Iranian Journal of Fisheries Sciences 21(6) 1397-1416 2022
DOI: 10.22092/ijfs.2022.351005.0

**Research Article**

# Effect of enzyme type, enzyme to substrate ratio, temperature and hydrolysis time on the yield and antioxidant effect of *Saurida tumbil* protein hydrolysate

**Bahram S.[1*]; Khezri M.[2]; Javadian S.R.[1]**

Received: May 2020 Accepted: December 2020

**Abstract**

It has become a hot topic to make optimal use of fisheries products and make them value added as well as find natural food substances which can effectively inhibit oxidative stress and improve human health. Given this, *Saurida tumbil* muscle was hydrolyzed by Alcalase and Papain at two concentrations of 2 and 4%, and at two hydrolysis times of 90 and 180 min and their antioxidant properties were compared. Antioxidant activity of the protein hydrolysates were investigated by ferrous chelating and ferric reducing, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydroxyl free radical scavenging activity tests. Samples hydrolyzed by Papain showed higher protein recovery than those hydrolyzed by Alcalase (59.9% versus 20.06%). The protein hydrolysate prepared by Papain enzyme showed significantly higher activity in removal of DPPH ($IC_{50}$=2.44 mg/mL) and hydroxyl (IC50=3.93 mg/mL) free radicals than those prepared by Alcalase enzyme (DPPH IC50=2.80 mg/mL, hydroxyl IC50=8.59 mg/mL). ABTS radical scavenging activity did not show any significant difference between hydrolyzed samples with Papain or Alcalase enzymes. Hydrolyzed samples with Alcalase enzyme showed higher ferrous chelating activity (IC50=5.33 mg/mL) and ferric reducing activity (optical absorption=0.13±00 at 700 nm) than those hydrolysate prepared by Papain enzyme. Generally, the type of enzyme, its concentration and the time of hydrolysis affected the yield and antioxidant properties of *Saurida tumbil* muscle protein hydrolysates and hydrolysis with papain at concentration of 4% for 180 min provided the best properties.

**Keywords:** Alcalase, Papain, Fish Protein Hydrolysate, *Saurida tumbil*, Antioxidative peptides

1-Islamic Azad University, Department of Fisheries, Qaemshahr Branch, Qaemshahr, Iran.
2-Department of Fisheries, Natural Resources Faculty, University of Kurdistan, Sanandaj, Kurdistan, Iran.
*Corresponding author's Email: Bahramsomi@gmail.com

## Introduction

Today new insights on food are emerged and food is perceived not only as a source of necessary nutrients for humans but also as a carrier of healthy compounds that have a positive role on improving physical and mental well-being and preventing nutrition related diseases (Siro *et al*., 2008; Rabiei *et al*., 2017; Shaviklo *et al*., 2019; Tkaczewska *et al*., 2020). Protein hydrolysis is a process in which the protein is broken down into small peptides and short amino acid chains with wide physiological functions including antioxidant, antimicrobial, anticancer, immunomodulatory, hypolipidemic, and hypoglycemic effects. Moreover, these peptides have low molecular weight, high absorption rate, low sensitivity, and high stability under various conditions, which offer major potential for incorporation into functional foods and nutraceuticals (Taheri *et al*., 2013; Rabiei *et al*., 2019b). Many of the physiological and functional properties of proteins are related to the bioactive peptides present in their structure, however, these peptides are inactive in the precursor protein sequence until released by proteolytic enzymes (Rabiei *et al*., 2019a).

Moreover, the production of reactive oxygen species such as hydroxyl, hydrogen peroxide, hydroproxyl radicals and etc. are unavoidable consequences of respiration in aerobic organisms. These free radicals are the most important initiators of the oxidation chain reaction, causing serious negative problems for human health and also resulting in economical loss due to the oxidation and quality loss of high-fat foods during storage. So, studying and using antioxidant compounds are of hot topics in the fields of human food and health. Since synthetic antioxidants may act as mutagenic and carcinogenic agents, there has been a growing interest in replacing artificial and synthetic antioxidants with natural ones (Kim, 2011; Khezri *et al*., 2016).

Researches have shown that fish protein hydrolysates (FPHs) have nutritional, antioxidant, antimicrobial, anticoagulant, anticancer, antihypertensive and several other physiological activities (Slizyte *et al*., 2016; Halim *et al*., 2018). The antioxidant activity of these hydrolysates are related to their ability to remove free radicals, act as a metal chelating agent, oxygen extinguisher or hydrogen donor, and prevent the intrusion of initiators of lipid oxidation through the formation of a layer around oil droplets (Elavarasan *et al*., 2014).

Marine resources accounting for about half of the world's biodiversity, are a valuable source of bioactive compounds that can be used in production of functional foods (Rabiei *et al*., 2019b). Less popular fish, by-catch species and fisheries processing wastes are ideal raw material for fish protein hydrolysate (FPH), which can be used as natural bioactive and preservative compounds, food binders, emulsifiers, gelling agents, and nutritional supplements as well as cryoprotectant and nutritional additive in liquid

fertilizer and aquafeed (Lima and Porto, 2016).

Enzymatic hydrolysis has been used as the most common and effective method to produce peptides with better nutritional and functional properties (Kim, 2011; Raftani Amiri *et al*., 2016; Halim *et al*., 2018; Noman *et al*., 2019). Interestingly, it is possible to produce peptides of specific size and better biological properties by controlling the enzymatic hydrolysis parameters including type of protease enzyme, enzyme to substrate ratio, substrate concentration, incubation duration, temperature and pH (Noman *et al*., 2019; Tkaczewska *et al*., 2020).

So far, many studies have been conducted on the producing of protein hydrolysates from wastes and muscle of different commercial, less popular or by-catch fish species. Also, the effect of hydrolysis conditions on the characteristics of fish protein hydrolysates has been investigated. For example, Lima *et al*. (2019) prepared protein hydrolysates from whitemouth croaker (*Micropogonias furnieri*) using the enzyme Alcalase (2/100 enzyme/protein) at 2, 4 and 8 h of hydrolysis. They reported that the highest degree of hydrolysis and anti-oxidation activity were observed for the longest hydrolysis time (Lima *et al*., 2019). In another study conducted by Hassan *et al*. (2019) the antioxidant properties of *Pangasius* viscera protein hydrolysate prepared by using enzymatic (papain and pepsin), and chemical methods (hydrochloric acid and sodium hydroxide) were evaluated. They concluded that the visceral protein hydrolysate prepared with pepsin had better overall quality and antioxidant properties and papain in nutritional point of view (Hassan *et al*., 2019). Parihanto *et al*. (2019) studied FPH prepared from parrotfish (*Chlorurus sordidus*) heads using different pHs (5, 7 and 9), durations (12 and 24 h) and sample ratio. The researchers found that the pH and duration of hydrolysis significantly affected the characteristics of FPH and the highest yield of hydrolysate and antioxidant activity was obtained at pH 9 after 24 h incubation (Parihanto *et al*., 2019). Mehregan Nikoo *et al.* (2014) studied the effect of temperature, hydrolysis time and enzyme-to-substrate ratio on the degree of hydrolysis and antioxidant activity of protein hydrolysate of *Carassius carassius* (Mehregan Nikoo *et al*., 2014). Tkaczewska *et al*. (2020) studied the effects of hydrolysis condition (pH, temperature and incubation duration of the hydrolysis) on the antioxidant activity of protein hydrolysate from *Cyprinus carpio* skin gelatin. The researchers reported that protein hydrolysates obtained using Protamex showed potent antioxidant properties, which differed depending on the hydrolysis parameters. The carp skin gelatin hydrolysates with the highest antioxidant properties were obtained using Protamex at pH 7 and a temperature of 50°C for 3 h (Tkaczewska *et al.*, 2020).

*Saurida tumbil*, also known as Kijar-e-bozorg (in Persian), Hasoom (in Arabic), Lizardfish (in English), Anoli

tumbil (in French), Lagarto tumbil (in Spanish), is a relatively abundant, small and less popular fish that is also reported as an important by-catch in shrimp trawlers in the Indo-Pacific waters. The annual catch of Greater lizardfish in 2018 was 21514 tons for Asia's marine waters and 6372 tons for southern waters of Iran (Persian Gulf and Oman Sea) (FAO, 2016). The proximate composition of *Saurida tumbil* is reported as: moisture 76.93%, Crude Protein 17.95%, Crude Fat 2.30% and Ash 1.60% (Joshi *et al*., 2015). Due to the low price and less popularity of *Saurida tumbil* as well as its high protein content (approximately 17-20% of the raw weight) (Jitesh *et al*., 2011), greater lizardfish can be considered as an inexpensive source of complete protein for producing FPH and other functional compounds.

Although many studies have carried out on production of fish protein hydrolyates, no study has been conducted on the producing of protein hydrolysates from *Saurida tumbil.* Therefore, the aim of this study was to investigate the effect of hydrolysis condition including enzyme type, enzyme concentrations and hydrolysis time on the antioxidant properties of *Saurida tumbil* protein hydrolysate.

## Materials and methods

*Fish preparation*

The present study was performed in laboratory of Mazandaran Science and Technology Park, Sari, Mazandaran, Iran and in seafood science laboratory of Islamic Azad University of Ghaemshahr, Mazandaran, Iran in 2017. Fresh *Saurida tumbil* was purchased from fish local market in Bushehr, Iran, then freezed at – 20 °C and transported on crushed ice to the laboratory. After thawing at refrigerator, fish were washed, deboned and filleted. Deboned skin off fiellets were washed again and then minced twice (mince yield=44.6%), then were kept in several plastic zip top bags at -20°C until use.

*Enzymatic hydrolysis of fish protein*

*Saurida tumbil* mince were thawed in the refrigerator, a portion of 50 gr was mixed with sodium phosphate buffer (pH=8 for Alcalase enzyme, pH=6 for Papain enzyme) at a ratio of 1: 2 (w:v), homogenated and then heated at 85°C for 20 min to inactivate the endogenous enzymes using shaker water bath. Then, the mixture was cooled and to which the Alcalase and Papain enzymes at ratios of 2% and 4% (based on the protein content of the sample) were added and hydrolysis was carried out for 90 and 180 min at 55°C in shaker water bath. The pH of the hydrolysis mixture was kept at 8 (optimum pH of Alcalase) and 6 (optimum pH of Papain) by adding respectively diluted NaOH and HCl. In the next step, the mixture was heated at 95°C for 15 min to stop the enzymatic reaction. Then, the resulting mixture was centrifuged at 8000g for 30 min at 4°C and the supernatant was collected and freeze dried. The resulting protein hydrolysates was weighed and kept in plastic zip top bags at -20 °C until use (Ovissipour *et al*., 2009; Rabiei *et al*., 2019b).

*Measurement of chemical composition of fish*

Moisture, fat, and ash contents were measured by AOAC (2002) and protein content was measured by AOAC (2005) method.

*Determination of protein hydrolysis yield*

In order to determine the protein hydrolysis yield, the amount of soluble protein in the supernatant obtained from enzymatic hydrolysis was determined using Biuret method (Layne, 1975). The protein hydrolysis yield was calculated using the following formula (James, 2013):

Protein hydrolysis yield (%)=(Supernatant soluble protein content/(total protein of fish mince) × 100

*Evaluation of antioxidant activity*

*DPPH radical scavenging activity*

Briefly, 1 mL of DPPH solution (0.1 mM DPPH prepared in 95% ethanol) was added to 1 mL of FPH at different concentrations of 0.5, 1 and 2 mg/mL and incubated for 15 min in the dark condition at room temperature. Then the absorbance of the mixture was recorded at 517 nm against blank sample (distilled water instead of the FPH) (Alemán *et al*., 2011). DPPH radical scavenging activity was calculated using the following formula (Gülçin, 2006):

DPPH radical scavenging activity (%) = $[(A_{blank}-A_{sample}) /A_{blank}]\times 100$

The $IC_{50}$ value ($IC_{50}$ value is defined as the concentration of the sample required to inhibit 50% of free radicals) was obtained by drawing a graph of the protein hydrolysate concentration (X-axis) against the scavenging activity (Y-axis) (Rabiei *et al*., 2019b).

*Ferric reduction activity test*

To measure the reducing power of ferric ion ($Fe^{3+}$) by protein hydrolysates, 2 mL of the hydrolyzed protein sample was combined with 2 mL of phosphate buffer (0.2 M, pH=6.6, to which 2 mL of 1% sodium ferricyanide was added. The mixture was kept at 50°C for 20 min, and to which 2 mL of trichloroacetic acid solution (10%) was added. The mixture was centrifuged at 1650 g for 10 min. 2 mL of the top layer of the solution was mixed with 2 mL of distilled water and 4 mL of 0.1% FeCl3 solution. After incubation for 10 min at room temperature, the absorption of the samples at 700 nm was recorded (Alemán *et al*., 2011).

*Hydroxyl radical scavenging activity*

Briefly, one mL of working solution (1.865 mM 1, 10-phenanthroline) was added to 2 mL of FPH solution. The mixture was homogenized and to which 1 mL of $H_2O_2$ (3% v/v) was added. The mixture was kept for 60 min at 37 °C in

a water bath, then the absorbance was recorded at 536 nm. The same prepared sample without added $H_2O_2$ was regarded as blank and the same prepared sample without added FPH was regarded as negative control. Hydroxyl radical scavenging activity was measured using the following formula (Gülçin, 2006):

Hydroxyl radical scavenging activity (%) = $[(A_{sample}-A_{negative\text{-}control})/(A_{blank}-A_{negative\text{-}control})]\times 100$

The $IC_{50}$ value was obtained by drawing a graph of the protein hydrolysate concentration (X-axis) against the scavenging activity (Y-axis) (Rabiei *et al*., 2019b).

*Ferrous chelating activity*

1 mL of hydrolyzed protein solution was mixed with 0.1 mL of 2 mM solution of $FeCl_2$ and 0.2 mL of ferrozine 5 mM and mixed with distilled water to a volume of 5 ml. The mixture was kept at room temperature for 20 min and then the absorption was read at 562 nm. Instead of the sample, distilled water was used in the control. The inhibitory activity was obtained through the following equation (Alemán *et al*., 2011):

$Fe^{2+}$ chelating activity (%) = [(Acontrol - Asample)/Acontrol] ×100

The $IC_{50}$ value was obtained by drawing a graph of the protein hydrolysate concentration (X-axis) against the scavenging activity (Y-axis) (Rabiei *et al*., 2019b).

*ABTS radical scavenging activity*

Freshly prepared ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6- sulphonic acid) solution (reacting 10 mL of 7.4 mM ABTS with 10 mL of 2.6 mM potassium persulfate for 12h in the darkness at room temperature) was diluted with methanol to obtain an absorbance of 1.1±0.02 at 734 nm. Then, 2850 µl of ABTS solution was added to 150 µl of FPH solution at different concentrations and after incubation at 23±2°C for 1 h, the absorbance was recorded. Blank sample was prepared using 150 µL of distilled water instead of the FPH. ABTS scavenging activity was determined using the following formula (Gülçin, 2006):

ABTS scavenging activity (%) = $[(A_{blank}-A_{sample})/A_{blank}]\times 100$

The $IC_{50}$ value was obtained by drawing a graph of the protein hydrolysate concentration (X-axis) against the scavenging activity (Y-axis) (Rabiei *et al*., 2019b).

*Statistical analysis*

All experiments were carried out in triplicate and the results were presented as mean±standard deviation. Data were analyzed using SPSS 22 software. The normal distribution of data was checked

using Kolmogorov-Smirnov test. Analysis of Variance (ANOVA) followed by Duncan's test was used to identify statistical differences between means. P value less than 0.05 was considered statistically significant.

## Results

*The approximate composition of Saurida tumbil muscle*

The approximate composition of *Saurida tumbil* muscle (%) was as follows: Moisture=74.80±1.99; protein= 19.71 ± 1.68; lipid = 4.41 ± 0.09 and ash = 1.29 ± 0.01

*The yield of Saurida tumbil muscle protein hydrolysis*

As presented in Figure 1, the yield of protein hydrolysate was significantly higher for Papain compared to Alcalase enzyme ($p$<0.05). In both Alcalase and Papain hydrolysis, with the increase in enzyme concentration and the duration time of hydrolysis the yield of hydrolyzed protein was significantly increased ($p$<0.05). The highest protein yield (59.9%) was observed for 4% Papain enzyme for 180 min ($p$<0.05). These results indicated the high efficiency of the Papain enzyme in the hydrolysis of fish mince.

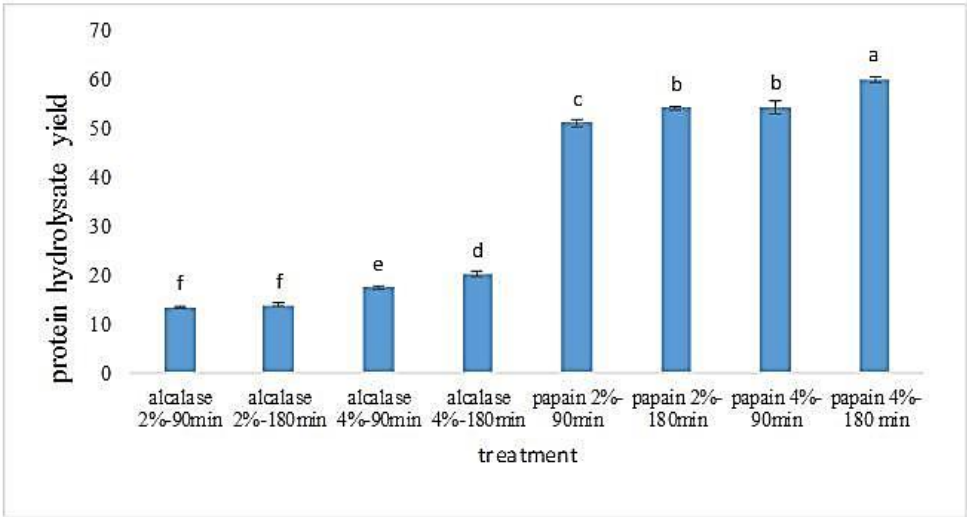


**Figure 1: The yield of protein hydrolysate of *Saurida tumbil* muscle.**

*Inhibitory activity of DPPH free radicals*

As presented in Figure 2-A, for both Alcalase and Papain treatments, the inhibitory activity of different concentrations of protein hydrolysates against DPPH showed significant increase with increase in enzyme concentration from 2 to 4 % and also with increase in hydrolysis time from 90 min to 180 min ($p$<0.05). The most inhibitory activity was observed for protein hydrolysis at concentration of 2 mg/mL prepared by 4% Papain for 180 min (43.72%) followed by 4% Alcalase for 180 min (40.77 %) ($p$<0.05). Moreover, 4% papain treatment provided the least $IC_{50}$ (2.44 mg/mL) for inhibition of DPPH radicals ($p$<0.05) (Fig. 2-B).

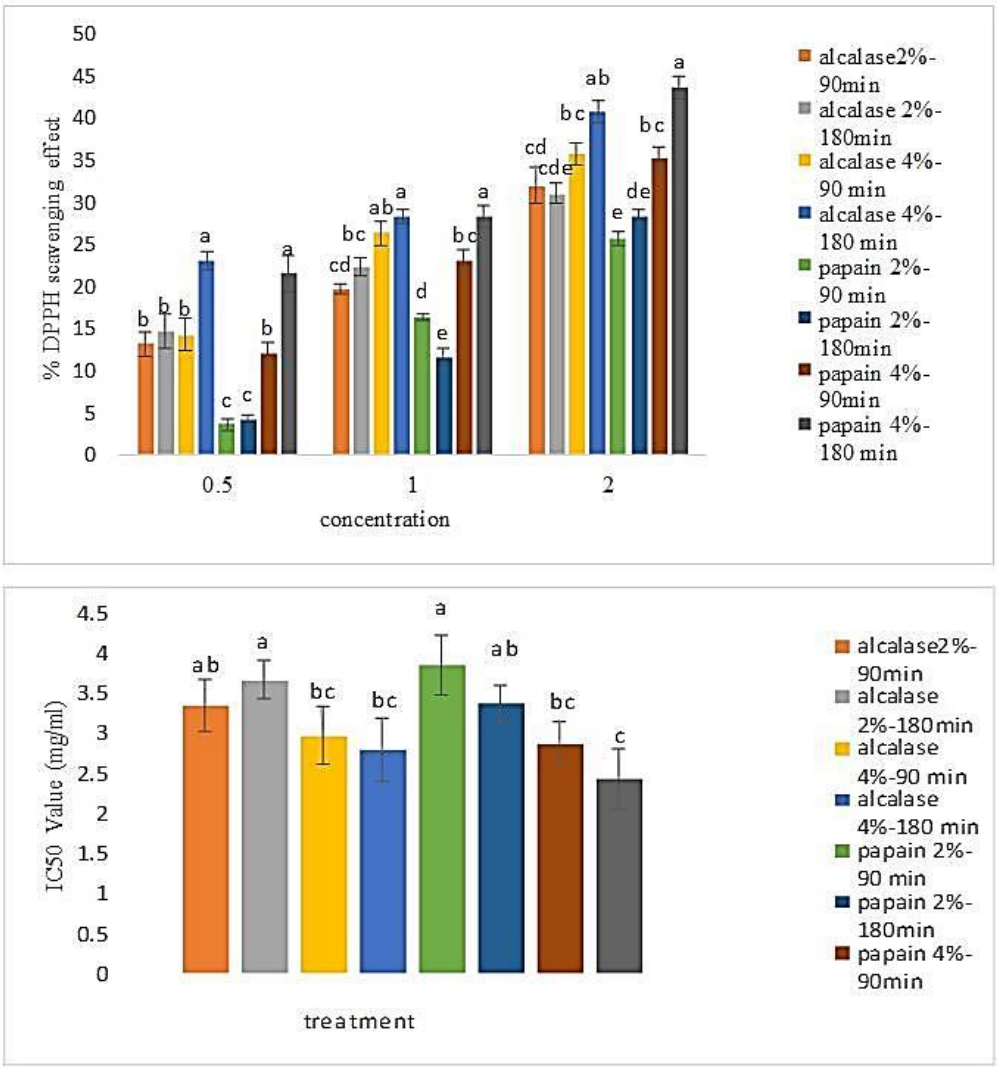


**Figure 2: DPPH free radical inhibitory activity (A) and $IC_{50}$ of DPPH free radical (B) of *Saurida tumbil* muscle protein hydrolysate.**
**$IC_{50}$ values of BHT as standard against DPPH free radical=0.047±0.01 mg/mL**

*Inhibitory activity of hydroxyl free radicals*

As presented in Figure 3-A, in all concentrations of peptide the Papain hydrolysis provided higher activity for inhibition of hydroxyl free radicals in comparison with Alcalase hydrolysis ($p$<0.05). The results of Figure 3-B also showed that Papain hydrolysis provided lower $IC_{50}$ compared to the Alcalase hydrolysis and the lowest $IC_{50}$ of hydroxyl radicals (3.93 mg/mL) was observed for 4% Papain for 180 min. Moreover, this treatment showed the highest hydroxyl radical scavenging activity (35.32 %) ($p$<0.05).

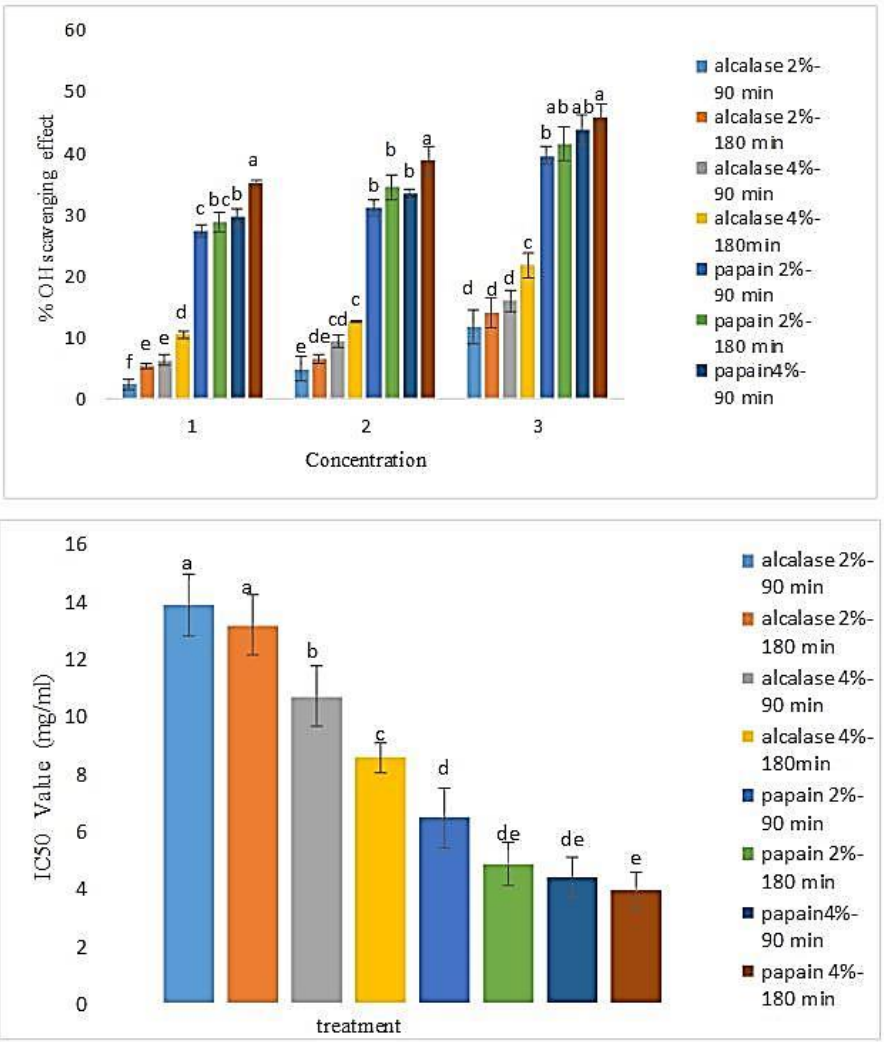


**Figure 3: Hydroxyl free radical inhibitory activity (A) and $IC_{50}$ of Hydroxyl free radical (B) of *Saurida tumbil* muscle protein hydrolysate.**
**$IC_{50}$ values of BHT as standard against Hydroxyl free radical= 0.24±0.02 mg/mL**

*Inhibitory activity of ABTS free radicals*

The results of inhibitory activity of ABTS free radicals are shown in Figure 4. With the increasing of the concentration of peptide from 0.5 to 2 mg/mL the inhibitory activity against ABTS free radicals was significantly increased ($p$<0.05). Overall, protein hydrolysis with Alcalase enzyme showed higher ABTS free radical inhibitory activity compared to protein hydrolysis with Papain enzyme (Fig. 4-A). Moreover, the lowest $IC_{50}$ of ABTS free radical (1.14 mg/mL) was observed for protein hydrolysis produced by 4% Alcalase for 90 min (Fig. 4-B).

*Ferrous (Fe 2+) chelating activity*

As shown in Figure 5-A, in all concentrations of peptide the Alcalase hydrolysis provided peptides with higher ferrous chelating activity compared to the Papain enzyme ($p$<0.05).

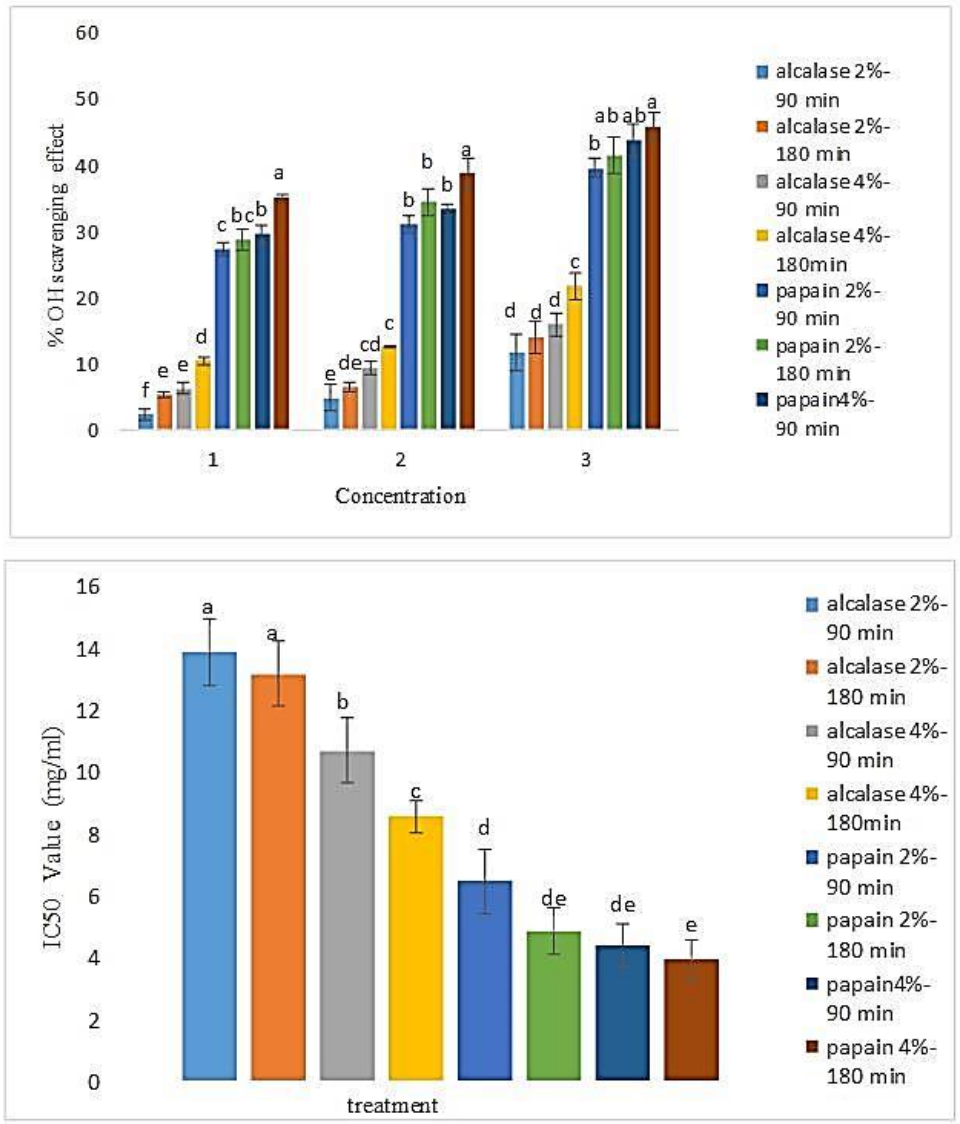


**Figure 4: ABTS free radical inhibitory activity (A) and $IC_{50}$ of ABTS free radical (B) of *Saurida tumbil* muscle protein hydrolysate.**
**$IC_{50}$ values of BHT as standard against ABTS free radical = 0.021±0.009 mg/mL**

Also, the highest ferrous chelating activity was observed for concentration of 3 mg/mL peptide provided by 2% Alcalase (26.95 and 27.89% for 90 and 180 min of hydrolysis time, respectively). Moreover, the treatment of 2% Alcalase in both hydrolysis times of 90 and 180 min showed the lowest $IC_{50}$ of ferrous free radicals ($p$<0.05, Fig. 5-B).

*Ferric ($Fe^{3+}$) reducing activity*

As shown in Figure 6, the ferric reducing activity was higher in samples hydrolyzed with Alcalase compared to the Papain enzyme ($p$<0.05). The highest ferric reducing activity was observed for protein hydrolyzed using 2% Alcalase for 180 min.

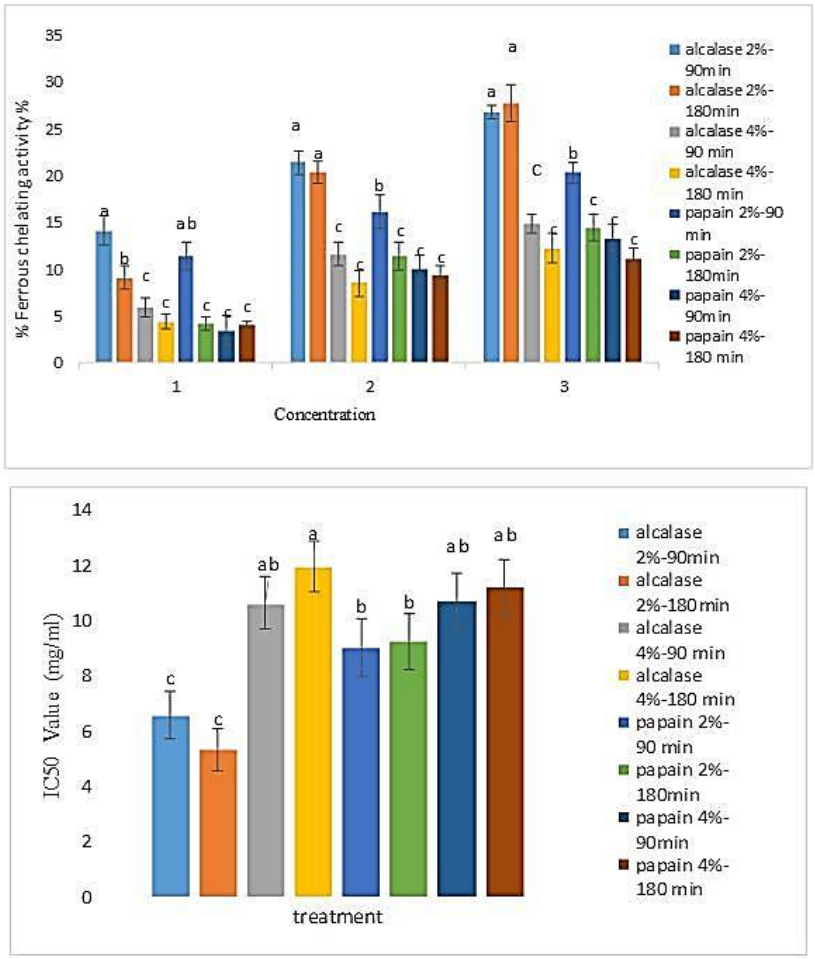


**Figure 5: Ferrous chelating activity (A) and $IC_{50}$ of ferrous chelating (B) of *Saurida tumbil* muscle protein hydrolysate.**
**$IC_{50}$ values of BHT as standard $Fe^{2+}$ chelating activity = 0.057±0.01 mg/mL**

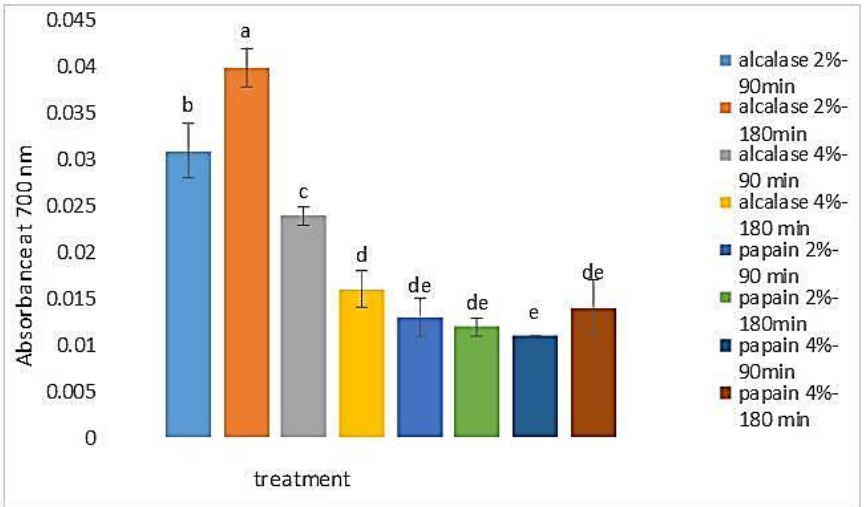


**Figure 6: Ferric reducing activity of protein hydrolysis of *Saurida tumbil* muscle at concentration of 10 mg/mL.**
**Optical absorbance of BHT as standard in ferric reducing activity = 1.16±0.09 mg/mL**

**Discussion**

The type of protease enzyme, enzyme to substrate ratio, substrate concentration, hydrolysis time, temperature and pH are of the most important factors affecting the characteristics of protein hydrolysates (Noman *et al*., 2019; Tkaczewska *et al*., 2020). In the present study, the effects of two enzymes of Alcalase and Papain at two concentrations of 2 and 4% and two hydrolysis times of 90 and 180 min were investigated on the yield and antioxidant activity of protein hydrolysates prepared from *Saurida tumbil* muscle.

In our study, with increasing of the enzyme concentrations and hydrolysis time in both Alcalase and Papain enzymes, the yield of protein hydrolysates increased and the highest protein hydrolysate yield (59.9%) was observed in hydrolyzed samples with 4% Papain enzyme for 180 min. This may be due to the increase of the solubility of proteins in the solvent by increasing the concentration of the enzyme and hydrolysis time. Protein hydrolysate yield indicates the ability of an enzyme to separate the proteins of a substance and depends on the characteristics of the substrate, the conditions of hydrolysis and the activity of the enzyme (Rabiei *et al*., 2019a; Rabiei *et al*., 2019b). It is quite clear that the different enzymes have different capabilities in protein extraction according to their specific nature, concentration and process time. Yasemi *et al*. (2013) investigated the effects of enzyme (Alcalase, Papain and Protamex), temperature and hydrolysis duration on the yield of protein hydrolysates prepared from viscera of *Aristichthys nobilis* and found that with increasing of the temperature and duration of hydrolysis the yield of protein hydrolysates increased which are in line with our study. However, the researchers reported that the highest yield was observed for protein hydrolysates prepared by Alcalase followed by Papain and Protamex (Yasemi *et al*., 2013), which are in contradictory to our results. On the other hand, the higher protein hydrolysate yield provided by Papain compared to Alcalase in our study was in line with the results of Noman *et al.* (2020) who reported that Papain was the more effective than Alcalase in providing the highest degree of hydrolysis and yield of protein hydrolysate from Chinese sturgeon (*Acipenser sinensis*). The different results of published researches may be due to the difference in substrate properties, enzyme properties and hydrolysis conditions.

In the present study, the inhibitory activity of DPPH and hydroxyl free radicals increased with the increasing in the peptide concentration. In consistent with our study, a direct link between increasing peptide concentrations and increasing the inhibitory activity of free radicals was reported in many studies. The researchers attributed this result to an increase in the degree of protein hydrolysis and its direct association with increased antioxidant activity (Centenaro *et al*., 2011; Zhang *et al*., 2015; Noman *et al.*, 2020). Moreover, in our study with the increase in

concentration of enzymes (Alcalase or Papain) and hydrolysis time the inhibitory activity against DPPH and hydroxyl free radicals increased. The highest inhibitory activity against DPPH and hydroxyl free radicals was observed for FPH prepared by 4% Papain enzyme for 180 min. The increasing inhibitory activity against free radicals with increasing hydrolysis time and enzyme concentration can be related to the more hydrolysis of proteins and producing smaller peptides (Halim *et al*., 2018; Lima *et al*., 2019; Parihanto *et al*., 2019). In another study, Esmaeili Kharyeki *et al*. (2018) reported higher DPPH scavenging activity of *Skipjack tuna* head protein hydrolysate prepared by Alcalase with the increasing of the hydrolysis time from 14 to 240 min. They attributed this effect to an increase in the degree of hydrolysis and smaller size of the peptides, and stated that low molecular weight peptides and amino acids have very high antioxidant activity (Esmaeili Kharyeki *et al*., 2018). Also, Slizyte *et al.* (2016) conducted a study on salmon bone protein hydrolysate using different enzymes and reported that samples hydrolyzed with a mixture of Papain and Bromelain enzymes had the highest DPPH radical inhibition activity and the ability to inhibit free radicals increased with increasing hydrolysis time from 20 minutes to 120 minutes (Slizyte *et al*., 2016), which is consistent with the results of the present study. Moreover, it is reported that by increasing the concentration of the enzyme to a certain extent, due to the increase in the reaction rate, the amount of antioxidant activity increases (Khajavi *et al*., 2016). In the present study, with increasing enzyme concentrations from 2% to 4% in all hydrolyzed protein concentrations, the percentage of DPPH and hydroxyl free radicals inhibition increased, which is in line with the results of other studies in this field.

Lower values of $IC_{50}$ indicate higher antioxidant activity. In the present study, the lowest $IC_{50}$ of DPPH and hydroxyl free radical scavenging (2.44 and 3.93 mg/mL, respectively) was observed in hydrolyzed samples with 4% Papain enzyme for 180 min time (Zhang *et al*., 2015). In our study, the low $IC_{50}$ of DPPH and hydroxyl free radical in samples hydrolyzed with Papain enzyme confirmed the higher antioxidant activity of these samples compared to the samples hydrolyzed with Alcalase enzyme. During the hydrolysis process, many small peptides and free amino acids are formed depending on the type of enzyme used. On the other hand, changes in the size, level and composition of free amino acids as well as small peptides affect antioxidant activity. In this regard, Halim *et al.* (2018) in a study on *Monopterus* sp. protein hydrolysates with different molecular weights showed that lower molecular weight peptides had a higher ability to inhibit DPPH free radicals due to further donation of hydrogen atoms (Halim *et al*., 2018). Noman *et al*. (2019) showed in a study that more than 97% of sturgeon (*Acipenser sinensis*) protein hydrolysates prepared with Papain enzyme at different times of hydrolysis

had lower molecular weight than 1000 Daltons and with increasing hydrolysis time more proteins with lower molecular weight were produced (Noman *et al*., 2019). Differences in the ability to inhibit DPPH and hydroxyl free radicals between protein hydrolysates prepared by different Alcalase and Papain enzymes could be due to differences in the type and composition of amino acids as well as peptides with different length created during the hydrolysis process of these two enzymes (Kim, 2011).

Kim (2011) reported $IC_{50}$ values of 2.64 mg/mL for DPPH free radical scavenging in fish protein hydrolysate (*Misgurnus anguillicaudatus*), which is in line with the results of the present study. Also, Kim (2011) studied the antioxidant activity of different hydrolysates (*Styela clava)* prepared using different enzymes and they found that the $IC_{50}$ values for DPPH free radical scavenging of samples hydrolysated using Alcalase, Termoase and Pepsin were 0.35, 0.75 and 0.0015 mg/mL, respectively.

In the present study, hydrolyzed samples with Papain enzyme showed the lowest ferrous chelating activity, the lowest ferric reduction activity and the lowest removing of ABTS radicals, while these samples showed the highest DPPH and hydroxyl free radical inhibition activity. This suggests an inverse relationship between DPPH and hydroxyl inhibitory activity with $Fe^{2+}$ chelating, $Fe^{3+}$ reducing activity and ABTS inhibition activity. In line with the present study, it was reported in conducted studies that the protein hydrolysates with the highest DPPH free radical inhibition activity, showed the lowest ABTS inhibition activity (Rabiei *et al.*, 2019a) and the lowest Fe chelating activity (Slizyte *et al*., 2016).

In the present study, with increasing the peptide concentration from 0.5 mg/mL to 2 mg/mL, the ABTS free radical inhibition activity increased and the highest free radical inhibition activity (79.21%) with $IC_{50}$ value of 1.14 was observed in the hydrolyzed sample prepared using 4 % Alcalase enzyme for 90 min, although it did not show a significant difference with the values of samples obtained by Papain enzyme. In a study conducted by Nahvi *et al*. (2017), the most activity of ABTS free radical scavenging by *Clupeonella cultriventris* protein hydrolysates was 85.32% (Nahvi *et al*., 2017) and this value was reported for about 60% for *Sardinella longiceps* protein hydrolysate in study of Jeevitha *et al*. (2014) and they stated that this rate was observed at maximum peptide concentration of 5 mg/mL(Jeevitha *et al*., 2014), which is consistent with the result of this study.

In the present study, with increasing of peptide concentration from 1 mg/mL to 3 mg/mL the ferrous chelating activity increased. With increasing peptide concentrations, the carboxyl and amine groups are likely to increase in the side chains of acidic and alkaline amino acids, which are the main inhibitors of metal ions increased (Giménez *et al*., 2009). Jeevitha *et al*. (2014) also reported that increasing the concentration of *Sardinella longiceps* protein hydrolysate from 1 to 5 mg/mL

resulted in the increase of percentage of ferrous chelating activity (Jeevitha *et al*., 2014).

Also, with increasing of the concentrations of Alcalase and Papain enzymes from 2% to 4%, the activity of ferrous chelating and ferric reduction activity decreased and their $IC_{50}$ values increased. Protein hydrolysates prepared by the Papain enzyme also showed lower ferrous chelating and ferric reduction activity than Alcalase enzyme, and increasing the hydrolysis time from 90 to 180 min did not have a significant effect on this rate. In line with these results, Slizyte *et al*. (2016) reported that salmon bone protein hydrolysate prepared with a mixture of Bromelain and Papain enzymes for 20 minutes showed the highest ferrous chelating activity and the lowest DPPH free radical inhibition activity. They stated that larger peptides have a better ability to chelate ferrous ions, and this property decreases as the size of the peptide decreases over hydrolysis time (Slizyte *et al*., 2016). Halim *et al*. (2018) also reported that higher molecular weight peptides of *Monopterus* sp. protein hydrolysates had a higher ability to chelate ferrous ion (Halim *et al*., 2018). In the present study, protein hydrolysate prepared with 2% Alcalase enzyme for 180 min showed the highest ferrous chelating activity (27.89%) and the lowest $IC_{50}$ (5.33). The reason for this could be the production of larger peptides in hydrolyzed samples with Alcalase enzyme than those obtained by Papain enzyme. However, a definite statement in this regard requires determining the molecular weight of the peptides and its relationship to the antioxidant activity of the peptide.

Although *Saurida tumbil* protein hydrolysates showed promising antioxidant activity in our study, further studies are required to achieve peptides with promising anti-bacterial, anti-viral, anti-inflammation, anti-cancer and etc. activities. This is especially of great necessity because of the emergence of bacterial antibiotic resistance due to the widespread use of conventional synthetic antimicrobials and also the adoption of survival mechanisms such as viable but non-culturable (VBNC) state by multiple bacteria under stressful conditions and the lack of convenient and accessible diagnostic techniques for these bacterial states (Khezri *et al*., 2020; Zolfaghari *et al*., 2020). Also, due to the change in the lifestyle of human societies and various daily stresses and the increase of various inflammatory, cancer, and etc. diseases, it is necessary to conduct studies with the aim of achieving effective natural compounds to prevent these diseases.

In conclusion, the type of enzyme, its concentration and the time of hydrolysis affected the yield and antioxidant properties of *Saurida tumbil* muscle protein hydrolysates. Samples hydrolyzed by Papain showed higher protein recovery and also DPPH and hydroxyl free radicals scavenging activity than those prepared by Alcalase enzyme. Moreover, hydrolyzed samples with Alcalase enzyme showed higher ferrous chelating and ferric reducing activity than those hydrolysate prepared by Papain enzyme. ABTS radical

scavenging activity did not show any significant difference between hydrolyzed samples with Papain or Alcalase enzymes. Generally, hydrolysis with papain at concentration of 4% for 180 min provided the best properties, which can be considered in the formulation of functional foods.

**Acknowledgement**

The authors appreciate the Qaemshahr Branch, Islamic Azad University for the financial support of this project.

**Referencs**

**Alemán, A., Giménez, B., Pérez-Santin, E., Gómez-Guillén, M. and Montero, P., 2011.** Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate. *Food Chemistry,* 125**,** 334-341. DOI:10.1016/j.foodchem.2010.08.058.

**AOAC, 2002.** Official Methods of Analysis, (Sixteenth Ed.) Association of Official Analytical Chemists, Washington DC.

**AOAC, 2005.** Official Methods of Analysis (16th ed.). Washington: Association of Official Analytical Chemists.

**Centenaro, G.S., Centenaro, M.S. and Hernandez, C.P., 2011.** Antioxidant activity of protein hydrolysates of fish and chicken bones. http://repositorio.furg.br/handle/1/1730.

**Elavarasan, K., Naveen Kumar, V. and Shamasundar, B., 2014.** Antioxidant and Functional Properties of Fish Protein Hydrolysates from Fresh Water Carp (C atla catla) as Influenced by the Nature of Enzyme. *Journal of Food Processing and Preservation,* 38**,** 1207-1214. DOI: 10.1111/jfpp.12081.

**Esmaeili Kharyeki, M., Rezaei, M., Khodabandeh, S. and Motamedzadegan, A., 2018.** Antioxidant Activity of Protein Hydrolysate in Skipjack tuna Head. *Fisheries Science and Technology*, 7**,** 57-64. [In persian]. DOI: 20.1001.1.23225513.1396.7.1.3.8.

**FAO, 2016.** The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 P.

**Giménez, B., Alemán, A., Montero, P. and Gómez-Guillén, M., 2009.** Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. *Food Chemistry,* 114**,** 976-983. DOI: 10.1016/j.foodchem.2008.10.050.

**Gülçin, İ., 2006.** Antioxidant activity of caffeic acid (3, 4-dihydroxycinnamic acid). *Toxicology*, 217, 213-220. DOI: 10.1016/j.tox.2005.09.011.

**Halim, N.R.A., Azlan, A., Yusof, H.M. and Sarbon, N.M., 2018.** Antioxidant and anticancer activities of enzymatic eel (*Monopterus* sp) protein hydrolysate as influenced by different molecular weight. *Biocatalysis and Agricultural Biotechnology,* 16**,** 10-16. DOI: 10.1016/j.bcab.2018.06.006.

**Hassan, M.A., Xavier, M., Gupta, S., Nayak, B.B. and Balange, A.K., 2019.** Antioxidant properties and instrumental quality characteristics of spray dried Pangasius visceral protein hydrolysate prepared by chemical and enzymatic methods. *Environmental Science and Pollution Research*, 26(**9**),8875-84. DOI:10.1007/s11356-019-04144-y.

**James, C.S., 2013.** *Analytical chemistry of foods*, Springer Science & Business Media.

**Jeevitha, K., Mohana, P. and Khora, S., 2014.** Antioxidant activity of fish protein hydrolysates from *Sardinella longiceps*. *International Journal of Drug Development and Research*, 6**,** 137-45.DOI: not found.

**Jitesh, S.B., Syed, Z.M., Hitendra, P.L., Ashok, D.R., Anil, K.S. and Balakrishnan, G., 2011.** Effect of egg albumen (protein additive) on surimi prepared from lizardfish (*Saurida tumbil*) during frozen storage. *Aquaculture, Aquarium, Conservation & Legislation*, 4(**3**), 306-12.

**Joshi, B.N., Koli, J.M. and Sharangdher, S.T., 2015.** Development of edible texturised dried fish granules from low-value fish (*Saurida tumbil*). *International Journal of Sciences and Applied Research*, 2(**8**), 69-77. DOI: Not Found.

**Khajavi, S., Zakipour Rahimabadi, E., Ghaffari Moghaddam, M., Shahraki, H., Mir, L. and Zand Karimi, M., 2016.** The effects of hydrolysis condition on the antioxidant activity of protein hydrolysate from Schizothorax zarudnyi's waste. *Journal of Fisheries (Iranian Journal of Natural Resources)*, 69**,** 351-58. [In persian]. DOI:10.22059/jfisheries.2016.61630

**Khezri, M., Rezaei, M., Rabiey, S. and Garmsiri, E., 2016.** Antioxidant and antibacterial activity of three algae from Persian gulf and Caspian sea. *Ecopersia*, 4(**2**),1425-35. DOI:10.18869/modares.Ecopersia.4. 2.1425.

**Khezri, M., Rezaei, M., Mohabbati Mobarez, A. and Zolfaghari, M., 2020.** Viable but non culturable state and expression of pathogenic genes of *Escherichia coli* O157: H7 in salted silver carp. *Journal of Food Safety*, 40(**5**), e12843. DOI: 10.1111/jfs.12843.

**Kim, S.M., 2011.** Antioxidant and anticancer activities of enzymatic hydrolysates of solitary tunicate (Styela clava). *Food Science and Biotechnology*, 20**,** 1075. DOI: 10.1007/s10068-011-0146-y.

**Layne, E., 1975.** Spectrophotometric and turbidimetric methods for measuring proteins. *Methods in Enzymology*, 3: 447-454. DOI: 10.1016/S0076-6879(57)03413-8.

**Lima, R. and Porto, A., 2016.** Marine Enzymes Biotechnology: Production and Industrial Applications, Part I—Production of Enzymes.

**Lima, M.M., Vanier N.L., Dias, A.R., Zavareze, E., Prentice, C. and Moreira, A.D., 2019.** Whitemouth croaker (*Micropogonias furnieri*) protein hydrolysates: chemical

composition, molecular mass distribution, antioxidant activity and amino acid profile. *International Food Research Journal*, 26(**1**), 247-254. http://www.ifrj.upm.edu.my/26%20(01)%202019/(27).pdf.

**Mehregan Nikoo, A., Sadeghi Mahoonak, A., Ghorbani, M., Taheri, A., Alami, M. and Kamali, F., 2014.** Effect of hydrolysing condition on antioxidant activity of protein hydrolysate from Crucian carp (*Carassius carassius*). *Journals of Research and Innovation in Food Science and Technology (JRIFST)*, 2**,** 351-64. [In persian]. DOI: 10.22101/JRIFST.2014.03.01.245.

**Nahvi, Z., Hosseini, S. and Zandi, M., 2017.** Production of hydrolyzed protein from Kilka by enzymatic hydrolysis and evaluation of its bioactive properties. *Aquatic Physiology and Biotechnology*, 5**,** 39-58. [In persian]. DOI: 10.22124/JAPB.2017.2565.

**Noman, A., Qixing, J., Xu, Y., Ali, A.H., Al-Bukhaiti, W.Q., Abed, S.M. and Xia, W., 2019.** Influence of degree of hydrolysis on chemical composition, functional properties, and antioxidant activities of chinese sturgeon (*Acipenser sinensis*) hydrolysates obtained by using alcalase 2.4 L. *Journal of Aquatic Food Product Technology*, 28**,** 583-597. DOI: 10.1080/10498850.2019.1626523.

**Noman, A., Ali, A.H., Al-Bukhaiti, W.Q. and Xia, W., 2020.** Effect of enzyme type on the properties of chinese sturgeon (*Acipenser sinensis*) protein hydrolysates produced by enzymatic hydrolysis process. *American Journal of Food Science and Nutrition Research*, 6(**1**), 10-6. http://www.openscienceonline.com/author/download?paperId=5424&stateId=8000&fileType=3.

**Ovissipour, M., Taghiof, M., Motamedzadegan, A., Rasco, B. and Molla, A.E., 2009.** Optimization of enzymatic hydrolysis of visceral waste proteins of beluga sturgeon Huso huso using Alcalase. *International Aquatic Research*, 1**,** 31-38. https://journals.iau.ir/article_673236_c26c62b230887aceaadf650e7b987966.pdf.

**Prihanto, A.A., Nurdiani, R. and Bagus, A.D., 2019.** Production and characteristics of fish protein hydrolysate from parrotfish (*Chlorurus sordidus*) head. *Peer J*, 7, e8297. DOI: 10.7717/peerj.8297.

**Rabiei, S., Nikoo, M., Rezaei, M. and Rafieian-Kopaei, M., 2017.** Marine-Derived Bioactive Peptides with Pharmacological Activities-A Review. *Journal of Clinical & Diagnostic Research*, 1,11(**10**), 1-6. DOI:10.7860/JCDR/2017/28672.10753.

**Rabiei, S., Rezaei, M., Asgharzade, S., Nikoo, M. and Rafieia-Kopai, M., 2019a.** Antioxidant and cytotoxic properties of protein hydrolysates obtained from enzymatic hydrolysis of Klunzinger's mullet (Liza klunzingeri) muscle. *Brazilian Journal of Pharmaceutical Sciences*,

55. 55, 1-10. DOI: 10.1590/s2175-97902019000218304.

**Rabiei, S., Rezaie, M., Abasian, Z., Khezri, M., Nikoo, M., Rafieian-Kopaei, M. and Anjomshoaa, M., 2019b.** The protective effect of *Liza klunzingeri* protein hydrolysate on carbon tetrachloride-induced oxidative stress and toxicity in male rats. *Iranian Journal of Basic Medical Sciences,* 22**,** 1203-1210. DOI: 10.22038/ijbms.2019.33201.7927.

**Raftani Amiri, Z., Safari, R., Bakhshandeh, T. 2016.** Functional properties of fish protein hydrolysates from Cuttlefish (*Sepia pharaonis*) muscle produced by two commercial enzymes. *Iranian Journal of Fisheries Sciences*, 15(**4**),1485-99. DOI: 20.1001.1.15622916.2016.15.4.23.3.

**Shaviklo, A.R., Mozafari, H., Motamedzadegan, A. and Damavandi-Kamali, N., 2019.** Oxidative stability, biochemical indices and characteristics of the fatty acid composition of fish protein isolated from tuna (*Thunnus albacares*) canning by-product stored at-24 ˚C for 6 months. *Iranian Journal of Fisheries Science,* 18(**4**), 699-715. DOI: 20.1001.1.15622916.2019.18.4.29.0.

**Siro, I., Kápolna, E., Kápolna, B. and Lugasi, A., 2008.** Functional food. Product development, marketing and consumer acceptance—A review. *Appetite,* 51**,** 456-467. DOI: 10.1016/j.appet.2008.05.060.

**Slizyte, R., Rommi, K., Mozuraityte, R., Eck, P., Five, K. and Rustad, T., 2016.** Bioactivities of fish protein hydrolysates from defatted salmon backbones. *Biotechnology Reports,* 11**,** 99-109. DOI: 10.1016/j.btre.2016.08.003.

**Taheri, A., Anvar, S. A., Ahari, H. and Fogliano, V., 2013.** Comparison the functional properties of protein hydrolysates from poultry by-products and rainbow trout (*Onchorhynchus mykiss*) viscera. *Iranian Journal of Fisheries Sciences,* 12(**1**),154-69. DOI: 20.1001.1.15622916.2013.12.1.13.6.

**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,* 117**,** 108616. DOI: 10.1016/j.lwt.2019.108616.

**Yasemi, M., Ghomi Marzdashti, M.R., Darnahal, T., Mohammadzadeh, B. and Amini, H., 2013.** Yelid of protein recovery and degree of hydrolysis associated protein hydrolysates from Bighead Carp (*Aristichthys nobilis*) by using enzymes. *Iranian Scientific Fisheries Journal,* 22**,** 149-156. [In persian]. DOI: 10.22092/ISFJ.2017.110110.

**You, L., Zhao, M., Regenstein, J.M. and Ren, J., 2010.** Purification and identification of antioxidative peptides from loach (*Misgurnus anguillicaudatus*) protein hydrolysate by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Research*

*International*, 43, 1167–1173. DOI: 10.1016/j.foodres.2010.02.009.

**Zhang, L., Liu, Y., Tian, X. and Tian, Z., 2015.** Antimicrobial Capacity and Antioxidant Activity of Enzymatic Hydrolysates of Protein from R ushan Bay Oyster (*Crassostrea gigas*). *Journal of Food Processing and Preservation*, 39**,** 404-412. DOI: 10.1111/jfpp.12245.

**Zolfaghari, M., Rezaei, M., Mohabbati Mobarez, A., Forozandeh Moghaddam, M., Hosseini, H. and Khezri, M., 2020.** Virulence genes expression in viable but non-culturable state of *Listeria monocytogenes* in fish meat. *Food Science and Technology International*, 26(**3**), 205-212. DOI: 10.1177/1082013219877267.