics has shown an excellent antioxidant property, among them following studies are worth mentioning: scallops (*Patinopecten yessoensis*) and abalone (*Haliotis discushannai* Ino) muscle protein hydrolysates (Zhou *et al.*, 2012), Pacific cod (*Gadus macrocephalus*) gelatin hydrolysate (Ngo *et al.*, 2011), tilapia (*Oreochromis niloticus*) skin gelatin hydrolysates (Zhang *et al.*, 2012) and Alaska pollock skin gelatin hydrolysates (Kim *et al.*, 2001).

Changes in hydrolysis conditions, such as temperature, time and enzyme to substrate ratio, lead

to changes in properties of the final product. Therefore, achieving best reaction conditions is essential for ensuring the highest antioxidant properties. Optimisation by modern experimental designs is a way to achieve the best results with reduced analysis costs (Yang *et al.*, 2009).

Marine catfish *Netuma thalassina* (Ruppell 1837) (= *Arius thalassinus*) caught heavily in trawls in the Chabahar Bay (Iran) is not edible in Iran and is mostly used by fishmeal industries. However, the skin of this fish species has the potential for extraction of gelatin with antioxidant properties. Therefore, this study was conducted to optimise marine catfish skin gelatin hydrolysate production by response surface methodology (RSM) and the antioxidant activity was used as a response.

Materials and methods

Fish preparation

Fresh fish catch of *N. thalassina* collected from the Chabahar Fishing Port, Iran was used for this study. Samples (stored in ice during transportation) were moved to the Biotechnology Laboratory of the Chabahar Maritime University and stored at -20°C until further use. All chemicals such as enzymes, citric acid, sulfuric acid and sodium hydroxide were purchased from Sigma-Aldrich (Iranian branch, Tehran).

Fish gelatin production

Fish samples were defrosted at refrigerator temperature. Skin tissue was removed and cut into small pieces (1×1 cm), then washed under running tap water to remove superfluous material. Gelatin was extracted from the skin, according to the method described by Koli *et al.* (2012), with slight modifications. Briefly, the skin samples were immersed in 0.2% (w/v) sodium hydroxide, sulfuric acid 0.2% (w/v) and citric acid 1.0% (w/v), respectively. In all treatments, acid and base solution was replaced every 40 min with a total time of 2 h for each treatment. After each treatment, skin samples were washed with distilled water and normal pH of 7 was obtained before conduction to the new solution. The ratio of skin to acid or base solution was 1: 6 (w/v) for each treatment. The skin tissues were then treated in 76% ethyl alcohol to remove fat. At all stages, Shaker Incubator (Fan Azma Gostar, Model: K_m 55, Iran) with continuous gentle stirring was used. The skin samples were then washed with distilled water.

The gelatin extraction was performed in 3 volumes of distilled water at 45°C for 12 h with continuous stirring in a shaker incubator. The mixtures were then filtered using a Buchner funnel with Whatman filter paper (no. 1). Then, the filtrate was freeze-dried (Jaldeb, Iran) for 20 h. Dried gelatin was stored at 20°C until use.

Hydrolysis of skin gelatin

The skin gelatin was hydrolysed with the enzyme Protamex under controlled conditions, as shown in Table 1. The enzyme with different ratios (Table 1) was added to 10 ml gelatin (1.0 %, w/v), and the mixture was incubated with stirring at desired temperature and times. Subsequently, the enzyme was deactivated by heating at 90°C for 10 min and then centrifuged at 3000 rpm for 15 min (Centurion Scientific, Model: K241R, England). The supernatant was collected as gelatin hydrolysate and freeze-dried (Alemán *et al.*, 2011b).

Antioxidant properties

The Fe²⁺ chelating activity was estimated by the method of Intarasirisawat *et al.* (2002) with some modification. FeCl₂ (0.1 ml, 2 mM) was mixed with the diluted test sample (3.7 ml). Then, ferrozine solution (0.2 ml, 5 mM) was added to the mixture. The absorbance was measured at 562 nm using a spectrophotometer (Unicon spectrophotometer, Model: S2100 SUV, USA) after 10 min at room temperature. Distilled water instead of the sample and EDTA were used as blank and control, respectively.

The chelating activity was calculated according to the following formula:

$$\text{Fe}^{2+}\text{chelating activity (\%)} = [(B - A) / B] \times 100$$

where A is the absorbance of the sample and B is the absorbance of blank.

Mathematical model

Response surface methodology (RSM) was used to optimise enzymatic hydrolysis conditions (Guerard *et al.*, 2007; Ren *et al.*, 2008). The effect of independent variables, including temperature (°C, A), time (min, B) and enzyme to substrate ratio (% , C), was evaluated at 3 levels (Table 2). Percentage of chelating activity was selected as the dependent variable. The central composite design consisted of 20 treatments including eight factorial points, six axial points and six replicates around the central point (to estimate the experimental error), for the optimisation process (Table 1). Three-dimensional plots were used to study the trends. Design Expert 7.00 software was used for data analysis.

Based on the experimental data, multiple regression modeling in the form of a second-order polynomial was used as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \dots \dots \dots (1)$$

where Y is the estimated response, β_0 the constant-coefficient, β_1 , β_2 , β_3 are linear coefficients, β_{11} , β_{22} , β_{33} the quadratic coefficients and β_{12} , β_{13} , β_{23} the interaction coefficients.

Table 1. Experimental design for optimising the hydrolysis condition of sea catfish (*N. thalassina*) skin gelatin

Temperature (°C)	Time (min)	Enzyme-substrate ratio (e/s, %)
40	240	1
65	150	3
40	60	5
52.5	150	3
52.5	240	3
52.5	150	3
65	60	1
40	240	5
52.5	150	5
52.5	150	3
65	240	5
52.5	150	1
52.5	150	3
40	150	3
40	60	1
52.5	150	3
52.5	150	3
65	60	5
52.5	60	3
65	240	1

Table 2. Independent variables and their level for optimising the hydrolysis condition of sea catfish (*N. thalassina*) skin gelatin

Independent variables	Symbol	Code value levels		
		1+	0	1-
Temp (°C)	A	65	52	40
Time (min)	B	240	150	60
e/s (%)	C	5	3	1

Results and discussion

In this study, hydrolysis conditions were optimised by RSM model to produce skin gelatin peptides from *N. thalassina* with high antioxidant properties. Analysis of variance for the linear, the quadratic and the interaction of the three variables (A, B and C) on the response values (Y) are shown in Table 3. Each of the linear coefficients of explanatory variables was significant at 5% level; temperature (p value <0.0001), time (p value = 0.0008) and enzyme to substrate ratio (p value <0.0001). Among the quadratic coefficients, time and enzyme to substrate ratio showed significance at 5% level.

Transition metals such as Fe, Cu, and Co in foods can accelerate autoxidation and break hydrolytic to volatile compounds (Klompong *et al.*, 2007). They also can catalyse the reactive oxygen species generation, such as hydroxyl radical (OH[•]) and superoxide anion (O₂^{•-}) and then activate the lipid peroxidation chain reaction.

Therefore, the chelation of metal ions by antioxidants reduces the oxidation reaction (Farvin *et al.*, 2014). It is believed that acidic or basic amino acids through carboxyl and amino groups in their side chains have an essential role in the chelation of metal ions (Rajapakse *et al.*, 2005).

The first issue in optimising the response surface is performance of the suggested model regression. In this regard, various statistical factors must be determined. In the present study, a determination coefficient R²=0.9796 represents a very good fit of the regression model to the data. When the R² value is close to one, the model predicts the response better (Muhamad *et al.*, 2013). The statistical model with the lowest difference between modified R² and predicted R² is the most appropriate (Liu *et al.*, 2013). In this study, the modified R² was 0.9612 and R² predicted was 0.9521 with the difference between them being very low. Signal to noise ratio in response surface models must be greater than 4 (Ren *et al.*, 2008). For this model, signal to noise ratio was 27.124, which represents a desired signal and model performance. The lack of fit examines the remaining error to absolute error. Lack of fit was not significant in the model (p>0.05), which indicates the model had a reasonable fit. The results of the interaction between temperature and time (p<0.0001) and interaction between time and enzyme to substrate ratio (p<0.0001) were also significant. However, the interaction between temperature and enzyme to substrate ratio was not significant (p>0.05). The regression equation for the coded values and the actual values are given in Equations 2 and 3 below:

$$\text{Chelating activity} = + 65.02 + 8.50 X_1 + 4.90 X_2 + 11.90 X_3 - 7.05 X_1^2 - 15.05 X_2^2 + 4.95 X_3^2 + 8.25 X_1 X_2 - 2.50 X_1 X_3 - 9.25 X_2 X_3 \dots\dots\dots (2)$$

$$\text{Chelating activity} = - 132.75051 + 4.61455 X_1 + 0.38085 X_2 + 11.47652 X_3 - 0.045091 X_1^2 - 1.8574 X_2^2 + 1.23864 X_3^2 + 7.33333 X_1 X_2 - 0.1 X_1 X_3 - 0.051389 X_2 X_3 \dots\dots\dots (3)$$

Normal probability distribution of data and comparison of the model predictions and actual results are shown in Figs. 1 and 2. Fig. 1 indicates the normal distribution of the experimental data. Fig. 2 indicates the good fit of the final model since the numerical predictions from the model and the experimental values fell on a straight line. This graph shows reasonable fitness of experimental point with the model's predicted value and high accuracy of the statistical model regarding the chelating activity of marine catfish skin (*N. thalassina*) gelatin hydrolysis.

The effects of temperature (A) and time (B) are illustrated in Fig. 3. At an enzyme to substrate ratio of 3%, an increase in temperature up to 52.5°C increased the percentage of chelating linearly, beyond that, it decreased.

Table 3. Analysis of variance of the response surface quadratic model of sea catfish (*N. thalassina*) skin gelatin

Source	Sum of squares	Degrees of freedom	Mean square	F value	p (Prob > F)
Model	5139.77	9	571.09	53.28	< 0.0001
Temp (A)	722.50	1	722.50	67.41	< 0.0001
Time (B)	240.10	1	240.10	22.40	0.0008
e/s (C)	1416.10	1	1416.10	132.12	< 0.0001
AB	544.50	1	544.50	50.80	< 0.0001
AC	50.00	1	50.00	4.66	0.0561
BC	684.50	1	684.50	63.86	< 0.0001
A ²	136.51	1	136.51	12.74	0.0051
B ²	622.51	1	622.51	58.08	< 0.0001
C ²	67.51	1	67.51	6.30	0.0309
Residual	107.18	10	10.72		
Lack of Fit	7.85	5	1.57	0.079	0.9927
Pure Error	99.33	5	19.87		
Correction total	5246.95	19			

Likewise, increasing the time up to 150 min, increased chelating activity. At a duration of more than 150 min, the percentage of chelation reduced. Simultaneously increasing temperature and time causes increase in the percentage of metal chelation. This increase occurred at temperatures lower than 52.5°C at a duration more than 150 min whereas at higher temperatures at durations of more than 195 min chelating activity showed a decreasing trend.

Fig. 4 shows the percentage of the Fe²⁺ chelation as a function of temperature and enzyme to substrate ratio, where the time was constant at 150 min, indicated that the percentage of chelation increased when enzyme to substrate ratio increased. However, this increase was not noticeable up to the 3% level of the enzyme, while there was considerable chelating activity at enzyme levels higher than 3%. Increasing the percentage of enzymes along with temperatures increased the percentage of chelation through interaction effects, but this rising trend continued only up to 58.75°C and at temperatures above 58.75°C, each of the enzyme's levels caused reduction in the chelation percentage.

As shown in Fig. 5, at constant temperature of 52.5°C and increase in time up to 150 min linearly increased the chelation percentage and beyond 150 min there was no tangible effect on the percentage of chelation. Increasing the percentage of enzyme caused linear increase in chelation. The increase of enzyme to substrate ratio along with an increase in the duration of hydrolysis up to 150 min, resulted in increasing the percentage of chelation as interaction. At high hydrolysis temperature, increasing the enzyme to substrate ratio caused decrease in the percentage of chelation. The maximum chelating activity was 73.30% observed at enzyme level of 5% and hydrolysis time of 150 min. Optimal conditions for the

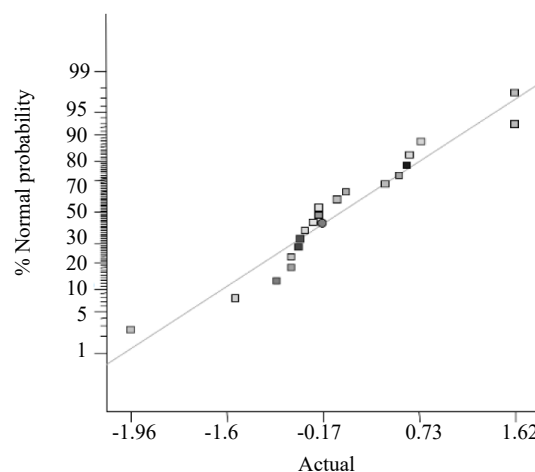


Fig 1. Normal probability distribution data of catfish skin gelatin hydrolysate

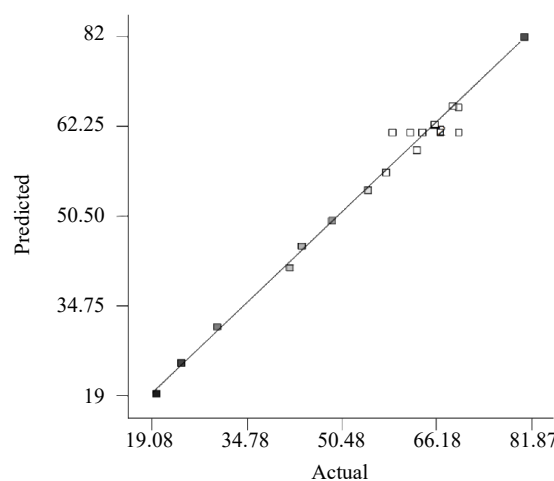


Fig 2. Comparison model predictions against experimental values of catfish skin gelatin hydrolysate

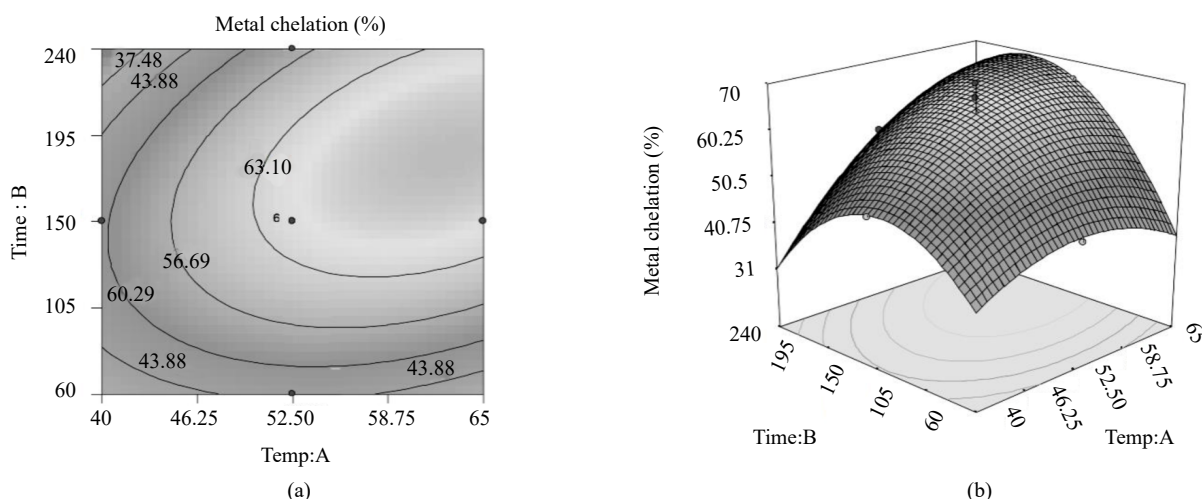


Fig. 3. Contour plot and response surface for the effect of (a) temperature A and (b) time B on the chelating activity of sea catfish (*N. thalassina*) skin gelatin

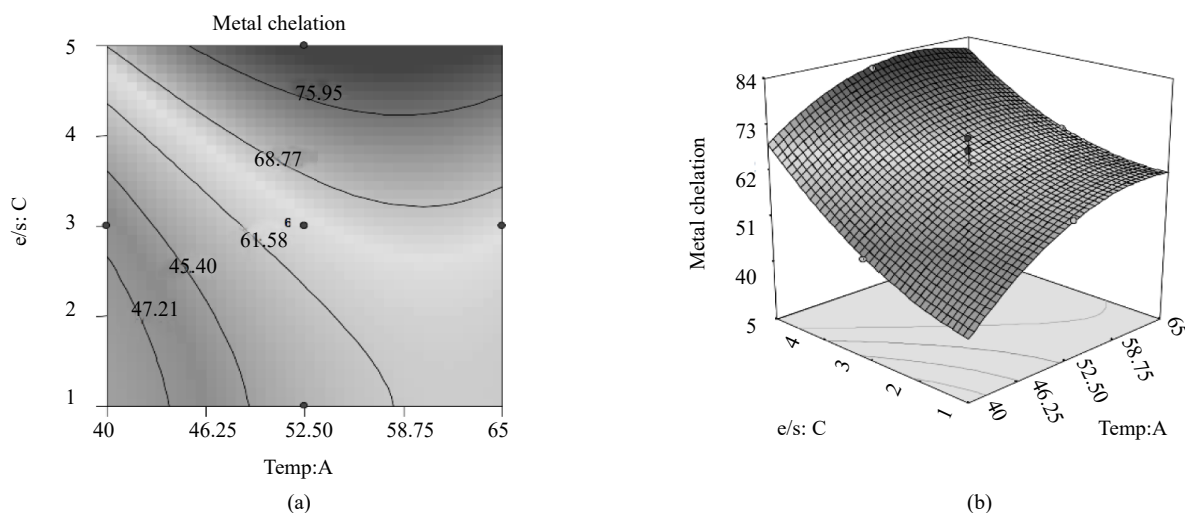


Fig. 4. Contour plot and response surface for the effect of (a) temperature A and (b) e/s C on the chelating activity of sea catfish (*N. thalassina*) skin gelatin

production of gelatin hydrolysate with the highest activity of Fe^{2+} chelation based on the confirmed model were as follows: Temperature - 56.55°C, enzyme to substrate ratio - 4.93% and time -149.23 min with metal ion chelating activity of 82.33%.

Contour plots and the response surface of the effects of temperature and time on percentage of chelation shows that by the beginning of hydrolysis process, production of peptides that had chelating activity and capable of oxidation of metal ions starts and its optimal range is at temperature of 52.5-65°C and time duration of 150-195 min which decreased beyond this optimal range. At the beginning of the reaction, hydrolysis releases the antioxidant peptide which increases the antioxidant

activity of hydrolysate. More reactions, out of the optimal range, can cause excessive hydrolysis of antioxidant peptides, their break down to free amino acids and reduce the inhibition activity of the hydrolysate (Ren *et al.*, 2008).

Unlike many other studies that reported increased antioxidant activity with increased hydrolysis temperature and time; in this study, very high temperature for long periods decreased antioxidant activity. This indicated the presence of an optimal temperature range for optimal performance of Protamex™ (E.C. 3.4.21.62/ 3.4.24.28) enzymes. Similar results were found in the study of Ren *et al.* (2008) on protein hydrolysis of grass carp, where the inhibitory activity increased from 1 h to about 6 h. However, more treatment time (6 to 12 h) decreased

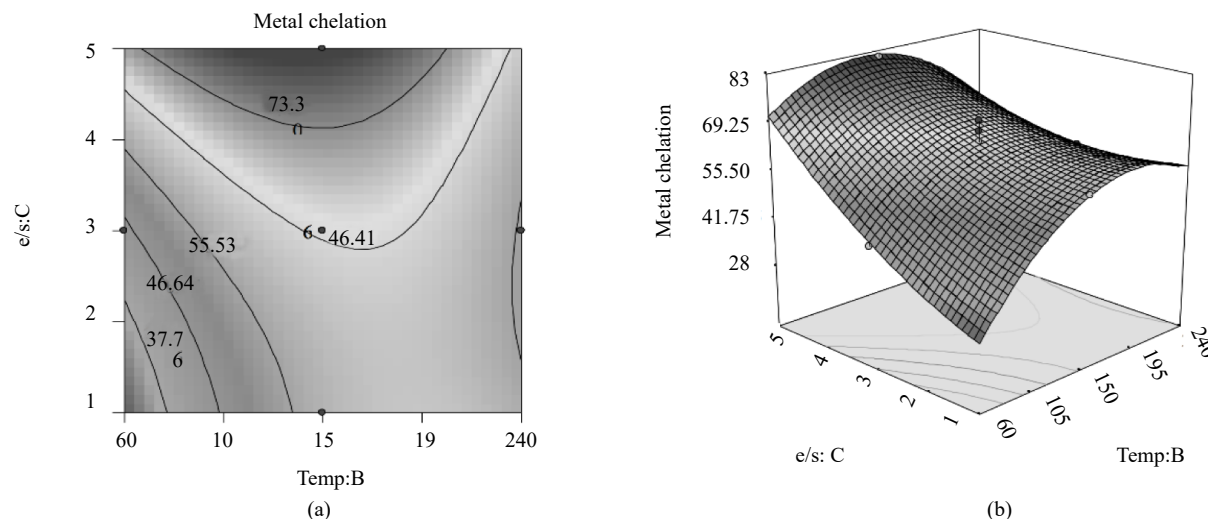


Fig. 5. Contour plot and response surface for the effect of (a) time B and (b) e/s C on the chelating activity of sea catfish (*N. thalassina*) skin gelatin

inhibitory activity that was not expected (Ren *et al.*, 2008). In a study conducted by Fang *et al.* (2012) on squid protein hydrolysis, increase in the hydrolysis temperature and time caused increased free radical scavenging, which then reduced with further increase of temperature and time. Also, Limpisophon *et al.* (2020), reported that proteolytic activity of the enzyme increased at above 30 to 60°C and then declined beyond 65°C due to enzyme denaturation. Differences in optimal temperatures at different studies were probably due to different substrates and conditions used for hydrolysate production.

According to the response surface and contour plots of the effects of temperature (A) and enzyme to substrate ratio (C) on the Fe²⁺ chelation (Fig. 4), increasing the temperature beyond a certain level decreased the chelating activity. This issue can be due to the instability of enzyme at high temperatures and also quicker enzyme denaturation at high temperatures, which reduces its biological activity. These results showed that enzyme to substrate ratio and temperature have an optimum limit to produce peptides with antioxidant properties.

Fang *et al.* (2012) in their study on squid protein hydrolysis using papain enzymes reported that the temperature in the range of 45-51°C increased the free radical scavenging activity, however, higher temperatures can reduce the inhibitory activity. In a study of Wangtueai *et al.* (2016), on lizardfish scale gelatin hydrolysis, when the e/s ratio was increased, the (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging increased. The optimal temperature and time of hydrolysis were between 45 to 55°C and 1 to 2 h. But at this time, ferric reducing ability of plasma (FRAP)

decreased and when the time reached 2 to 3 h, it increased. The effects of temperature and e/s ratio for FRAP were the same as ABTS test.

Our results indicated that the percentage of chelation increased with increase in the enzyme to substrate ratio which may be due to more hydrolysis of gelatin when adding more protease; in this case, a high proportion of enzyme to substrate ratio is desired to enhance the chelation reaction. It is also possible that as time increases the stability of the enzyme is reduced and the reaction does not follow the laws of thermodynamics and aggregation of product reached to such an extent that have an inhibition property (Styer, 1988). This also might explain the reduction in chelation percent in the longer time duration (Fig. 5). In a study of Fang *et al.* (2012), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability increased gradually from 45 to 51°C while the activity reduced in the range of 51-55°C. Similar results were reported by Ren *et al.* (2008) in their study on grass carp muscle sarcoplasmic protein with papain enzyme, that at constant temperature, increasing the enzyme to substrate ratio increased the inhibitory activity. Several factors, including protein extraction conditions, the degree of hydrolysis and proteolytic enzyme type, can impact the antioxidant activity of bioactive peptides (Liu and Chiang, 2008). Moreover, the e/s ratio had the most significant effect on the antioxidant properties of jellyfish collagen hydrolysate (Zhuang *et al.*, 2009). Also, You *et al.* (2010), reported that DPPH free radical scavenging activity increased significantly at hydrolysis time between 1 to 2 h ($p < 0.05$).

In the present study, optimum conditions of the predicted model was at a reaction temperature of 56.55°C, an enzyme to substrate ratio of 4.93% and reaction time of 149.23 min with a Fe²⁺ chelation rate of 82.3349% that was very close to the experimental data. Zhuang *et al.* (2009), reported an optimal pH of 7.75, temperature of 48.77°C and e/s ratio of 3.50% for jellyfish collagen hydrolysate production. You *et al.* (2010), worked on fish gelatin hydrolysate preparation with antioxidant properties. They reported an e/s ratio of 3% at 52.1°C and 2.65 h as optimum conditions. The DPPH and ABTS free radical scavenging activity were 97.3% and 9.86 mM Trolox, respectively. Moreover, the optimum conditions for lizardfish (*Saurida* spp.) scale gelatin hydrolysate with antioxidant activity were 3% e/s at 55°C for 3 h, and the FRAP activity was 61.7% (Wangtueai *et al.*, 2016). The optimum conditions for shark skin gelatin hydrolysate with antioxidant activity were 60°C, pH 7.5 and enzyme of 2.70 Au kg⁻¹ (Limphisophon *et al.*, 2020). Optimum conditions for fish gelatin hydrolysate thus depend on the kind of substrate and enzyme and for each study, the optimal conditions are different.

The molecular structure and mass of bioactive peptides obtained by the hydrolysis of gelatin are influenced by the reaction conditions. Gelatin peptides contain hydrophobic amino acids that could act as antioxidant agents. So gelatin peptides impart more antioxidant effects than other peptide sequences. Also, several studies have reported that aromatic amino acids and histidine play a critical role in these activities (Mendis *et al.*, 2005a, b). Based on published literature, it is known that the hydrolysis process produces a mix of amino acids and peptides that have reduction power by donating the electron. Peptides by their higher electron donating ability, have better reducing power, and are excellent antioxidant agents (Pezeshk *et al.*, 2018; Tkaczewska *et al.*, 2020).

In the present study, catfish skin gelatin hydrolysate (*N. thalassina*) showed a good Fe²⁺ chelating activity. The hydrolysis at optimum conditions of 56.55°C, e/s ratio of 4.93% and at 149.23 min, showed metal-chelation of 82.33%. These results indicated that antioxidant peptides derived from catfish skin gelatin hydrolysate can be a potential candidate in the pharmaceutical industry and in the development of functional foods with antioxidant potential. However, additional *in vivo* model system studies for food and medical purposes are necessary.

Acknowledgments

Authors are grateful to the authorities for providing research facilities at the Chabahar Maritime University, Iran.

References

- Aewsiri, T., Benjakul, S., Visessanguan, W., Wierenga, P. A. and Gruppen, H. 2010. Antioxidative activity and emulsifying properties of cuttlefish skin gelatin-tannic acid complex as influenced by types of interaction. *Innov. Food Sci. Emerg. Technol.*, 11(4): 712-720.
- Aleman, A., Gimenez, B., Montero, P. and Gomez-Guillen, M. C. 2011a. Antioxidant activity of several marine skin gelatins. *J. Food Sci. Technol.*, 44(2): 407-413. DOI: 10.1016/j.lwt.2010.09.003.
- Aleman, A., Perez-Santín, E., Bordenave-Juchereau, S., Arnaudin, I., Gomez-Guillen, M. C. and Montero, P. 2011b. Squid gelatin hydrolysates with antihypertensive, anticancer and antioxidant activity. *Food Res. Int.*, 44(4): 1044-1051. DOI: 10.1016/j.foodres.2011.03.010.
- Badii, F. and Howell, N. 2006. Fish gelatin: Structure, gelling properties and interaction with egg albumen proteins. *Food Hydrocoll.*, 20(5): 630-640. <https://doi.org/10.1016/j.foodhyd.2005.06.006>.
- Duan, R., Zhang, J., Xing, F., Konno, K. and Xu, B. 2011. Study on the properties of gelatins from skin of carp (*Cyprinus carpio*) caught in winter and summer season. *Food Hydrocoll.*, 25: 368-373. DOI: 10.1016/j.foodhyd.2010.07.002.
- Fang, X., Xie, N., Chen, X., Yu, H. and Chen, J. 2012. Optimisation of antioxidant hydrolysate production from flying squid muscle protein using response surface methodology. *Food Bioprod. Process.*, 90(4): 676-682. DOI:10.1016/j.fbp.2012.04.001.
- Farvin, S. K. H., Andersen, L. L., Nielsen, H. H., Jacobsen, C., Jakobsen, G., Johansson, I. and Felleming, J. 2014. Antioxidant activity of cod (*Gadus morhua*) protein hydrolysates: *In vitro* assays and evaluation in 5% fish oil-in-water emulsion. *Food Chem.*, 149: 326-334.
- Gimenez, B., Aleman, A., Montero, P. and Gomez-Guillen, M. C. 2009. Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. *Food Chem.*, 114(3): 976-983. <https://doi.org/10.1016/j.foodchem.2008.10.050>.
- Gomez-Guillen, M. C., Gimenez, B., Lopez-Caballero, M. E. and Montero, M. P. 2011. Functional and bioactive properties of collagen and gelatin from alternative source: A review. *Food Hydrocoll.*, 25(8): 1813-1827. <https://doi.org/10.1016/j.foodhyd.2011.02.007>.
- Guerard, F., Sumaya-Martinez, M., Laroque, D., Chabeaud, A. and Dufosse, L. 2007. Optimisation of free radical scavenging activity by response surface methodology in the hydrolysis of shrimp processing discards. *Process Biochem.*, 42(11): 1486-1491. DOI:10.1016/j.procbio.2007.07.016.
- Intarasirisawat, R., Benjakul, S., Visessanguan, W. and Wu, J. 2012. Antioxidative and functional properties of protein

- hydrolysate from defatted skipjack (*Katsuwonus pelamis*) roe. *Food Chem.*, 135: 3039-3048. doi: 10.1016/j.foodchem.2012.06.076.
- Karim, A. and Bhat, R. 2009. Fish gelatin: Properties, challenges and prospects as an alternative to mammalian gelatins. *Food Hydrocoll.*, 23(3): 563-576. DOI: 10.1016/j.foodhyd.2008.07.002.
- Kim, S., Kim, Y., Byun, H., Nam, K., Joo, D. and Shahidi, F. 2001. Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska pollack skin. *J. Agric. Food Chem.*, 49(4): 1984-1989. doi: 10.1021/jf000494j.
- Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chem.*, 102(4): 1317-1327. DOI:10.1016/j.foodchem.2006.07.016.
- Koli, J. M., Basu, S., Nayak, B., Patange, S., Pagarkar, A. and Gudipati, V. 2012. Functional characteristics of gelatin extracted from skin and bone of tiger-toothed croaker (*Otolithes ruber*) and pink perch (*Nemipterus japonicus*). *Food Bioprod. Process.*, 90(3): 555-562. DOI:10.1016/j.fbp.2011.08.001.
- Limpisophon, K., Shibata, J., Yasuda, Y., Tanaka, M. and Osako, K. 2020. Optimization of hydrolysis conditions for production of gelatin hydrolysates from shark skin byproduct and evaluation of their antioxidant activities. *J. Aquat. Food Prod. Technol.*, 29(8): 739-749.
- Liu, B. L. and Chiang, P. S. 2008. Production of hydrolysate with antioxidative activity and functional properties by enzymatic hydrolysis of defatted sesame (*Sesamum indicum* L.). *J. Appl. Sci. Eng.*, 6(2): 73-83.
- Liu, Y., Wei Shoulian and Liao, M. 2013. Optimization of ultrasonic extraction of phenolic compounds from *Euryale ferox* seed shells using response surface methodology. *J. Ind. Crop Product.*, 49: 837-843. https://doi.org/10.1016/j.indcrop.2013.07.023.
- Mendis, E., Rajapakse, N. and Kim, S. 2005a. Antioxidant properties of a radicals scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *J. Agric. Food Chem.*, 53(3): 581-587. doi: 10.1021/jf048877v.
- Mendis, E., Rajapakse, N., Byun, H. G. and Kim, S. K. 2005b. Investigation of jumbo squid (*Dosidicus gigas*) skin gelatin peptides for their *in vitro* antioxidant effects. *J. Life Sci.*, 77(17): 2166-2178. doi: 10.1016/j.lfs.2005.03.016.
- Muhamad, M. H., Sheikh Abdullah, S. A., Mohamad, A. B., Abdul Rahman, R., Kadhum, H. and Amir, A. 2013. Application of response surface methodology (RSM) for optimization of COD, NH₃-N and 2,4-DCP removal from recycled paper wastewater in a pilot-scale granular activated carbon sequencing batch biofilm reactor (GAC-SBBR). *J. Environ. Manage.*, 121: 179-190. DOI:10.1016/j.jenvman.2013.02.016.
- Najafian, L. and Babji, A. 2012. A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment and applications. *Peptides*, 33(1): 178-185. doi: 10.1016/j.peptides.2011.11.013.
- Ngo, D. H., Ryu, B., Vo, T. S., Himaya, S. W., Wijesekara, I. and Kim, S. K. 2011. Free radical scavenging and angiotensin-I converting enzyme inhibitory peptides from Pacific cod (*Gadus macrocephalus*) skin gelatin. *J. Biol. Macromol.*, 49(5): 1110-1116. DOI: 10.1016/j.ijbiomac.2011.09.009.
- Pezeshk, S., Ojagh, S. M., Rezaei, M. and Shabanpour, B. 2018. Fractionation of protein hydrolysates of fish waste using membrane ultrafiltration: Investigation of antibacterial and antioxidant activities. *Probiot. Antimicrob. Proteins*, 11(3): 1015-1022.
- Phanturat, P., Benjakul, S., Visessanguan, W. and Roytrakul, S. 2010. Use of pyloric caeca extract from bigeye snapper (*Priacanthus macracanthus*) for the production of gelatin hydrolysate with antioxidative activity. *J. Food Sci. Technol.*, 43: 86-97. DOI:10.1016/j.lwt.2009.06.010.
- Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y. and Kim, S. K. 2005. Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *J. Food Res. Int.*, 38(2): 175-182. https://doi.org/10.1016/j.foodres.2004.10.002.
- Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., Kakuda, Y. and Xue, J. 2008. Optimization of antioxidant peptide production from grass carp sarcoplasmic protein using response surface methodology. *J. Food Sci. Technol.*, 41(9): 1624-1632.
- Sarmadi, B. H. and Ismail, A. 2010. Antioxidative peptides from food proteins: A review. *Peptides*, 31(10): 1949-1956. doi: 10.1016/j.peptides.2010.06.020.
- Tkaczewska, J., Borawska-Dziadkiewicz, J., Kulawik, P., Duda, I., Morawska, M. and Mickowska, B. 2020. The effects of hydrolysis condition on the antioxidant activity of protein hydrolysate from *Cyprinus carpio* skin gelatin. *LWT - Food Sci. Technol.*, 117: 108616.
- Wangtueai, S., Siebenhandl-Ehn, S. and Haltrich, D. 2016. Optimisation of the preparation of gelatin hydrolysates with antioxidative activity from lizardfish (*Saurida* spp.) scales gelatin. *Chiang Mai J. Sci.*, 43(1): 68-79.
- Yang, J. L., Liang, W. S., Chow, C. J. and Siebert, K. J. 2009. Process for the production of tilapia retorted skin gelatin hydrolysates with optimised antioxidative properties. *Process Biochem.*, 44(10): 1152-1157.
- You, L., Regenstein, J. M. and Liu, R. H. 2010. Optimisation of hydrolysis conditions for the production of antioxidant peptides from fish gelatin using response surface methodology. *J. Food Sci.*, 75(6): 582-587.
- Zhang, Y., Duan, X. and Zhuang, Y. 2012. Purification and characterisation of novel antioxidant peptides from

- enzymatic hydrolysates of tilapia (*Oreochromis niloticus*) skin gelatin. *Peptides*, 38(1): 13-21. doi: 10.1016/j.peptides.2012.08.014.
- Zhou, D. Y., Tanga, Y., Zhu, B. W., Qin, L., Li, D. M., Yang, J. F., Lei, K. and Murata, Y. 2012. Antioxidant activity of hydrolysates obtained from scallop (*Patinopecten yessoensis*) and abalone (*Haliotis discus hannai* Ino) muscle. *Food Chem.*, 132(2): 815-822.
- Zhuang, Y. L., Zhao, X. and Li, B. F. 2009. Optimization of antioxidant activity by response surface methodology in hydrolysates of jellyfish (*Rhopilema esculentum*) umbrella collagen. *J. Zhejiang Uni. Sci. B*, 10(8): 572-579.