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Evaluation the effect of hydrolysis conditions and type of protease on the degree of hydrolysis and antioxidant properties of the protein hydrolysate from the skipjack fish (*Katsuwonus pelamis*) viscera by the response surface methodology

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ABSTRACT

Oxidation in living organs causes dangerous diseases, including cancer, and in food, it causes spoilage and heavy economic losses. Synthetic antioxidants have adverse and dangerous effects on human health, therefore identifying natural antioxidant compounds is one of the main needs of the food industry. In fish processing industries, about 50-70% of fish, which are potential sources of valuable nutritional com such as essential amino acids, are produced as waste. Therefore, finding a way to optimally use these wastes and produce healthy compounds with high added value such as bioactive peptides has great importance. In this research, the effect of hydrolysis conditions (time: 30-300 min and enzyme concentration 0.5-3 %) and type of protease (alcalase and pancreatin) on the degree of hydrolysis and antioxidant properties (DPPH radical scavenging activity, Fe chelating activity, nitric oxide radical inhibition, total antioxidant capacity and Fe reducing power) of protein hydrolysate from skipjack viscera was investigated using the response surface methodology. The results showed that the optimum conditions for achieving the most antioxidant properties with alcalase and pancreatin were: hydrolysis time of 146.9 and 171.67 minutes and enzyme concentration of 1.94 and 2.17%; in these conditions, the degree of hydrolysis of the produced protein hydrolysates was 25.12% and 20.35%, respectively. Comparing the antioxidant properties of hydrolysates produced by both proteases showed that the alcalase enzyme led to the production of protein hydrolysates with stronger antioxidant properties than pancreatin. Therefore, it can be concluded that the protein hydrolysate of the skipjack fish viscera using alcalase enzyme as a healthy and value-added product can be used in the production of functional products and health supplements.

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1- Introduction

Free radicals play an essential role in the oxidation of lipids and the breakdown of unsaturated fatty acids, which lead to the occurrence of rancidity and the reduction of the shelf life of many foods (1). On the other hand, free radicals cause changes in DNA, protein and other intracellular molecules, which result in the occurrence of dangerous diseases such as cardiovascular diseases, nervous system disorders, diabetes and even Alzheimer's. Therefore, preventing the formation of free radicals and the occurrence of oxidation in the body and food is of particular importance. Now some combinationsAntioxidant Synthetic like[1]PG ، [2]BHA and BHT[3] They are usually used to deal with free radicals in food and biological systems; But due to their pathogenic potential, the use of these compounds in the food industry is limited (2). This issue has increased attention to the identification of natural antioxidant compounds such as bioactive peptides (3). Consumption of natural antioxidants with increasing potentialAntioxidant The body increases the level of human health (4). Also, these compounds lead to the delay of lipid oxidation in foods, which in addition to improving the nutritional quality of food products, also contribute to their stability, color, taste, and texture (5-7). Studies have shown that bioactive peptides usually contain 2-20 amino acids and have several health-related properties, including: antihypertensive (8), antioxidant (9), antibacterial (10) and immune system regulator (11). In general, bioactive peptides can be produced during food processing by spontaneous hydrolysis of food proteins using endogenous digestive enzymes or by providing hydrolysis conditions and using commercial proteases. In addition, bioactive peptides can be released during gastrointestinal digestion of ingested food. Studies have shown that the type of protease enzyme, hydrolysis conditions and protein source play an important role in the health-giving properties of the resulting peptides (12). In recent years, bioactive peptides have been produced from various plant and animal sources such as fenugreek (13), orange core (14) and chicken meat (15). Meanwhile, peptidesAntioxidant Produced from the waste of aquatic processing such as sardine fish head (16), skin and intestines and entrails of whitefish.[4] (17), skin and fish head leather jacket[5] (18) have received a lot of attention because it has been determined that these peptides have a significant health potential and can be used in the production of useful products. On the other hand, in the fish processing industry, only their white meat is used in the production of canned fish, and about 50-70% of primary fish (head, intestines and viscera, bones, skin, liver and dark meat) which are potential sources of valuable compounds. Nutrients such as proteins and essential amino acids are thrown away as waste, which leads to environmental pollution and heavy economic losses to producers. Considering the limitation of biological resources, environmental pollution and economic efficiency of aquatic processing units, finding a solution for better management and use of these wastes and turning them into products with high added value is essential. Fish intestines and viscera are one of the most important by-products of aquatic processing industries, which contain valuable protein and lipid fractions, as well as vitamins and minerals (19). About 4 million tons of tuna fish are caught in the world every year, among which is Hoover Muscat with the scientific name *Katsuwonus the suitor*It has the highest catch rate and is

[1]- n-propyl gallate

[2] -Butylated hydroxytoluene

[3] -Butylated hydroxyanisole

[4]- cuttlefish

[5] -leatherjacket

mainly used to produce canned fish; Therefore, it constitutes a large part of the waste of canned fish factories (20). Therefore, according to the mentioned cases, the purpose of this research is to investigate the effect of hydrolysis time and the type of protease (alcalase and pancreatin) on the degree of hydrolysis and its characteristics.Antioxidant (Radical scavenging activity[6]DPPH, iron ion reduction, iron ion chelation, nitric oxide radical inhibition, etcAntioxidant total) hydrolyzed protein obtained from the intestine and viscera of Hoover Muscati fish.

2- Materials and methods

2-1- Materials

Pancreatin, alkalase, trichloroacetic acid, DPPH, Coomassie Brilliant Blue (G250), ferric chloride, potassium ferricyanide, iron dichloride and ferrosin were obtained from Merck, Germany. Other chemical substances were also obtained with laboratory purity from Sigma Company of America.

2-2- sample preparation

FirstThe intestines and viscera of Hoover Muscati fish obtained from the processing waste of Sahil Said Kanarak (Tafeh) were washed with distilled water and completely minced. Then, to deactivate the internal enzymes of the fish, for a period of time20 They were heated at 95°C for 10 minutes in a bain-marie and placed in an ice water bath for 10 minutes to reduce the temperature. The samples were dried by placing them in a 30°C oven for 24 hours. In the next step, the samples are milled and They were passed through a sieve with 40 mesh. Finally, the samples were stored in polyethylene bags in a freezer at -20 degrees Celsius for further tests. (21).

2-3- Fat removal

In order to degrease, the powders were mixed with hexane at a ratio of 1:10 (weight/volume) and stirred for 5 hours at room temperature. Then, hexane was separated using a Buchner funnel and dried in a 30°C oven for 5 hours. The resulting powder was ground again and passed through a 40 mesh sieve (21).

2-4- protein extraction

For protein extraction, defatted powdermixed with distilled water at a ratio of 1:10 andpH It was adjusted to pH=10 using 1 normal soda and stirred for 1 hour using a magnetic stirrer. Then the resulting solution was centrifuged at 7000×rpm for 20 minutes and the resulting supernatant was collected. ThenpH Supernatant to 4=pH (pH isoelectric) and centrifuged at 8000×rpm for 20 minutes to precipitate the proteins. Finally, the resulting protein precipitate was washed twice with distilled water and rpm×7000, was centrifuged again for 5 minutes. The resulting protein isolate is dried using a dry freeze dryer and kept in the freezer until the tests are performed-20 degrees Celsiuswas maintained (22).

5-2- Preparation of hydrolyzed protein

The resulting protein isolate was dissolved in a concentration (w/v) of 5% in 0.2 M M phosphate buffer, pH = 7.4 for pancreatin and pH = 8 for alkalase and was stirred for 30 minutes at room temperature to fully hydrate it. Then it was placed in a shaker incubator at 200 rpm at a temperature of 40°C for pancreatin and 50°C for alkalase. Enzyme was added to the samples in the proportions mentioned in Table 1 and hydrolysis was done according to the times specified in this table. Finally, to inactivate the enzyme, the erlens were placed in a 90°C water bath for 10 minutes and cooled to ambient temperature using an ice water bath. Then the samples were centrifuged at 8000 × rpm for 20 minutes. The supernatant was separated and dried using a freeze dryer and kept at -20°C until the next tests (13).

[6]- 1,1-diphenyl-2-picrylhydrazyl

6-2-Measurement of chemical compounds

The amount of moisture, protein, ash and fat was measured using the AOAC 2000 method (22).

2-7- Determining the degree of hydrolysis

Hydrolyzed protein suspension and trichloroacetic acid (M 0.44) were mixed in a volume ratio of 1:1 and incubated for 15 minutes at 4°C. Then, the mixture was centrifuged at 10000 rpm for 10 min. The amount of protein in the supernatant containing trichloroacetic acid M 0.22 was determined by the Bradford method (9). Finally, the degree of hydrolysis was determined using formula 1:

(1)
$$Degree\ of\ hydrolysis(\%) = \frac{protein\ \ in\ supernatant\ containing\ TCA}{protein\ in\ hydrolysate\ suspantion} \times 100$$

2-8- Examining the antioxidant properties of hydrolyzed protein

2-8-1- DPPH free radical inhibition activity

First, the resulting hydrolyzates were dissolved in distilled water (10 mg/ml), then 1.5 ml of each sample was mixed with 1.5 ml of DPPH ethanol solution (mM0.15) were mixed and vortexed for 20 seconds. In the next step, the resulting mixture was kept in the dark for 30 minutes and then centrifuged at 4000 rpm for 10 minutes. The absorbance of the supernatant solution was read at a wavelength of 517 nm (18). Radical scavenging percentagefreeDPPH was calculated using formula 2:

(2) $$I\ (\%) = [\frac{Ablank - Asample}{Ablank}] \times 100$$

2-8-2- Determining the reducing power of iron ion

To determine the reducing power of iron ions of hydrolyzed samples, 0.5 ml of the sample dissolved in distilled water (at a concentration of 10 mg/ml) with 0.5 ml of 0.2 M phosphate buffer (pH=6.6) and 0.5 ml 1 liter of potassium ferricyanide (W/V) was mixed and incubated at 50°C for 20 minutes. Then, 0.5 ml of 10% trichloroacetic acid solution was added to the mixture and centrifuged at 2500 rpm for 10 minutes. Finally, 1 ml of supernatant was mixed with 1 ml of distilled water and 0.2 ml of 0.1% ferric chloride (W/V). The absorbance of the sample at 700 nm was read after keeping the mixture for 10 minutes at ambient temperature. An increase in the absorption of the reaction mixture indicates an increase in the reducing power (23).

2-8-3- Iron ion chelating activity

To evaluate the iron ion chelating activity, 1 ml of the sample dissolved in distilled water (concentration 40 mg/ml) was mixed with 0.05 ml of iron dichloride solution (Mm2) and 1.85 ml of double distilled water. Then, 0.1 ml of ferrozine solution (5 Mm) was added and the mixture was vigorously stirred. Absorbance was read at 562 nm after 10 minutes of keeping the mixture at room temperature (24). Double distilled water was used as a control sample. The chelating activity of the samples was calculated using equation 3**:**

(3)Chelating effect (%) $= \frac{(Acontrol - Asample)}{Acontrol} \times 100$

2-8-4- CapacityAntioxidant Total

This method is based on the reduction of 6-valent molybdenum to 5-valent molybdenum, which is associated with the formation of a green phosphomolybdenum complex in an acidic environment. In this method, 0.1 ml of the sample dissolved in distilled water (concentration 10 mg/ml) with 1 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was poured into an Eppendorf tube and It was placed in a 90°C water bath for 90 minutes. After cooling, the absorbance of the samples was read at 695 nm. Double distilled water was used as control sample. More absorption indicates capacityAntioxidant The total is more (13).

2-8-5- nitric oxide radical inhibition activity

For this purpose, 60 µl of sodium nitroperoxide in PBS buffer[7] It was mixed with 60 µl of sample dissolved in distilled water (concentration 10 mg/ml). Then it was incubated at 25°C for 150 minutes. Then 120 microliters of Grease reagent was added to it and the absorbance of the resulting solution was read at 546 nm (25). The nitric oxide radical scavenging activity of the samples was calculated using equation 4:

(4)

$$NO\ chelating\ activity = \frac{(Acontrol - Asample)}{Acontrol} \times 100$$

9-2- Statistical analysis

Optimization of hydrolysis conditions was done with Design expert software version 7 and response surface method in the form of central compound design. The independent variables of time (30-300 minutes) and the ratio of enzyme to substrate (0.5-3%) and the investigated response are iron ion reduction power, DPPH radical scavenging activity, capacityAntioxidant total, iron ion chelating activity, nitric oxide radical scavenging activity and hydrolysis degree were considered. To compare the average of fat, moisture, ash and protein, Duncan's multi-range test was used at the 95% confidence level. All tests were performed in three repetitions.

3. Results and Discussion

3-1- Chemical compounds

The amount of ash, moisture and protein of non-defatted and defatted powder and the resulting protein isolate are listed in Table 1. The amount of fat in non-defatted powder was 21.25%, which decreased to 7.06% and 4.10% after defatting and extracting the protein. Also, the amount of moisture and ash has decreased and after the extraction of better protein, they decreased from 8.03% and 8.4% to 5.2% and 3.15%. On the other hand, the amount of protein in non-defatted powder was 43.75%, which increased to 87.5% after the extraction process. The amount of hydrolyzed protein fat was significantly lower than the original powder of intestines and fish viscera. The decrease in fat content can be due to the processes of defatting and protein extraction in isoelectric pH. The low amount of fat (less than 5%) in the protein isolate is the result of the good efficiency of hexane in degreasing the intestines and viscera of fish. The moisture reduction process during the protein extraction process can also be the result of using an oven with a temperature of 30 degrees Celsius after the degreasing process in order to remove the remaining hexane and also using a freeze dryer to dry the protein isolate. The increase of the isolated protein compared to the primary intestines and viscera powder, in addition to the reduction of the amount of fat during the degreasing process, can be due to the removal of a large part of non-protein compounds at pH=10. On the other hand, reducing the amount of ash can also be due to the protein extraction process, which removes many non-protein organic compounds and mineral salts [13].

[7]- *Phosphate-buffered saline*

Table 1. chemical composition of Undefatted, defatted powder and protein isolate of

	Ash (%)	Fat (%)	Moisture (%)	Protein (%)
Undefatted powder	0.16 [a] ±5.4	0.12[a]±21.25	0.35[a]±8.03	0.12[c]±43.75
Defatted powder	0.12 [b] ±4.36	0.11[b]±7.06	0.26[b]±7.2	0.16[b]±61.25
Protein isolate	0.11 [c] ±1.15	0.23[c]±4.10	0.25[c]±5.3	87.5±0.24[a]

Means with the same letters in each column show no significant difference at the 0.05 level

2-3- The effect of time, ratio of enzyme to substrate and type of protease on radical scavenging activityDPPH

Variance analysis results of the effect of time, enzyme to substrate ratio and protease type on radical scavenging activityDPPH It is mentioned in Table 2. Effect of time, ratio of enzyme to substrate and A variables^2 and B^2 on radical scavenging activityDPPH The hydrolyzates obtained from the activity of both alkalase and pancreatin enzymes were significant, while the effect of AB variable only on the activityAntioxidant The hydrolyzates obtained from alkalase were significant ($p<0.05$). Correlation of radical scavenging activityDPPH with quadratic independent variables and the amount of correlation coefficient in the case of hydrolyzates obtained from alkalase and pancreatin, respectively, R 0.9995^2= and R 0.9521^2= which indicates the high ability of the model in predicting the effect of process variables on radical scavenging activityDPPH is. On the other hand, the non-significance of the misfit index[8], indicates that the model was able to describe the changes in the amount of data well. Radical scavenging relationshipDPPH With the variables of the hydrolysis process with alkalase and pancreatin, it follows equations (5) and (6).

(5)

$$DPPH\ radical\ inhibition\ activity = +49.86 + 1.66\ A + 4.60\ B - 0.47\ AB - 11.05A^2 - 8.10\ B^2$$

(6)

$$DPPH\ radical\ inhibition\ activity = +37.25 + 3.62\ A + 5.10\ B + 1.03\ AB - 3.89A^2 - 4.89\ B^2$$

[8] - Lack of fit

Table 2. ANOVA of DPPH radical scavenging activity and Total antioxidant capacity of protein hydrolysate with alcalase and pancreatin

	Sum of squares	df	value P	Estimate the coefficient
				Hydrolysis with alcalase Hydrolysis with pancreatin
ANOVA of DPPH radical scavenging activity				
Model	1026.18	5	0.0001>	49.86
	0.489	5	0.0026	37.25
A-time	22.02	1	0.0001>	1.66
	104.91	1	0.0058	3.62
B- E/S	169.00	1	0.0001>	4.60
	207.91	1	0.0013	5.10
AB	0.88	1	0.0328	-0.47
	4.22	1	0.3968	1.03
A^2	688.92	1	0.0001>	-11.05
	85.38	1	0.0088	-3.89
B^2	370.29	1	0.0001>	-8.10
	134.80	1	0.0034	-4.89
Lac of fit	0.49	3	0.0828	
	24.59	3	0.0651	
ANOVA of total antioxidant capacity				
Model	0.35	5	0.0004	0.93
	2.94	5	0.0111	0.46
A-time	3.606	1	0.1884	-0.021
	1.71	1	0.0027	-0.46
B- E/S	0.29	1	0.0001>	0.19
	0.010	1	0.6882	-0.036
AB	3.721	1	0.1826	0.031
	2.916	1	0.8288	-0.027
A^2	0.047	1	0.0027	-0.092
	1.21	1	0.0056	0.46
B^2	1.565	1	0.3619	0.017
	0.15	1	0.1614	0.16
Lac of fit	6.568	3	0.2238	
	0.28	3	0.0625	

* A: hydrolysis time B: enzyme to substrate ratio

According to Figure 1 A, in constant enzyme amounts (less than 1.75%), increasing the hydrolysis time up to 182 minutes increases the ability of the resulting hydrolyzates to inhibit radicals.DPPH It was 48.12, but in enzyme amounts higher than 1.75, increasing the hydrolysis time up to 182 minutes increased the activity.Antioxidant up to 50.4% and further increase in hydrolysis time had a negative effect. On the other hand, at fixed times of hydrolysis, increasing the concentration of the enzyme up to 2% increases the activityAntioxidant up to 50.4% and increasing the concentration of the enzyme decreased the ability of the hydrolyzates to inhibit radicalsDPPH tour According to the graph, in the hydrolysis time of 173.49 minutes and enzyme concentration of 1.98%, the highest amount of activityAntioxidant 50.55% is obtained. In the case of hydrolyzates resulting from pancreatin activity (Figure 1B), at constant hydrolysis times and less than 165 minutes, increasing the enzyme concentration up to 2.3% increases the radical scavenging activity.DPPH up to 36.10%, but in hydrolysis

times longer than 165 minutes, increasing the enzyme concentration up to 2.3% increased the antioxidant activity up to 39.6%. On the other hand, in constant enzyme concentrations, with increasing time up to 230 minutes, radical scavenging activityDPPH The hydrolysates of pancreatin increased, but at longer hydrolysis times, increasing the concentration of the enzyme had a negative effect on the activityAntioxidant had In general, according to the graph, the maximum amount of radical scavenging activityDPPH (39.45 percent) in the hydrolysis time of 235.64 minutes and the enzyme concentration is 2.40 percent. Similar to these findings, Uysipour et al. (2009) reported that by hydrolyzing salmon waste for 180 minutes, the radical scavenging activityDPPH The resulting hydrolyzed protein increased to 40%, but increasing the hydrolysis time had a negative effect on this property [26]. Also, in accordance with these findings, Kaveh et al. (2018) reported that in the hydrolysis of fenugreek protein with pancreatin, increasing the concentration of the enzyme up to 2% increases the abilityAntioxidant The hydrolyzed products were obtained, but increasing the concentration of the enzyme had a negative effect.27[. Probably, the excessive increase in the hydrolysis time causes the progress of the hydrolysis process and the effect of proteases on the produced antioxidant peptides and their destruction, which leads to a decrease in the abilityAntioxidant It is hydrolyzed with increasing time [28]. On the other hand, reducing radical scavenging activityDPPH By increasing the concentration of the enzyme, it can be caused byHydrolysis is excessive, which causes the complete release of hydrophilic amino acids, which inhibits the interaction of active amino acids with lipid-soluble radicals.DPPH It faces a problem [29]. More radical scavenging activityDPPH The hydrolyzates obtained from alkalase compared to pancreatin show the very favorable effect of alkalase enzyme as an endopeptidase compared to pancreatin in releasing peptides with the ability to inhibit fat-soluble radicals.DPPH has it. In general, studies have shown that the type of protease and the degree of hydrolysis have a significant effect on the propertiesAntioxidant It has bioactive peptides [24].

3-3- Effect of time, ratio of enzyme to substrate and type of protease on total antioxidant capacity

Total antioxidant capacity is a method to evaluate the total antioxidant power of a compound, which includes the antioxidant power of fat- and water-soluble compounds. This method is based on the reduction of 6-valent molybdenum to 5-valent molybdenum, which leads to the formation of a green phosphomolybdenum complex in an acidic environment [30]. According to the results of variance analysis of the total antioxidant capacity mentioned in Table 2, only the effect of variable A^2 It was significant on the total antioxidant capacity of both hydrolyzates obtained from alkalase and pancreatin, and enzyme concentration only had a significant effect on the antioxidant activity of hydrolyzates obtained from alkalase and hydrolysis time on the total antioxidant activity of hydrolysates obtained from pancreatin ($p<0.05$). The response surface analysis showed that the reaction parameters with the total antioxidant capacity of both the hydrolyzates obtained from alkalase and pancreatin had quadratic relationships, and their regression coefficients were better than R^2= 0.9785. and R^2= 0.9128 was The non-significance of the misfit index also shows the high ability of the model in predicting the range of valuesis under investigation. The relationship between the total antioxidant capacity and the investigated variables in the hydrolysis with alkalase and pancreatin better follows the relationship (7) and (8).

(7)

$$Total\ antioxidant\ capacity = +0.021 - 0.93\ A + 0.19\ B + 0.031\ AB - 0.092A^2 + 0.017\ B^2$$

(8)

$$Total\ antioxidant\ capacity = +3.26 - 0.021\ A - 0.72\ B - 3.20\ AB + 5.08A^2 + 0.21\ B^2$$

According to Figure 1C, at fixed values of enzyme concentration by increasing the hydrolysis time with alcalase enzyme up to 165 minutes, the capacityAntioxidant The total increased a little, but a further increase in time had a negative effect. In general, capacity changesAntioxidant Total with the change of hydrolysis time was insignificant, which indicates that the hydrolysis time is not significant on the capacityAntioxidant The hydrolyzates obtained from alkalase (Table 2). On the other hand, in constant amounts of hydrolysis time, increasing the concentration of the enzyme has a positive effect on capacityAntioxidant and with increasing enzyme concentration from 0.86% to 2.63%, capacityAntioxidant Total increased. According to the maximum capacity diagramAntioxidant Total (absorbance at 695 nm, 1.14) in alcalase hydrolysis in 168 minutes, the enzyme concentration was 2.63%. On the other hand, as seen in Figure 1D, time generally has a negative effect on capacityAntioxidant The total hydrolyzates obtained from pancreatin had, so that in all constant concentrations of the enzyme, increasing the time decreases the capacity.Antioxidant The total hydrolyzates, but as mentioned earlier, the effect of enzyme concentration on capacityAntioxidant The total hydrolyzates obtained from pancreatin were not significant. Maximum capacityAntioxidant Total (absorbance 1.5 at 695 nm) was obtained in hydrolysis with pancreatin enzyme in hydrolysis time of 73.27 minutes and enzyme concentration was 0.88%. In general, it can be concluded that pancreatin and alkalase enzymes have been able to release peptides with electron-donating properties by hydrolyzing the peptide bonds in the intestinal protein and viscera of Hoover Muscat fish, which have caused the conversion of free radicals into stable compounds and finally led to an increase in the total antioxidant capacity. Is. However, excessive increase in hydrolysis time or enzyme concentration has led to the intensification of the hydrolysis process and as a result the hydrolysis of peptides with electron-donating properties. In this regard, pancreatin had a better performance in producing peptides with electron-donating properties. These results were in accordance with the findings of Kaveh et al. (2022) in the hydrolysis of fenugreek protein and Mazloumi et al. (2018) in the hydrolysis of orange core protein [31 and 9].

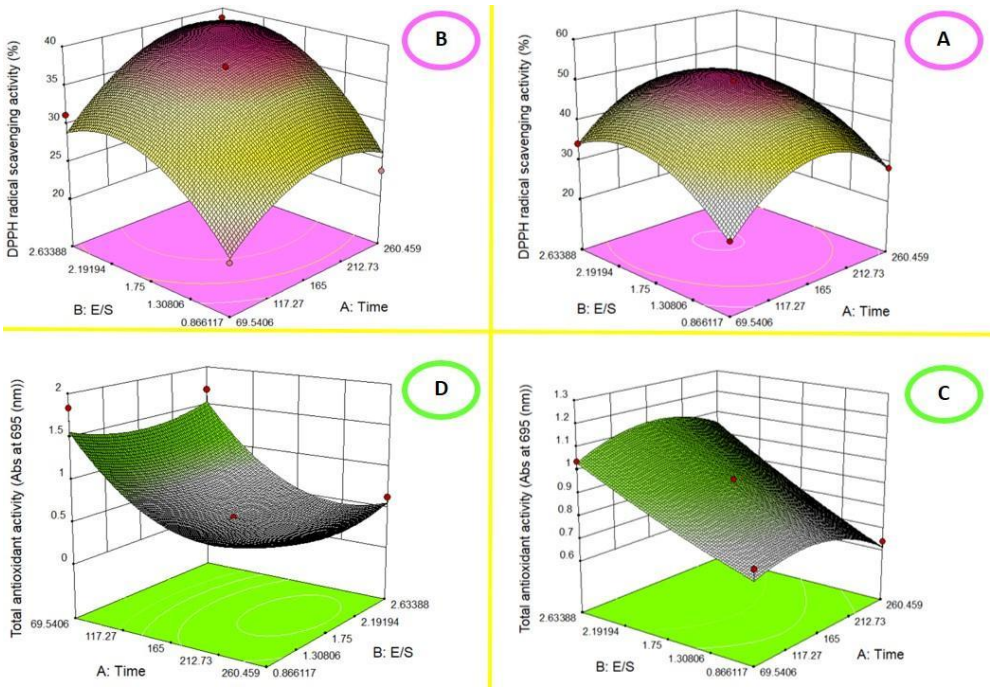

Figure 1- The effect of hydrolysis time, enzyme to substrate ratio and proteases type on DPPH radical scavenging activity and total antioxidant capacity of protein hydrolysate of skipjack viscera (A and C: hydrolysis with alcalase, B and D: hydrolysis with pancreatin)

4-3- The effect of time, ratio of enzyme to substrate and type of protease on nitric oxide radical inhibition activity

Nitric oxide is a free radical that is formed through the oxidation of arginine to citrulline byNitric oxide-Synthetase is produced [3]. The results of variance analysis mentioned in Table 3 show that the effect of enzyme concentration variables, A^2 and B^2 It was significant on the nitric oxide radical scavenging activity of the hydrolyzates obtained from alkalase and pancreatin, and the variables of hydrolysis time and AB did not have a significant effect. (05/0>p). Nitric oxide radical scavenging activity with quadratic relationship reaction parameters with regression coefficient9994/0R^2= for hydrolyzates obtained from alkalase and817/0R^2= for hydrolyzates obtained from pancreatin. The high regression coefficient and non-significance of the misfit factor indicate the appropriate ability of the model to predict the impact of process variables.Nitric oxide radical inhibition activity and the variables of the hydrolysis process with alkalase and pancreatin better follow the relationship (9) and (10).

(9)

$$\textit{Nitric oxide radical inhibition activity} = +58.26 - 0.31\,A + 7.77\,B - 0.33\,AB - 17.50A^2 - 12.20\,B^2$$

(10)

$$\textit{Nitric oxide radical inhibition activity} = +45.61 + 2.71\,A + 7.04\,B - 0.31\,AB - 9.55A^2 - 11.03\,B^2$$

Table 3. ANOVA of NO radical scavenging activity and reducing power of protein hydrolysate with alcalase and pancreatin

	Sum of squares	df	P value	Estimate the coefficient
				Hydrolysis with alcalase Hydrolysis with pancreatin
ANOVA of NO radical scavenging activity				
Model	2520.63	5	0.0001>	58.26
	1389.32	5	0.0186	45.61
A-time	0.78	1	0.1639	-0.31
	58.93	1	0.2436	2.71
B- E/S	482.60	1	0.0001>	7.77
	396.89	1	0.0186	7.04
AB	0.44	1	0.2785	0.33-
	0.38	1	0.9192	-0.31
A^2	1729.66	1	0.0001>	-17.50
	514.73	1	0.0113	-9.55
B^2	839.99	1	0.0001>	-12.20
	687.62	1	0.0063	-11.03
Lac of fit	1.39	3	0.0803	
	168.58	3	0.0743	
ANOVA of reducing power				
Model	0.10	5	0.0077	0.84
	0.092	5	0.026	0.75
A-time	7.73	1	0.0854	0.031
	5.38	1	0.1982	-0.026
B- E/S	0.030	1	0.0082	0.062
	0.039	1	0.0102	0.070
AB	6.25	1	0.9539	1.25
	0.022	1	0.0294	-0.075
A^2	0.028	1	0.0096	-0.07
	0.020	1	0.0362	-0.059
B^2	0.056	1	0.0022	-0.099
	0.012	1	0.0786	-0.046
Lac of fit	8.092	3	0.0623	
	0.012	3	0.0839	

* A: hydrolysis time B: enzyme to substrate ratio

As can be seen in Figure 2 A, at constant times of hydrolysis, increasing the enzyme concentration up to 2.4% led to an increase in the nitric oxide radical inhibition activity of hydrolyzed alkalase, but a further increase in enzyme concentration had a negative effect. On the other hand, as mentioned in Table 3, the effect of time on the nitric oxide radical inhibition activity of the hydrolysates obtained from alkalase was not significant, but in general, at constant enzyme concentrations, increasing the time up to 165 minutes increased the nitric oxide radical inhibition activity, but the increase was more Hydrolysis time decreases this propertyAntioxidant The resulting hydrolyzates were treated with alkalase. In general, according to the graph, the highest nitric oxide radical inhibition activity of hydrolyzed alkalase products (59.5%) was obtained in 165.7 minutes and the enzyme concentration was 2%. In the case of hydrolyzates obtained from pancreatin (Figure 2 B), at constant enzyme concentrations, increasing the hydrolysis time up to 180 minutes increased the nitric oxide radical inhibition activity, but increasing the time decreased their antioxidant activity, but

as shown in Table 3 The effect of time was not significant(05/0<p). On the other hand, in fixed times of hydrolysis, increasing the concentration of enzyme up to 2.2%, the ability of hydrolyzed pancreatin to inhibit nitric oxide radical increased and decreased with further increase in enzyme concentration. In general, according to the diagram, the highest nitric oxide radical inhibition activity (46.5%) was obtained at the enzyme concentration of 2.16% and the hydrolysis time was 164.5 minutes.Studies have shown that nitric oxide free radicals are produced in the reaction of nitrogen with reactive oxygen species and cause dangerous diseases such as diabetes, osteoporosis, cancer, and cardiovascular diseases [32]. Similar to these results, Lee et al. (2012) by examining the effect of eight types of proteases (fluorzyme, nutrease, protamax, alcalase, papain, pepsin, trypsin anda-Chymotrypsin) on nitric oxide radical scavenging activity of hydrolyzed oyster protein*Philippine rudimentary tapes* reported that the hydrolyzate resulting from the activity of alcalase enzyme had more inhibitory ability [34]. Also, Sadeghi Mahonek and Kaveh (1401) by hydrolyzing pumpkin meal protein with alcalase enzyme stated that the hydrophilicity of peptides has a direct relationship with their ability to inhibit nitric oxide radical and they reported the highest amount of nitric oxide radical inhibition at 52.8% [8]. . On the other hand, Shariat Alavi et al. (2017) hydrolyzed tomato seed protein using alcalase enzyme and reported that increasing the time had a positive effect on the nitric oxide radical scavenging activity of the hydrolyzed protein, but increasing the concentration of the enzyme reduced this ability [33]. [. In general, the nitric oxide radical inhibitory activity of hydrolyzed protein depends on the amino acid composition, degree of hydrolysis, type of enzyme and hydrolysis conditions [13].

3-5- The effect of time, ratio of enzyme to substrate and type of protease on the reducing power of iron ion

Iron ion reducibility test to evaluate the ability of a compound to convert Fe ion^{3+} to Fe^{2+} is used The results of the variance analysis of iron ion reduction power (Table 3) show that the effect of hydrolysis time on none of the hydrolysates obtained from alkalase or pancreatin had a significant effect, while the variables of enzyme concentration and A^2 They showed a significant effect. On the other hand, variable B^2 Only on the hydrolyzates obtained from alkalase and variable AB showed a significant effect only on the hydrolysates obtained from pancreatin.(05/0>p). The reducing power of both hydrolyzed iron ions obtained from alkalase and pancreatin with the reaction parameters of the quadratic interfaces and the regression coefficient9252/0R^2= And8825/0R^2= had The lack of fit factor was also not significant, which indicates the appropriate ability of the model in predicting the effect of process variables. Analysis of variance showed that the relationship between the reducing power of iron ionAnd the variables of the hydrolysis process with alkalase and pancreatin are in relation (11) and (12).

(11)

$$Iron\ ion\ reduction\ strength = +0.031 + 0.84\ A + 0.062\ B + 1.25\ AB - 0.070A^2 - 0.099\ B^2$$

(12)

$$Iron\ ion\ reduction\ strength = +0.026 - 0.75\ A + 0.070\ B - 0.075\ AB - 0.059A^2 - 0.046\ B^2$$

According to Figure 2C, at constant times of hydrolysis, increasing the enzyme concentration up to 2.2% increased the reductive power of the hydrolyzed products from alkalase, but a further increase had a negative effect. In the constant concentration

of the enzyme, increasing the hydrolysis time up to 190 minutes increased the iron ion reduction power, but increasing the time further reduced this power. But as mentioned in Table 3, the effect of time on this featureAntioxidant Alkalase hydrolyzates are not significant(05/0<p). In the case of hydrolyzates obtained from pancreatin (Figure 2D), at constant enzyme concentration, increasing the time up to 195 minutes increased the reducing power of iron ions, but a further increase in time resulted in a negative effect, and this negative effect occurred at enzyme concentrations greater than 2. 2 percent was much more. At constant times and less than 185 minutes, with increasing enzyme concentration, the reducing power of iron ion increased, but at times longer than 185 minutes, increasing enzyme concentration up to 2.2% had a positive effect on reducing power, but at higher enzyme concentrations, increasing time Hydrolysis decreased the power of hydrolyzates obtained from pancreatin in reducing iron ion. In general, the increase in the reductive power of hydrolyzed protein during hydrolysis and the appropriate enzyme concentration can be considered due to the release of amino acids with high reductive power such as methionine, tryptophan and lysine [24]. In agreement with these findings, Khantafant et al. (2011) with protein hydrolysis of redfish muscle[9] They reported the positive effect of increasing the time and degree of hydrolysis on the reductive power of the resulting hydrolyzates [34]. alsoAhmadi et al. (2019), By hydrolyzing the protein of carp intestines and viscera with alcalase enzyme, they stated that increasing the hydrolysis time up to 180 minutes significantly increased the regeneration power of the hydrolyzed products [35]. In general, studies have shown that the structure, size, composition and sequence of peptides change with the passage of time and the progress of the hydrolysis process, which has a significant effect on the reductive power of the hydrolyzed product [16].

9- Brown stripe red snapper

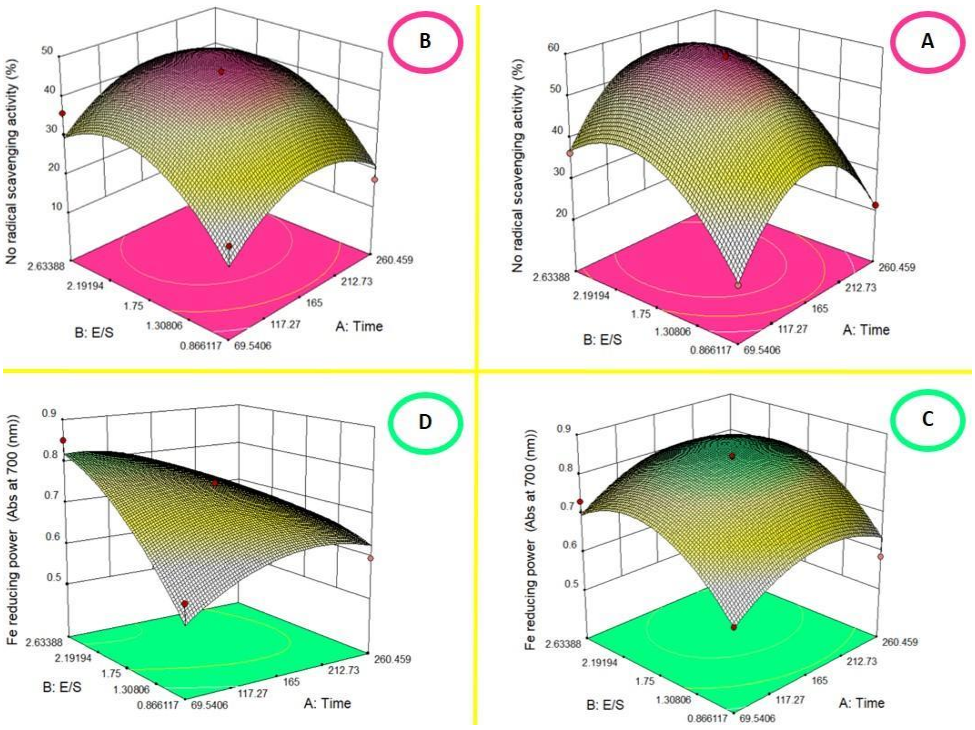

Figure 2- The effect of hydrolysis time, enzyme to substrate ratio and proteases type on NO radical scavenging activity and reducing power of protein hydrolysate protein hydrolysate of skipjack viscera (A and C: hydrolysis with alcalase, B and D: hydrolysis with pancreatin)

3-6- Effect of time, ratio of enzyme to substrate and type of protease on iron ion chelating activity

Fe ion^{2+} It acts as a catalyst in oxidation reactions, which causes rapid conversion of superoxide anion into dangerous hydroxyl radicals. Produced hydroxyl radicals can react with biological molecules and cause damage to cells and tissues [36]. According to the variance analysis results mentioned in Table 4, all the investigated variables had a significant effect on the iron ion chelating activity of the hydrolyzates obtained from alkalase, but only the time variables, A^2 and B^2 On this propertyAntioxidant Pancreatin hydrolyzates showed a significant effect(05/0>p). Reaction parameters with chelating activity of both hydrolyzates obtained from alkalase and pancreatin, quadratic and better interfaces with regression coefficient9999/0R^2= And9821/0R^2= they had. The non-significance of the misfit factor also indicates the high ability of the model in predicting the effect of process variables on the investigated response. According to the results of relational variance analysis of iron ion chelating activityAnd the variables of the hydrolysis process with alkalase and pancreatin are in relation (13) and (14).

(13)

$$Iron\ ion\ chelating\ activity = +53.61 + 1.77\ A + 5.37\ B + 0.81\ AB - 12.78A^2 - 10.38\ B^2$$

(14)

$$Iron\ ion\ chelating\ activity = +59.41 + 6.92\ A + 0.34\ B + 1.81\ AB - 6.57A^2 - 2.80\ B^2$$

As can be seen in Figure 3 A, at fixed times, with the increase of the enzyme concentration up to 1.9%, the chelating activity of the hydrolyzed alcalase increased,

but the further increase in the concentration of the enzyme had a negative effect and led to a decrease in the ability of the hydrolyzed alcalase to chelate. iron ion. On the other hand, in constant enzyme concentrations, iron ion chelating activity increased with increasing hydrolysis time up to 170 minutes and decreased with further increase in time. In general, according to the graph, in the hydrolysis with alcalase enzyme, the highest level of iron ion chelating activity (54.32%) was obtained in the hydrolysis time of 178.69 minutes and the enzyme concentration was 1.98%. In the hydrolysis with pancreatin enzyme (Figure 3B), no significant change in iron ion chelating activity of the hydrolyzed protein was created at constant times with increasing enzyme concentration. As mentioned in Table 4, enzyme concentration had no significant effect on the chelating activity of pancreatin hydrolyzates. On the other hand, at constant enzyme concentrations, increasing the hydrolysis time up to 210 minutes increased the chelating activity, but increasing the time had a negative effect. In general, the highest amount of iron ion chelating activity (61.38%) was obtained by hydrolysis with pancreatin in the hydrolysis time of 217.75 minutes and the enzyme concentration was 1.95%. Decreasing the iron ion chelation activity by increasing the hydrolysis time can be due to the decrease in the efficiency of the enzyme in the production of antioxidant peptides due to the productionEnzyme inhibiting compounds during hydrolysis are long; Also, the hydrolysis of iron ion chelating peptides that are produced early in the hydrolysis process can be another reason for the reduction of iron ion chelating activity during hydrolysis in long periods of time [26]. On the other handThe increase in the iron ion chelation activity of hydrolyzates produced by increasing the alkalase enzyme concentration up to 1.9% can be attributed to the increase in the active sites of the enzyme available for protein hydrolysis. The breakdown of more peptide bonds and ultimately the increase in the solubility of the produced peptides, which has increased the chelating activity of the produced peptides [37]. In general, the most important factors that depend on the chelating ability of hydrolyzed protein are: the nature and composition of the protein source, the type of protease used, and the degree of hydrolysis [38]. These findings are similar to the results of Klampang et al. (2007), Maqsoodlou et al. (2016) and Jamdar et al. (2010) respectively in protein hydrolysis of yellow tail fish.[10], bee and peanut pollen [34, 39 and 40].

10 -yellow stripe trevally

Table 4. ANOVA of Fe chelating activity and degree of hydrolysis of protein hydrolysate with alcalase and pancreatin

	Sum of squares	df	P value	Estimate the coefficient
		Hydrolysis with alcalase		
		Hydrolysis with pancreatin		
		ANOVA of Fe chelating activity		
Model	1452.04	5	0.0001>	53.61
	645.69	5	0.0002	59.41
A-time	25.20	1	0.0001>	1.77
	383.39	1	0.0001>	6.92
B- E/S	230.59	1	0.0001>	5.37
	0.95	1	0.5533	0.34
AB	2.66	1	0.0005	0.81
	13.18	1	0.0643	1.81
A^2	922.51	1	0.0001>	-12.78
	243.54	1	0.0002	-6.57
B^2	608.59	1	0.0001>	-10.38
	44.18	1	0.0075	-2.80
Lac of fit	0.17	3	0.3000	
	11.76	3	0.1237	
		ANOVA of degree of hydrolysis		
Model	514.81	5	0.0001>	24.42
	179.57	5	0.0432	22.37
A-time	97.37	1	0.0001>	3.49
	0.80	1	0.7420	-0.32
B- E/S	173.37	1	0.0001>	4.66
	0.75	1	0.7490	-0.31
AB	96.63	1	0.0001>	-4.92
	21.58	1	0.1303	-2.32
A^2	102.14	1	0.0001>	-4.25
	3.27	1	0.5127	0.76
B^2	88.46	1	0.0001>	-3.96
	127.62	1	0.0070	-4.75
Lac of fit	3.65	3	0.0504	
	32.84	3	0.0671	

* A: hydrolysis time B: enzyme to substrate ratio

7-3- The effect of time, the ratio of enzyme to substrate and the type of protease Degree of hydrolysis

The results of analysis of variance (Table 4) showed that all the investigated variables had a significant effect on the degree of hydrolysis of the hydrolyzed protein obtained by alcalase, but only variable B^2 It had a significant effect on the degree of hydrolysis resulting from the activity of pancreatin enzyme(05/0>p). The degree of hydrolysis had quadratic relationships with the reaction parameters of both alkalase and pancreatin enzymes, and their regression coefficient was the best.8449/0R^2= And9927/0R^2=. On the other hand, the non-significance of the lack of fit factor shows that the model has a high ability to predict the effect of the examined variables on the desired response. The results of analysis of variance showed that the relationThe variables of the hydrolysis process with alkalase and pancreatin and in the case of better hydrolysis follow the relationship (15) and (16).

(15)

$$Hydrolysis\ degree = +24.42 + 3.49\ A + 4.66\ B - 4.92\ AB - 4.25A^2 - 3.96\ B^2 \quad (16)$$

$$Hydrolysis\ degree = +22.37 - 0.32A - 0.31\ B - 2.32\ AB + 0.76A^2 - 4.75\ B^2$$

According to Figure 3C, at fixed times and less than 220 minutes, the degree of hydrolysis increased with increasing enzyme concentration, but at times longer than 220 minutes, with increasing enzyme concentration up to 2.3%, the degree of hydrolysis increased, but with further increase in enzyme concentration, it decreased. Found. On the other hand, in constant enzyme concentrations, increasing the time up to 240 minutes increased the degree of hydrolysis, but further increasing the hydrolysis time had a negative effect on this parameter. In general, the highest degree of hydrolysis (25.62%) was obtained with alcalase enzyme in 186.20 minutes and the enzyme concentration was 2.32%. In the case of hydrolyzates obtained from pancreatin (Figure 3D), at constant enzyme concentrations, changing the hydrolysis time did not have a significant effect on the degree of hydrolysis. On the other hand, at constant times, increasing the enzyme concentration up to 2.2% increased the degree of hydrolysis, and further increase had a negative effect. But in general, these changes were not noticeable because according to Table 4, the effect of time and enzyme concentration on the degree of hydrolysis resulting from pancreatin activity was not significant. The highest degree of hydrolysis (23.60%) was obtained at the hydrolysis time of 71.68 and the enzyme concentration was 1.95%. Therefore, the highest degree of hydrolysis was achieved using alcalase enzyme. Similar to these findings, Kaveh et al. (2007) and Kang et al. (2007) also reported better treatment with the hydrolysis of fenugreek protein and wheat gluten, and the degree of hydrolysis of alkalase was higher than that of pancreatin [42 and 41]. The decrease in the degree of hydrolysis with an excessive increase in time can be caused by excessive hydrolysis of the protein substrate and the inhibitory effect of the final peptides produced [43]. On the other hand, the decrease in the rate of enzymatic hydrolysis can be caused by the decrease in the number of peptide bonds available for hydrolysis, as well as the inactivation of the protease enzyme [44]. These results are similar to the findings of Sharaft et al. (2013), Yu et al. (2009) and Yasmi et al. (2013) on better treatment in the hydrolysis of waste protein after cooking hoover fish, tian fish and guts and viscera of carp fish, which report did, the degree of hydrolysis decreased with an excessive increase in the hydrolysis time [45-47].

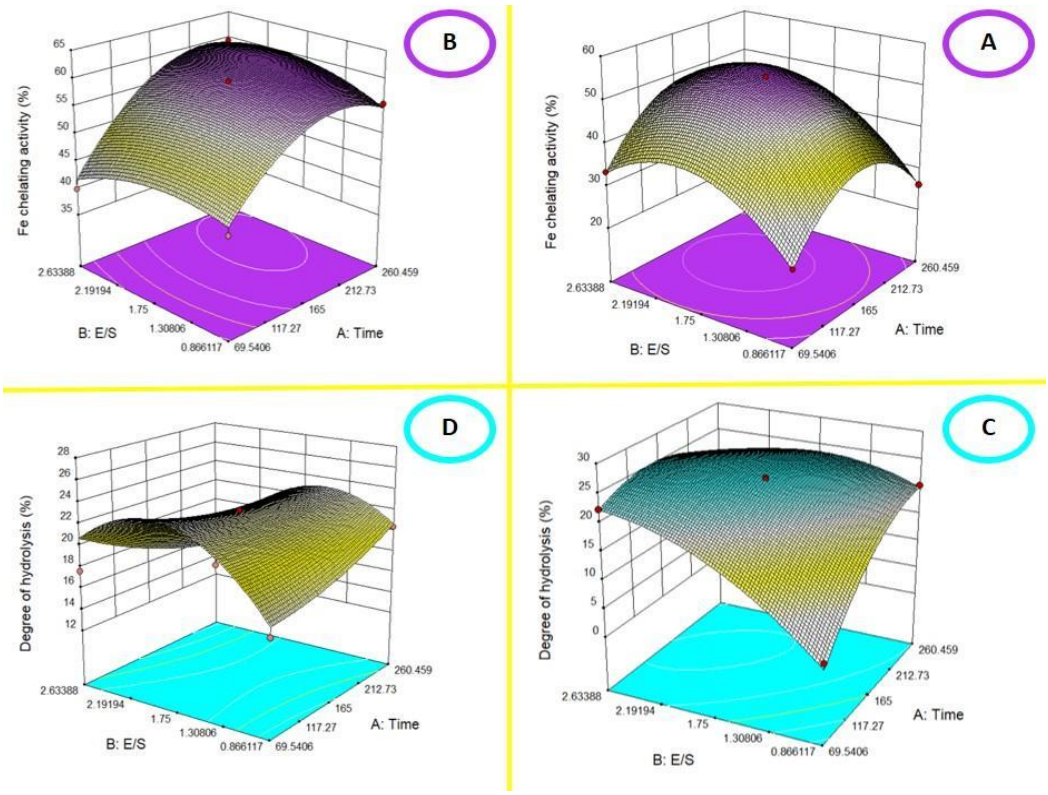

Figure 3- The effect of hydrolysis time, enzyme to substrate ratio and proteases type on Fe chelating activity and degree of hydrolysis of protein hydrolysate of skipjack viscera (A and C: hydrolysis with alcalase, B and D: hydrolysis with pancreatin)

8-3- Selection of optimal treatment and validation of the model

After analyzing the data, the software predicted that the highest DPPH radical inhibition activity (49.78 percent), total antioxidant capacity (0.971 absorbance at 695 nm), nitric oxide radical inhibition (58.82 percent), Reduction of iron ion (0.771 absorbance at 700 nm) with alkalase enzyme during hydrolysis time of 146.9 minutes and enzyme concentration of 1.94% and the highest rate of iron ion chelation (59.46%) with pancreatin enzyme during hydrolysis time is 67. 171 minutes and the enzyme concentration is 2.17%. In these conditions, the degree of hydrolysis resulting from alkalase activity was predicted to be higher than that of pancreatin at the rate of 24.65%. In order to evaluate the validity of the model, hydrolysis was performed under these conditions. DPPH radical scavenging activity, total antioxidant capacity, nitric oxide radical scavenging, reduction of iron ion with Alcalase enzyme Bettertib 47.56%, absorbance 0.850 at 695 nm, 55.82% and 0.812 absorbance at 700 nm and chelating activity The iron ionization of the hydrolyzed protein obtained from pancreatin was calculated to be 60.57%. The degree of hydrolysis resulting from alkalase activity was also higher than pancreatin and beteratib by 25.12% and 20.35%. These results indicate the very good ability of the model to predict the effect of time and enzyme concentration variables on the hydrolysis process.

4 - Conclusion

This research was conducted with the aim of investigating the effect of hydrolysis conditions and type of protease on the antioxidant properties and degree of hydrolysis of the hydrolyzed protein obtained

from the intestines and viscera of Muscat Hoover fish. The results showed that the antioxidant properties of hydrolyzed protein are influenced by the type of enzyme, hydrolysis time and enzyme concentration. Comparison of the antioxidant activity of hydrolyzed products from alkalase and pancreatin showed that alkalase enzyme as an endopeptidase led to the production of hydrolyzed protein with stronger antioxidant properties than pancreatin. Studies have shown that the antioxidant properties of hydrolyzed protein depend on the conditions of hydrolysis (temperature, time and enzyme concentration), the degree of hydrolysis and the sequence and amino acid composition of peptides. In general, according to the results of this research, the hydrolyzed protein obtained from the intestine and viscera of Hoover Muscat fish by alcalase enzyme has a very high antioxidant potential at the time of hydrolysis and the concentration of the enzyme, which can compete with synthetic antioxidant compounds widely used in the food industry and It is used in food formulations to produce beneficial products.

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بهینه سازی شرایط هیدرولیز و نوع پروتئاز بر درجه هیدرولیز و ویژگی‌های ضداکسایشی پروتئین هیدرولیز شده حاصل از امعاء و احشا ماهی هوور مسقطی(*Katsuwonus pelamis*) با استفاده از روش سطح پاسخ

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چکیده

اکسیداسیون در اندام‌های زنده باعث بیماری‌های خطرناکی از جمله سرطان و در مواد غذایی باعث فساد و ضررهای اقتصادی سنگینس می‌گردد. آنتی اکسیدان‌های سنتزی عوارض نامطلوب و خطرناکی بر سلامتی انسان دارند، از این رو شناسایی ترکیبات ضداکایشی طبیعی از نیازهای اصلی صنعت مواد غذایی است. در صنایع فرآوری ماهی‌ها حدود ۷۰-۵۰ ٪ ماهی اولیه که منابع بالقوه‌ای از ترکیبات با ارزش تغذیه‌ای مانند آمینو اسیدهای ضروری هستند به‌عنوان ضایعات تولید می‌شود. بنابراین یافتن راهی جهت استفاده‌ی بهینه از این ضایعات و تولید ترکیبات سلامتی‌بخش با ارزش افزوده‌ی بالا مانند پپتیدهای زیست فعال از اهمیت زیادی برخوردار است. در این پژوهش به بررسی تاثیر شرایط هیدرولیز (زمان: ۳۰۰-۳۰ دقیقه و غلظت آنزیم ۳-۰/۵ درصد) و نوع پروتئاز (آلکالاز و پانکراتین) بر درجه هیدرولیز و ویژگی‌های ضداکسایشی (مهار رادیکال DPPH، شلاته کنندگی یون آهن، مهار رادیکال نیتریک اکسید، ظرفیت ضداکسایشی کل و احیاء کنندگی یون آهن) پروتئین هیدرولیز شده حاصل از امعاء و احشا ماهی هوور مسقطی با استفاده از روش سطح پاسخ پرداخته شد. نتایج نشان داد که شرایط بهینه جهت دستیابی به بیشترین ویژگی‌های ضداکسایشی با آلکالاز و پانکراتین به‌ترتیب عبارت بودند از: زمان هیدرولیز ۱۴۶/۹ و ۱۷۱/۶۷ دقیقه و غلظت آنزیم ۱/۹۴ و ۲/۱۷ درصد؛ در این شرایط درجه هیدرولیز پروتئین‌های هیدرولیز شده‌ی تولیدی ۲۵/۱۲ درصد و ۲۰/۳۵ درصد محاسبه شد. مقایسه ویژگی‌های ضداکسایشی هیدرولیز شده‌های تولیدی با هردو پروتئاز نشان داد که آنزیم آلکالاز منجر به تولید پروتئین هیدرولیز شده با خواص ضداکسایشی قوی‌تری نسبت به پانکراتین شد. بنابراین می‌توان نتیجه گرفت که پروتئین هیدرولیز شده امعاء احشا ماهی هوور مسقطی با استفاده از آنزیم آلکالاز به‌عنوان یک محصول سلامتی بخش و با ارزش افزوده قابلیت کاربرد در تولید محصولات غذایی فراسودمند و مکمل‌های سلامتی بخش استفاده نمود.