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RESEARCH ARTICLE

Antioxidant and chelating activities from Lion fish (*Pterois volitans* L.) muscle protein hydrolysates produced by *in vitro* digestion using pepsin and pancreatin

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ABSTRACT

The aim of the present research was to determine the antioxidant, and Cu^{2+} and Fe^{2+} chelating activities, amino acid content and estimated molecular weight of proteins and peptides from lion fish muscle-derived hydrolysates obtained by enzymatic hydrolysis with pepsin and pancreatin. The fillets were freeze-dried and hydrolysis was performed, taking aliquots at different times. The degree of hydrolysis, antioxidant activity against $ABTS^{+}$ and DPPH, Cu^{2+} and Fe^{2+} chelating activity, as well as molecular weight estimated by SDS-PAGE and amino acid content by HPLC was determined. The highest degree of hydrolysis (DH) was at 180 min (37.75%). SDS-PAGE showed proteins with an estimated molecular weight of 45,259 and 42,487 Da, which could be associated with myofibrillar proteins. The progressive degradation of proteins by enzymes was also observed, detecting polypeptides with a EMW of 5,988 and 4,954 Da in the most representative hydrolysates. The H40 hydrolysate exhibited the highest ABTS and DPPH radical depuration activity, with values of 107.31 mM/mg protein and 54.27%, respectively. The iron and copper chelating activity was related to DH, since the highest values of iron and copper chelating activity were obtained in hydrolysates H120 and H140, with 90.83% chelation of Cu^{2+} and 56.33% chelation of Fe^{2+}, respectively with no significant differences compared to subsequent times. In addition, the antioxidant and chelating activities were possibly related to Trp, Cys, Lys, Pro and Arg content. Lion fish muscle hydrolysates could be a potential source of functional ingredients due to their *in vitro* antioxidant and chelating activity.

Keywords: Antioxidant activity; Chelating activity; Hydolysates; Lion fish; Pepsin-pancreatin

INTRODUCTION

The lion fish (*Pterois volitans* L.) is a fish of the Scorpaenidae family and is native to the Eastern Indian and Pacific oceans. Lion fish appeared in the Caribbean, possibly as a result of escape or intentional introduction, apparently from Florida (USA), in the 1980s and since then it spread through the Caribbean ocean (Green et al., 2012). In 2004, it had dispersed in the Bahamas (Whitfield et al., 2007) and by 2008 its sighting was already recorded in the Cayman Islands, Jamaica, the Dominican Republic and the Virgin Islands and Barbados. In 2009 the expansion of this species was already alarming since its territory already included Mexico, Belize, Honduras, Nicaragua, Costa Rica and Panama (Betancur et al., 2011). Currently, government agencies and ecological conservation organizations are implementing strategies to mitigate the invasion, including sport fishing. In addition, bromatological studies have been carried out, in order to recommend its consumption; in Mexico the National Commission of Natural Protected Areas (CONANP), has put in motion other proposals for its control, promoting the tasting of dishes made with lion fish. On the other hand, marketing and imports of the meat of this fish have been developed, as part of the control strategies.

Current studies agree with the global trend of the search for food that, in addition to its nutritional value, provide benefits to the physiological functions of the body, leading

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to the formation of a new area of research in the science of food and nutrition related to functional foods. Due to its importance as a nutrient, functional ingredients have become the center of attention of a large number of food scientists and technologists. The use of antioxidants in the food industry generates an interest in research since, oxidant compounds are produced in living organisms degrading proteins, lipids and DNA, decreasing their nutritional and functional value (Dávalos et al., 2004). Antioxidant peptides are those that have the ability to trap free radicals and form complexes with metal ions that catalyze the reactions of these radicals and act by preventing other molecules from binding to oxygen, preventing cell aging and delaying degenerative diseases (Chen et al., 1998)

Chelating peptides are a source of interest, for example in the case of peptides with copper chelating activity are essential to maintain a good antioxidant defense, fighting the oxidative stress of copper by chelating the metal ion (Villarroel et al., 2014). Iron, as well as copper, have the capacity to produce reactive oxygen species that cause the breakdown of the DNA chain and the oxidation of its bases (Burkitt, 2001). They can also be useful in organs such as the brain, where the oxidative process is involved in the development of certain diseases.

Today, fish and other marine sources have gained interest because they are a potential source of antioxidant peptides mainly due to the abundance of raw material from underutilized species or by-products. In addition, several investigations have demonstrated that fish protein hydrolysates as well as their peptides with a specific amino acid sequence exhibit antioxidant, antihypertensive, neuroactive immunomodulatory, antimicrobial, hormonal and mineral regulating, antithrombotic, and anti-carcinogenic properties, among others. Also, food-grade microbial enzymes such as alkalase®, flavourzyme®, and protamex™, from plant sources (such as papain) and animal sources (pepsin and trypsin) have been used to produce antioxidant peptides (Samaranayaka and Li-Chan 2011; Sierra-Lopera et al., 2018).

Several studies have established the relationship between biological activity and molecular weight of peptides, being the fractions with molecular weight between 1-4kDa of interest for pharmaceutical use (Sierra-Lopera et al., 2018).

Different technologies exist for the isolation and purification of bioactive peptides at the pilot plant level; for instance, by chromatographic methods or the use of membranes, antioxidant peptides have been obtained from the hydrolysis of by-products such as Salmon fins (*Salmo sp.*), heads of Bluefin leatherjacket (*Navodon septentrionalis*), skin of Grass carp (*Ctenopharyngodonidella*), viscera of Squid (*Ommastrephes bartrami*), muscle of Blue mussel (*Mytilus edulis*) and Patin (*Pangasius sutchi*). In addition, calcium chelating peptides have been obtained from scales and bones of Atlantic salmon (*Salmo salar L.*) and Nile tilapia (*Oreochromis niloticus*), and dietary supplements obtained from White fish (Brand: Seagest™), Molva (Brand: MOLVAL®), Cod (Brand: Norland Hydrolyzed Fish Collagen), sardine (Brand: Valtyron®) are already marketed in France, the United States and Japan (Sierra-Lopera et al, 2018).

Jensen et al. (2013) has reported that enzymatic hydrolyzates from hake, Hoki, blue mussel and Atlantic salmon have antioxidant capacity. Lin et. al. (2014), reports that Pacific mackerel, spanish mackerel, sablefish and Japanese anchovy, present iron chelating peptides which could be useful as food additives or pharmaceutical agents. It is important to carry out studies that allow know if the *Pterois volitans* L. from Mexican Caribbean Sea has potential as a raw material for obtaining functional ingredients such as enzymatic hydrolysates. Therefore, the aim of the present research was to determine the antioxidant, and Cu^{2+} and Fe^{2+} chelating activities, amino acid content and estimated molecular weight (EMW) of proteins and peptides from lion fish muscle-derived hydrolysates obtained by enzymatic hydrolysis with the commercial enzymes pepsin and pancreatin. The results would allow to extend the use and application of the meat of this fish, giving it an added value and mitigating the invasion.

MATERIALS AND METHODS

A general description of the methodology can be seen in Fig. 1.

Raw material production

Red lion fish *Pterois volitans* L. specimens were collected by divers near Cozumel Island, in the state of Quintana Roo, on the Caribbean coast of Mexico. Fish were gutted and filleted. The skinless fillets freeze-dried in the laboratory until use. The dried sample was pulverized and mixed until it was completely homogeneous. Finally, it was stored in freezing at -20 °C in a polyethylene bottle to carry out the tests. Fillet moisture and protein content were analyzed using AOAC methods: moisture (934.01), protein (954.01) (calculated as nitrogen x 6.25).

Protein hydrolysate preparation

A subsample of the freeze-dried fillet was used to obtain the hydrolysates, carrying out two replicates of the process. Protein hydrolysates were isolated from the fillets following the first step of the hydrolysis method described by Megías et al. (2004). Freeze-dried fish fillet/water (5 % w/v) were

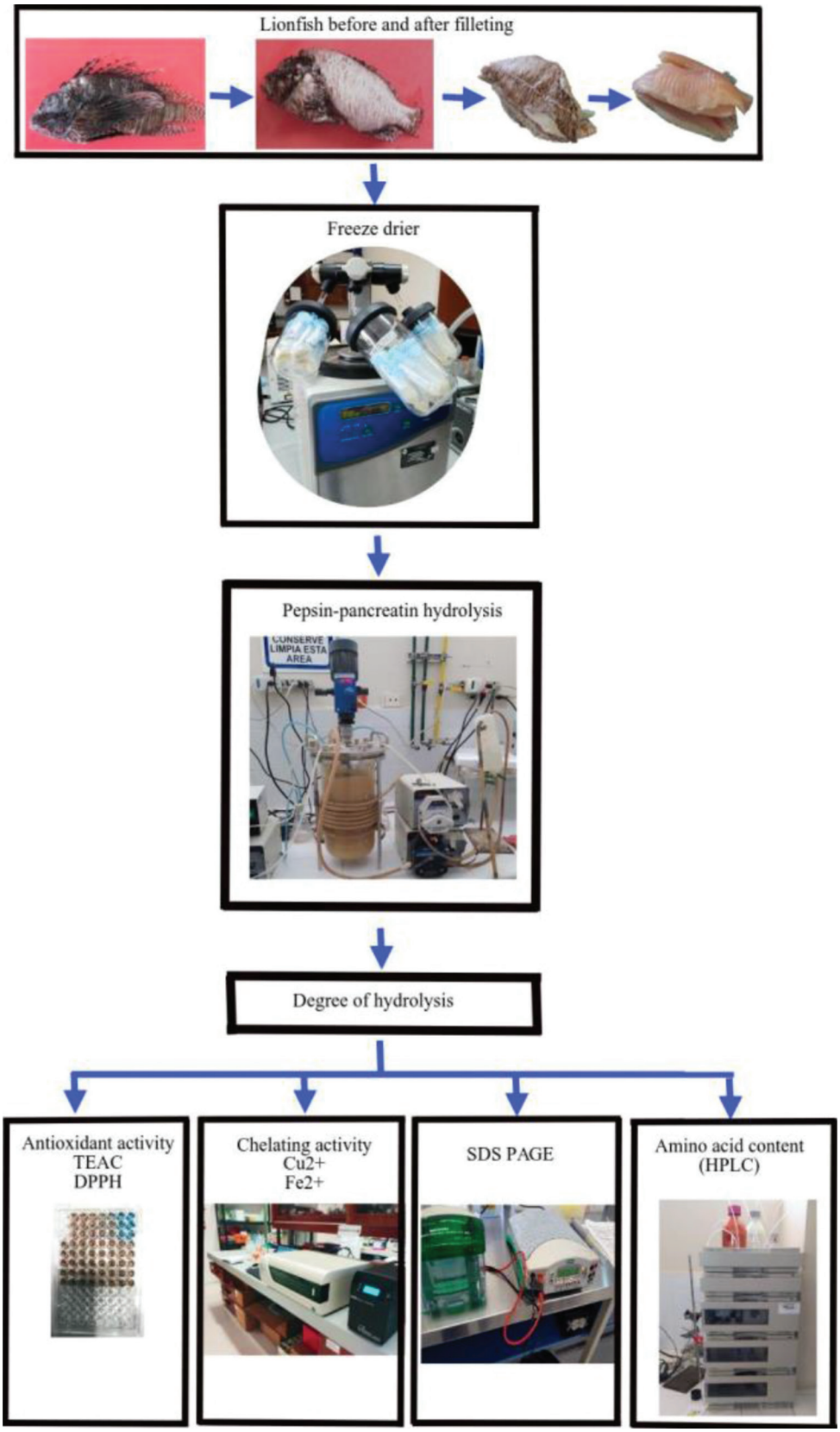

Fig 1. Overview of the methodology. The lionfish's bones, scales and viscera were removed; the fillets were freeze-dried. Hydrolysis was performed with pepsin for 60 minutes and pancreatin for 120 minutes, taking aliquots at different times. The degree of hydrolysis, antioxidant activity against ABTS+ and DPPH, chelating activity of Cu^{2+} and Fe^{2+}, as well as the molecular weight estimated by SDS-PAGE and amino acid content by HPLC were determined.

digested at 37 ºC using pepsin (SigmaP7000) at pH 2.5 for 60 minutes, and then with pancreatin (SigmaP1625) at pH 7.5 for 120 minutes. A 1/20 (w/w) enzyme to substrate ratio was used for both enzymes. Aliquots were taken at different times (10, 20, 40, 60 min in case of digestion using pepsin and 80, 100, 120, 160, 180 min in case of digestion using pepsin/pancreatin) and hydrolysis was stopped by heat inactivation at 80 ºC for 20 minutes. The resulting hydrolysates (H10, H20, H40, H60, H80, H100, H120, H160, H180) were clarified by centrifugation at 11227 x *g* for 30 minutes at 5 ºC, frozen at -20 ºC and stored at the same temperature until further use. Hydrolysate protein content was quantified following Lowry et al. (1951) and the result was used in all subsequent analyses.

Degree of hydrolysis

Degree of hydrolysis (DH) was calculated following Nielsen et al. (2001). The free amino groups were quantified with o-phthalaldehyde (Sigma P0657), in the presence of dithiothreitol, which forms a colored compound detectable at 340 nm in a spectrophotometer (Thermo Spectronic, Genesys 10UV). The cleaved peptide bonds were quantified using a calibration curve with L-serine (Sigma S4500) as a standard:

Amino acid analysis of the non-hydrolyzed protein (NHP) and hydrolysates derived from lion fish muscle

Amino acid analysis of NHP, H60 and H180 was carried out by acid hydrolysis and HPLC, after derivatization with diethyl ethoxymethylenemalonate (Aldrich D94208), according to the method described by Alaiz et al. (1992), using D,L-α- aminobutyric acid (Aldrich D94208) as internal standard.

Antioxidant and chelating activity of hydrolysates

Free radical scavenging activity

Free radical scavenging activity was measured according to Shimada et al. (1992) with modifications. Protein hydrolysates (1mg of protein) were added to DPPH (SigmaD9132) in methanol (100 µL, 100 µM) in 96-well plates. Plates were shaken and absorbance at 517 nm was measured after 30 min using a plate reader. DPPH mixed with 100 µL distilled water was used as control. As a standard, 5 µg of BHA (from a stock solution of 1mg of BHA/mL) was used with the same procedure described. Experiments were done in triplicate.

$ABTS^+$ (2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) decolorisation assay

Antioxidant activity in the hydrolysates was measured following Pukalskas et al. (2002). The protein hydrolysates of lion fish at a protein concentration of 1 mg/mL and commercial antioxidant butylated hidroxyanisole (BHA) (Sigma B1253) at the same concentration were analyzed. $ABTS^+$ radical cation was produced by reacting ABTS with potassium persulfate. Antioxidant compound content in the hydrolysates was determined by diluting the $ABTS^+$ solution with PBS to an absorbance of 0.800 ± 0.030 AU at 734 nm. After adding 990 µL of diluted $ABTS^+$ solution (A 734 nm = 0.800 ± 0.030) to 10 µL antioxidant compound or Trolox standard (final concentration 0.5-3.5 mM) in PBS, absorbance was read at ambient temperature exactly 6 min after initial mixing. Experiments were done in triplicate.

Copper chelating activity

Copper (Cu^{2+})-chelating activity was determined using the pyrocatechol violet reagent, according to Saiga et al. (2003). Protein hydrolysates (equivalent to 1 mg protein) were added to Eppendorf tubes containing 1 mL 50 mM sodium acetate buffer (pH 6.0), 25 µL 4 mM pyrocatechol violet (Sigma P7884), and 10 µg Cu ($CuSO_4$). Ethylenediaminetetraacetic acid (EDTA) (50 µg) was used as a positive control. Absorbance at 632 nm was measured after incubation for 1 min at room temperature. Experiments were done in triplicate.

Iron chelating activity

Iron (Fe^{2+}) chelating activity was measured based on formation of the Fe^{2+}-ferrozine complex, according to Carter (1971). Protein hydrolysates (equivalent to 1 mg protein) were added to Eppendorf tubes containing 1 mL 100 mM sodium acetate buffer (pH 4.9) and 100 µL $FeCl_2 \cdot 4\ H_2O$ solution (0.01 mg Fe/mL water). Again, EDTA (50 µg) was used as a positive control. Absorbance at 562 nm was measured after adding the ferrozine solution (50µL, 40 mM in water) (Sigma P5338) and incubating for 30 min at room temperature. Experiments were done in triplicate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

This analysis was done following Schägger and Jagow (1987), through 18% acrylamide gel and a 4% stacking gel. Peptide fractions (5-6 µg/µL of protein) were dissolved separately in a sample buffer and heated to 100 °C for 5 min. Runs were done at 40 mA for 1.5 h in a Miniprotean electrophoresis chamber (BIORAD, Hercules, California). Wells were loaded with 10 µg of the protein or one of the hydrolysates; a kaleidoscope polypeptide standard (BIORAD, USA, Cat. #1610325) was used which contained carbonic anhydrase (31.5 kDa); soy trypsin inhibitor (22.9 kDa); lysozyme (14.1 kDa), aprotinin (7.9 kDa) and insulin (3.2 kDa).

Statistical analysis

A one-way analysis of variance (ANOVA) with a 5% significance level was applied to the results using the Statgraphics Centurion XV program. The Duncan method was used to compare the means between hydrolysates DH values and *in vitro* activities.

RESULTS AND DISCUSSION

Moisture and protein determination

The lion fish freeze-dried fillet had 81.64±0.12% (d.b) of protein and 11.46±0.1% of moisture, similar to the 88.6±0.3% and 3.6±1.9%, respectively reported in northern Pacific hake (*Merluccius*) by Pacheco et al. (2008). According to Clemente et al. (1999) the lyophilized fillet could be considered as a protein concentrate since it has a percentage higher than 65%.

Degree of hydrolysis (DH)

The degree of hydrolysis ranged from 3.13±2.34% to 37.74±0.38%, as seen in the hydrolysis kinetics (Fig. 2). This could be due to lysosomal proteases within in the fish muscle, which contribute to the proteolytic breakdown (Samaranayaka et al., 2011). Fang et al. (2012) reported a DH of 5.30±0.3% in tilapia (*Oreochromis niloticus*) similar to that obtained in this study.

Characteristic hydrolysis kinetics is observed with the pepsin-pancreatin system. During the first stage, when pepsin is added, digestion begins, obtaining the highest DH at 60 min (19.19±2.2%). This may be due to that marine-derived proteins are more digestible than plant-derived proteins, and even some others from animals (He et al., 2013). In the second stage of digestion, the presence of pancreatin again leads to a considerable increase of DH with respect to time, since at 80 min the DH was 31.86±0.39% (Fig. 2), which could be attributed to the increased number of available substrates generated by previous digestion with pepsin (Montoya et al., 2008).

In H100 the DH was 32.33±4.42%, without statistical difference with respect to H80 ($p>0.05$), similar to what was found between H140 and H160, which had 35.33±0.41% and 35.46±0.74% of DH, respectively and without statistical differences ($p>0.05$).

This performance may also be due to the depletion of substrate during the digestion process, resulting in DH with no apparent difference (Eisenthal-Danson et al., 2002). You et al. (2010) report a DH higher than that obtained in the present work, with a DH of 46.6±0.3% in Dojo fish hydrolysates (*Misgurnus anguillicaudatus*) in conditions similar to those used in this work. Molla et al. (2011) reported a DH of 30% in the hydrolysate of the visceral protein of beluga (*Huso huso*) digested with the Protamex® enzyme, similar to that obtained in this study.

Electrophoretic profile

Electrophoretic analysis (Fig. 3) of the fillet (NHP) and protein hydrolysates (H20, H60, H80, H120 and H180 min) of *Pterois volitans* L. showed the presence of three majority bands (marked with arrows). The molecular weight of the band with the highest molecular weight could not be determined, however, the second and third bands had an estimated molecular weight of 45,259 and 42,487Da which could be associated to myofibrillary proteins (paramyosin), the second to tropomyosin and the third to actin, as reported by Sánchez-Sanchez et al. (2013). Benjakul et al. (1997) reported the presence of bands with weights similar to those found here, in Pacific hake (*Merluccius gayi*), with a weight between 112-82 KDa and 45 KDa, which are attributed to endogenous enzymes. Proteins were also detected in NHP with estimated molecular weights of 27,297, 24,055, 20,539, 19,281, 14,057, and 9,321Da.

A progressive degradation of proteins was also observed as polypeptides with an estimated molecular weight of

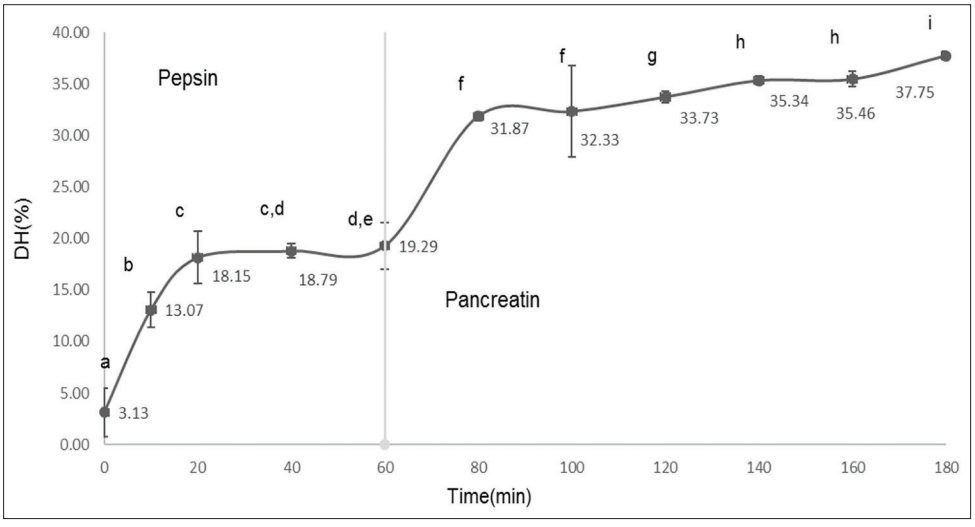

Fig 2. Hydrolysis degree kinetics of lion fish muscle protein treated with pepsin and pancreatin. Error bars indicate the standard deviation. Statistically significant differences are indicated by different letters ($p<0.05$).

5,988 and 4,954Da were found. Furthermore, a greater degradation is observed in H180, which remains unchanged until the end of digestion, since the greater the DH, the more likely it is to obtain smaller peptides. When assessing the behavior of the hydrolysates, it is observed a trend towards a decrease in the size of the proteins in comparison with the profile of the non-hydrolysed protein, therefore a better solubility and presence of bioactive peptides are expected (Sánchez-Sanchez et al., 2014).

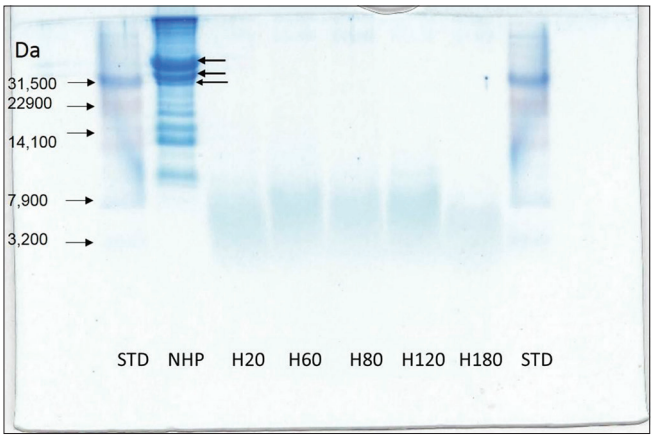

Fig 3. Electrophoretic profile of NHP and hydrolysates derived from lion fish muscle subjected to enzymatic hydrolysis with pepsin and pancreatin.

Antioxidant and chelating activity of protein hydrolysates derived from P. volitans L.

Determination of DPPH radical scavenging activity

Fig. 4A shows the DPPH free radical scavenging activity of lion fish muscle protein hydrolysates.

NHP had a DPPH free radical uptake capacity of 19.86±0.26%, given by the degree of hydrolysis (Thiansilakul et. al., 2007). When the commercial enzyme pepsin was added, there was an increase in antioxidant activity, from 45.32±0.41% to 54.27±0.53%, finding the

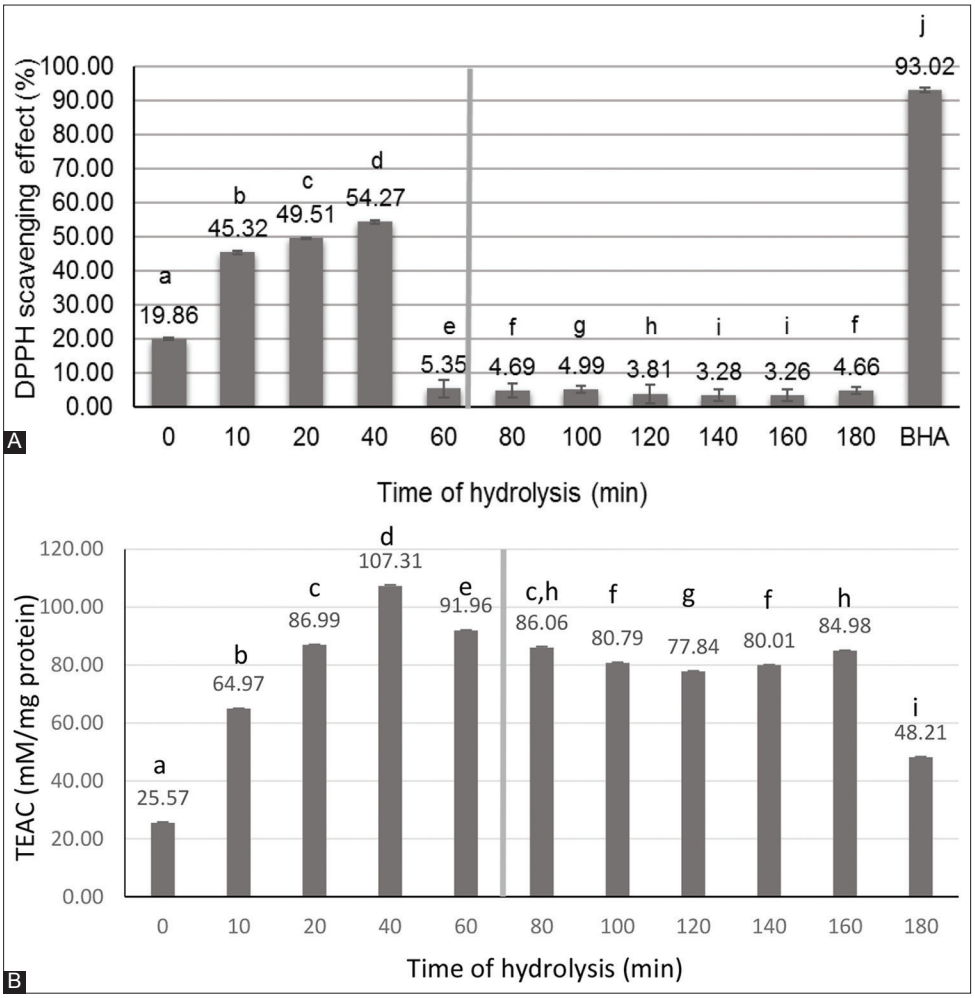

Fig 4. (A-B). Percentage of DPPH radical scavenging activity of fillet (0 min) and hydrolysates (1 mg protein) derived from lion fish muscle subjected to enzymatic hydrolysis with pepsin and pancreatin. Error bars indicate the standard deviation. Different letters indicate statistical difference ($p<0.05$). Standard 5 µg of BHA. Antioxidant activity (TEAC values) of NHP and hydrolysates (1 mg protein) derived from lion fish muscle subjected to enzymatic hydrolysis with pepsin and pancreatin. Error bars indicate the standard deviation. Different letters indicate statistical difference ($p<0.05$). Standard BHA (1mg/mL).

highest activity in 40 min, however an important decrease is observed at 60 minutes (5.35±2.59%). The addition of pancreatin at 80 minutes did not increase antioxidant activity and this behavior continued in subsequent minutes. The BHA standard had 93.02±0.75% of DPPH free radical uptake capacity.

Nakajima et al. (2008) which evaluated the antioxidant capacity of Atlantic salmon (*Salmo salar*), Coho salmon (*Onchorhynchus Kisutch*) and Alaska pollack (*Theragra chalcogramma*), which underwent similar sequential hydrolysis with pepsin (2 h)/pancreatin (3h), reported that the antioxidant capacity ranged from 20 to 30% during pepsin digestion, and significantly falls to approximately 5%, which is similar to the pattern found here. Changes in the size and composition of peptides, as well as the presence of free amino acids, generated during hydrolysis, affect antioxidant capacity (Klompong et al., 2007). This may explain the decrease in antioxidant capacity from 60 minutes.

In the amino acid profile (Table 1), it is observed the high-quality of the proteins of the freeze-dried lion fish fillet. A higher content of essential amino acids, such as His, Thr, Val, Ile, Leu, Phe, Lys and Trp, was found compared to the intake recommended by FAO (2007) for adults, except for Cys with a lower content. Morales et al. (2016) noted that low levels of Cys and Trp are usually found in fish proteins. It has also been reported that antioxidant activity is due to the presence of amino acids and peptides containing residues of Val, Leu, Ile, Ala, Phe, Cys or Lys in N-terminal. However, the presence of these amino acids in the hydrolysates H60 and H180 does not explain the behavior regarding the abrupt decrease in the activity detected in the hydrolysate obtained with pepsin (H60) and pepsin-pancreatin in the longest time (H180) (Table 1).

Determination of ABTS+ radical uptake activity

The trolox antioxidant equivalent coefficient (TEAC) is shown in Fig. 4B. The fillet of lyophilized lion fish (NHP) had a 25.57±0.26 of TEAC.

TEAC (mM/mg protein) increased gradually with the hydrolysates derived from pepsin digestion at minutes 10 to 40 (64.97 to 107.31); at 60 minutes, it had a slight decrease (91.96) that remained stable during pancreatin digestion and up to 160 minutes. A decrease in TEAC (48.21) was observed at 180 minutes after pancreatin digestion. The TEAC value of the antioxidant BHA was 373.49 mM/mg of protein.

It is worth noting that, even though the protein hydrolysate H180 had the highest DH (37.75±0.38%), the uptake value of the radical $ABTS^+$ was the lowest among the other hydrolysate. This performance was also observed in protein hydrolysates of ornate threadfin bream muscle (*Nemipterus hexodon*), generated with pepsin from Skipjack tuna stomach (*Katsuwonus pelamis*) (Nalinanon et al., 2011), where a TEAC of 150 mM/mg, 159 mM/mg and 151 mM/mg was reported in hydrolysates with DH of 10%, 20% and 30%, respectively. Zheng et al., (2016) reported that the amino acids Tyr, Trp, Cys, Met, Lys and Phe capure the radical $ABTS^+$, however the capacity decreases in the following order: Tyr > Trp > Cys, while the amino acids Met, Lys and Phe display a lower uptake of this radical. Therefore, as shown by the amino acid content in Table 1, the highest concentration of Tyr, Cys, Lys and Trp in H60 compared to H180, could be promoting the uptake activity of the radical $ABTS^+$. These results suggest that lion fish protein hydrolysates contain amino acids or peptides that are electron donors which can react with free radicals to convert them into stable products and stop the pro-oxidant chain reaction (Nalinanon et al., 2011).

Cu^{2+} chelating activity

NHP showed a chelating activity of 45.31±0.98%, while protein hydrolysates exhibited a higher activity (Fig. 5A). The chelating capacity of Cu^{2+} during pepsin hydrolysis increased over time; however, the addition of pancreatin increased this activity and also raised over time, reaching a maximum value at 120 minutes. This may be due to the

Table 1: Amino acid composition of lion fish fillet and its hydrolysates obtained at 60 min of hydrolysis with pepsin and 180 min with pepsin-pancreatin (g/100g protein)

Amino Acids	Lion fish muscle (NHP)	Pepsin H60	Pepsin-pancreatin H180	FAO (2007)
Asx[1]	9.95±0.002	7.29±0.08	7.89±0.25	
Glx[2]	15.35±0.56	12.25±0.78	11.84±1.36	
Ser	2.8±0.08	2.02±0.24	1.85±0.29	
His	1.75±0.09	1.52±0.03	1.66±0.10	1.5
Gly	4.37±0.40	4.77±0.09	4.53±0.42	
Thr	3.93±0.01	3.81±0.03	3.80±0.06	2.3
Arg	10.96±0.36	12.53±0.18	13.10±0.09	
Ala	2.92±0.04	2.81±0.03	2.89±0.17	
Pro	6.47±0.55	5.29±0.06	7.83±3.17	
Tyr	2.68±0.07	2.29±0.12	2.14±0.33	
Val	10.82±0.38	13.37±0.17	13.13±0.38	3.9
Met	2.58±0.04	1.89±0.08	2.21±0.17	1.6
Cys	0.13±0.01	1.30±0.70	0.03±0.01	2.2[d]
Ile	4.46±0.06	4.19±0.09	4.36±0.07	3.0
Leu	8.01±0.12	8.22±0.20	8.12±0.18	5.9
Phe	3.62±0.02	3.29±0.01	3.47±0.18	3.0[c]
Lys	9.20±0.09	9.30±0.40	8.83±0.56	4.5
Trp	2.63	3.84±0.26	2.30±0.92	0.6
Hydrophobic		42.91	44.31	
Neutral		14.20	12.36	
Hydrophilic		42.89	43.33	

Hydrophobic amino acids: Ala, Val, Met, Phe, Leu, Ile, Pro, Trp.
Neutral Amino Acids: Ser, Gly, Thr, Tyr, Cys; [c]Phe + Tyr. [d]Met + Cys
[1]Asx: Asp+Asn; [2]Glx: Glu+Gln

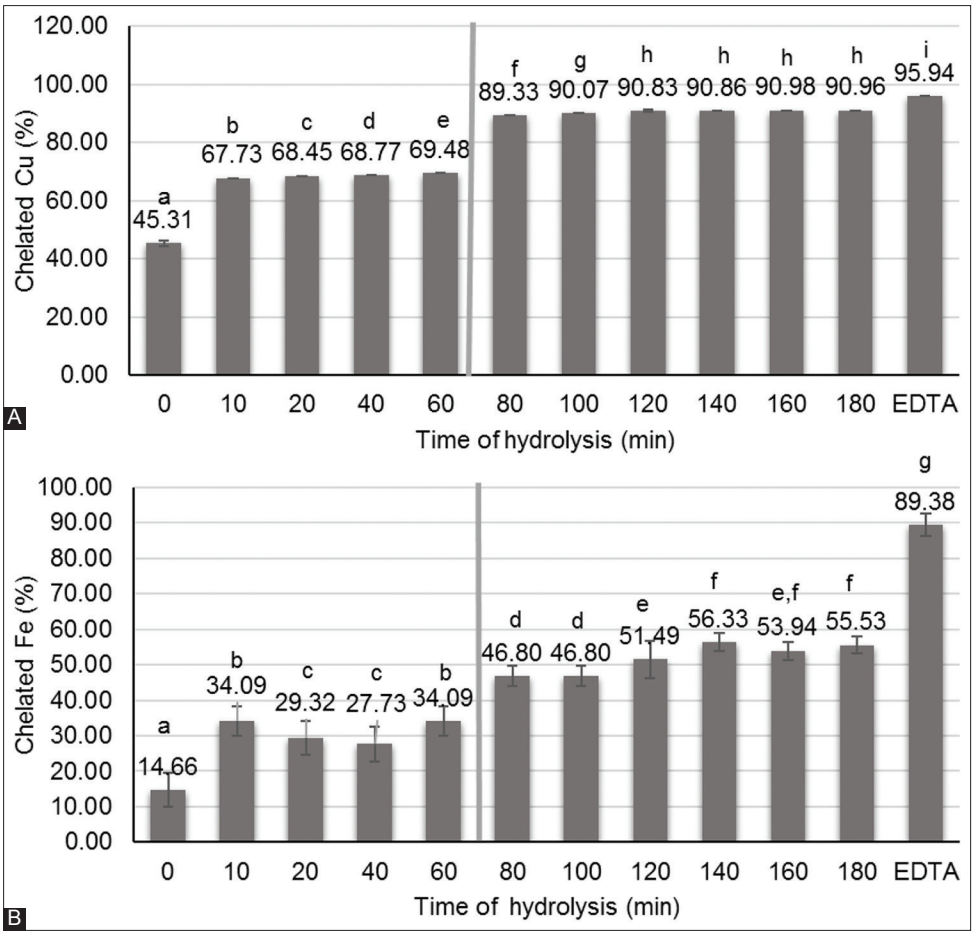

Fig 5. (A-B) Copper chelating activity of NHP and hydrolysates (1 mg protein) derived from lion fish muscle subjected to enzymatic hydrolysis with pepsin and pancreatin. Error bars indicate the standard deviation. Different letters indicate statistical difference ($p<0.05$). Standard 5 µg of EDTA. Iron chelating activity of NHP and hydrolysates (1 mg protein) derived from lion fish muscle subjected to enzymatic hydrolysis with pepsin and pancreatin. Error bars indicate the standard deviation. Different letters indicate statistical difference ($p<0.05$). Standard 50 µg of EDTA.

process of hydrolysis, as the protein structure changes over time to promote chelating activity (Eisenthal-Danson et al., 2002).

The Cu^{2+} chelating activity observed are higher than that reported by Zhuang et al., 2010 in jellyfish (*Rhopilema esculentum*) digested with a mixture Properasa E and trypsin (1 mg of protein evaluated), which had an activity of 40%. Conversely, Guo (2015) reported lower values (23.22±3.35%) in the Alaska Pollok fish (*Gadus chalcogrammus*) digested with trypsin (50°C, pH 8, 4h).

Estrella (2017) reported the presence of His in lion fish, which is an amino acid characterized by being a copper chelator, whereas Ghanbari et al. (2015) reported that the high chelating capacity of copper is associated to the presence of glutamic acid and aspartic, as well as basic amino acids such as Lys and Arg. As seen in table 1, NHP had a His content (g/100 g of protein) of 1.75, while H60 was 1.52, and H180 was 1.66. The Lys content in NHP was similar to that found in H60, while H180 was slightly lower. However, with respect to Arg (g/100 g of protein), H180 had the highest content (13.10), followed by H60 (12.53) and NHP (10.96). Therefore, it is possible that Arginine is the main amino acid that explain the differences observed. The activity of chelating peptides can prevent the oxidative activity of copper through the chelation of this metallic ion, so it could prevent the oxidative activity that occurs in the stomach, as well as to inhibit the oxidation of low-density lipoproteins induced by copper in the bloodstream (Burkitt, et al., 2001), and contribute to in the treatment of Wilson's disease, in which the liver is unable to eliminate copper properly (Jiménez et al., 2010).

Fe^{2+} chelating activity

NHP had 14.66±4.68% of iron chelation activity, and as shown in Fig. 5B, this activity increased in relation to time and degree of hydrolysis. The iron chelating capacity was increased when pancreatin was added, with the highest peak at 140 minutes. Girgih et al. (2013) reported iron chelation values about 30% on the fillet of salmon (*Salmo salar*) hydrolyzed with pepsin. However, Sila et al. (2014)

reported values higher than 80% in a digestion system with Alcalase® using shrimp (*P. longirostris*) as substrate. Klompong et al. (2007) analyzed the fish protein *Selaroides leptolepis* subjected to hydrolysis with Alcalase® and reported chelation values greater than 60%, similar to the highest iron chelation values reported in this work. The chelating activity of metals can be associated to the structure, molecular weight and composition of amino acids, being Gly and His, the amino acids that have been reported to have the highest iron chelating activity (Lin et al., 2014). Estrella (2017) found these amino acids in lion fish protein as well as protein hydrolysates obtained by treatment with alcalase®. However, chelating activity may also account for the presence of the amino acids Pro and Arg (Table 1). The importance of peptides with iron chelating capacity is that they can be used as antioxidants or for the treatment of diseases such as thalassemia or myelodysplastic syndrome, which are a class of anemia characterized by impaired hemoglobin production which is treated with constant blood transfusions, leading to accumulation of mitochondrial iron (Villegas, 2006). As it can be observed, the chelating activity of iron is less compared to that of copper, which is explained by the fact that iron has a greater number of coordination sites and therefore more peptides are required to chelate it in a greater proportion (Ruiz, 2009).

CONCLUSIONS

Lion fish (*Pterois volitans* L.) muscle hydrolysates were obtained using a pepsin-pancreatin sequential system, where the highest DH was 37.74±0.33% at 180 min of digestion. The enzymatic digestion generated peptides with an estimated molecular weight between 4,954 and 5,988 Da, which were not detected in the electrophoretic profile of the muscle. The highest antioxidant activity for both DPPH and $ABTS^+$ was found with pepsin digestion at minute 40, while the chelating activity of Cu^{2+} and Fe^{2+} was higher in the pepsin-pancreatin system at minute 120 and 140, respectively. Higher Trp, Cys and Lys content in pepsin hydrolysates at 60 min may be associated with higher antioxidant activity against $ABTS^+$, while higher Pro and Arg content in pepsin-pancreatin hydrolysates at 180 min may be associated with higher Cu^{2+} and Fe^{2+} chelating activity. Therefore, hydrolysates obtained from lion fish high-quality muscle protein are a potential source of ingredients with antioxidant and chelating activity, which in the future could be used as functional ingredients.

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Authors´ contributions

Luis Chel-Guerrero participated in the design of the research, acquisition and analysis of data, involved in drafting the paper for important intellectual content. David Cua-Aguayo and Azucena Chuc-Koyoc participated in the adaptation of some analytical techniques, acquisition and analysis of data. David Betancur-Ancona, participated in the analysis of data, involved in revising the paper critically. Irma Aranda-González participated in the acquisition and data analysis, involved in revising the paper critically. Translate the manuscript from Spanish to English. Santiago Gallegos-Tintoré participated in the design of the research (head of research project), acquisition and analysis of data, involved in drafting the paper for important intellectual content and revising the paper critically. Translate the manuscript from Spanish to English. All authors read and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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