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Antioxidant and angiotensin converting enzyme inhibitory activities of red lionfish (*Pterois volitans* L.) muscle protein hydrolysates obtained using pepsin-pancreatin system

[1]Chuc-Koyoc, A., [1]Chel-Guerrero, L., [1]Sosa-Crespo, I., [1]Betancur-Ancona, D., [2]Vioque, J. and [1]*Gallegos-Tintoré, S.

[1]Facultad de Ingeniería Química, Universidad Autónoma de Yucatán, Campus de Ciencias Exactas e Ingenierías, Periférico Norte, km 33.5, Tablaje Catastral 13615, Chuburná de Hidalgo Inn. C.P. 97203, Mérida, Yucatán, México
[2]Instituto de la Grasa (C.S.I.C.), Campus de la Universidad Pablo de Olavide, Edificio 46 Ctra. de Utrera, km.1, 41013, Sevilla, Spain

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Abstract

Despite being an invasive species and representing a threat in the area, red lionfish (*Pterois volitans* L.) meat is valued for its pleasant taste and high protein content. The objective of the present work was to evaluate the angiotensin converting enzyme inhibition (ACE-I) and the antioxidant activities of red lionfish muscle protein hydrolysates *in vitro.* Hydrolysates were obtained using the pepsin-pancreatin system, and their degree of hydrolysis (DH), electrophoretic, and amino acid profile were determined. Subsequently, their ACE-I and $ABTS^+$ radical scavenging activity were evaluated, selecting those with the highest response. The highest DH (66.1%) was found in the hydrolysate obtained at 120 min of reaction time (H_{120}); electrophoresis revealed the action of the enzymes on the muscle proteins. The highest bioactivities occurred during hydrolysis with pepsin for 60 min (H_{60}), obtaining the highest ACE-I of 68.8% (evaluated with 500 µg protein) with a DH of 16.8%, ACE-I related amino acid content of 33.6%, and hydrophobic amino acid content of 42.9%. With the 30 min hydrolysates (H_{30}), the highest Trolox equivalent antioxidant capacity of 60.3 mM/mg protein was obtained with 18.4% DH and 22.1% radical scavenger amino acids; its comparison with H_{120} proved that higher DH did not produce a greater response in $ABTS^+$ radical scavenging activity, DPPH, reducing power, and copper chelation assays; with β-carotene bleaching being the exception. Therefore, it can be concluded that red lionfish muscle hydrolysates could be a promising source of peptides with antihypertensive and antioxidant properties, and can be suitable as functional ingredients.

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Introduction

Chronic degenerative diseases affect the physical and mental health of patients who suffer from them; in most cases, acute drugs are necessary; in others, various chronic treatments are required. The consumption of these drugs mostly generates improvement in patients; however, some patients may experience side effects. In an attempt to counteract these effects, alternatives such as traditional and/or homeopathic medicine have been used, including the use of functional foods of animal-origin; the majority of them are land-based. However, in recent years, research has been done with marine species as their main source (He *et al*., 2013; Girgih *et al*., 2016).

Different studies have been conducted from marine sources, focusing on biomolecules such as starches, fats, and proteins, the latter being the most researched due to their high presence in this type of food. In addition to the nutritional benefits provided by the muscles of certain marine animals, bioactivities such as antioxidant or antihypertensive properties have been demonstrated (Chel-Guerrero *et al*., 2020), and have been studied based on the peptides obtained from the native protein from the muscles of these species. An option for the production of bioactive peptides from animal protein is the red lionfish (*Pterois volitans* L.), an invasive species in the Atlantic Ocean, Caribbean Sea, and Gulf of Mexico (Yandle *et al*., 2022) that competes for food

*Corresponding author.
Email: santiago.gallegos@correo.uady.mx

and space with populations important for fisheries and species with biological functions, representing a threat to coral reefs, and having ecological, economic, and social consequences. Despite this, research has shown that red lionfish fillets have flavour, colour, and texture characteristics that are well accepted compared to other commercial species such as snappers, groupers, and sea basses (Carreño-Montoya *et al.*, 2016).

Due to the benefits that this species could present, the purpose of the present work was to evaluate the inhibitory activity of the angiotensin converting enzyme, as well as the antioxidant activity of protein hydrolysates obtained from red lionfish muscle by treatment with the commercial gastrointestinal enzymes pepsin and pancreatin, suggesting that the hydrolysates obtained could be a promising source of peptides with antihypertensive and/or antioxidant properties, which could be used as functional ingredients.

Materials and methods

Raw material

Red lionfish samples were collected by divers near Cozumel Island in the state of Quintana Roo, on the Caribbean coast of Mexico. The fish were gutted and filleted. The skinless fillets were freeze-dried in the laboratory until further use. The dried sample was pulverized and mixed until it was completely homogeneous. Finally, it was stored at -20°C in a polyethylene bottle to perform the tests. To preserve the various biological materials, samples were freeze-dried (Labconco, FreeZone, 4.5) with a cooling system of -47°C at 0.06 - 0.07 mbar.

Protein hydrolysates preparation

A subsample of the freeze-dried fillet was used to obtain the hydrolysates; the process was performed in duplicate. Protein hydrolysates were isolated from the fillets following the first step of the hydrolysis method described by Megías *et al*. (2008). Freeze-dried fish at fillet:water ratio (5%, w/v) was digested at 37°C using pepsin (Sigma P7000) at pH 2.5 for 60 min, and then with pancreatin (Sigma P1625) at pH 7.5 for 180 min. A 1:20 (w/w) enzyme-to-substrate ratio was used for both enzymes. Aliquots were taken at different times (30 and 60 min for digestion using pepsin; and 90, 120, 150, and 180 min for digestion using pepsin/pancreatin), and hydrolysis was stopped by heat inactivation at 80°C for 20 min. The resulting hydrolysates (H_{30}, H_{60}, H_{90}, H_{120}, H_{150}, and H_{180}) were clarified by centrifugation at 11,227 *g* for 30 min at 5°C, frozen at -20°C until further use. Hydrolysate protein content was quantified following Lowry *et al*. (1951).

Degree of hydrolysis

Degree of hydrolysis (DH) of H_0 (non-hydrolysed protein), H_{30}, H_{60}, H_{90}, H_{120}, H_{150}, and H_{180} was calculated following Nielsen *et al*. (2001). The free amino groups were quantified using *o*-phthalaldehyde (Sigma P0657) in the presence of dithiothreitol, which formed a coloured compound detectable at 340 nm using a spectrophotometer (Thermo Spectronic, Genesys 10UV). The cleavage of peptide bonds was quantified using a calibration curve with L-serine (Sigma S4500) as a standard, using Eq. 1:

$$\% \text{ DH} = \frac{(h)}{(htot)} x\ 100 \qquad \text{(Eq. 1)}$$

where, *htot* = total number of peptide bonds per protein equivalent, and *h* = number of hydrolysed bonds. All the tests were performed in triplicate.

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

This analysis was done following Schägger and Jagow (1987) with 13% acrylamide gel and a 4% stacking gel. Hydrolysates (7.5 µg/µL of protein) were dissolved separately in a sample buffer, and heated at 100°C for 5 min. Runs were done at 40 mA for 1.5 h in a Miniprotean electrophoresis chamber (BIO-RAD, Hercules, California). Wells were loaded with 37.5 µg of the protein from each of the hydrolysates; a low-range standard (BIO-RAD, USA, Cat. #161-0304) was used which contained phosphorylase B (105.203 kDa), bovine serum albumin (84.174 kDa), ovalbumin (50.443 kDa), carbonic anhydrase (36.811 kDa), soybean trypsin inhibitor (29.059 kDa), and lysozyme (20.486 kDa).

Quantitative determination of amino acids of hydrolysates from lionfish muscle

Amino acid analysis of H_{30}, H_{60}, H_{90}, H_{120}, H_{150}, and H_{180} hydrolysates was performed by acid hydrolysis and HPLC after derivatisation with diethyl ethoxymethylenemalonate (Aldrich D94208), according to Alaiz *et al*. (1992) using DL-α-aminobutyric acid (Aldrich D94208) as internal standard.

Angiotensin converting enzyme inhibition (ACE-I)

Inhibitory activity was quantified for hydrolysates H_{30}, H_{60}, H_{90}, H_{120}, H_{150}, and H_{180} according to Cian *et al*. (2011) with some modifications. These modifications consisted of purifying the enzyme from the lung of a recently killed rabbit as follows: 1 g of the lung was extracted with a buffer containing 0.25 M sucrose and 0.1 M sodium anhydrous phosphate (pH 8.3; 1:5 p/v), and 5 µL PMSF (phenylmethylsulphonyl fluoride) was added. The mixture was centrifuged at 15,500 *g* for 10 min at 4°C. Then, 20 µL of sample, 20 µL of ACE-I, 20 µL of hippuryl-L-histidyl-L-leucine, 15 µL of 5 M NaCl (0.3%), and 175 µL of 0.1 M NaH_2PO_4 (pH 8.3) were added to the mixture. This was incubated at 37°C for 45 min, and the reactions were inactivated using 665 µL of 2,4,6-trichloride-triazine in 3% dioxane, and 1.1 mL of NaH_2PO_4 was added. The mixture was centrifuged at 15,500 *g* for 10 min at 4°C, and the absorbance was measured at 382 nm. The percentage of ACE-I inhibition was expressed as the ratio between the reactions with the sample and control, and calculated using Eq. 2:

$$\text{ACE inhibition (\%)} = 100 - \left[\frac{(AS-ABS)}{(AE-ABE)}\, x\, 100\right] \quad \text{(Eq. 2)}$$

where, AS = optical density of ACE-I with sample and substrate (enzyme-substrate-sample), ABS = optical density of ACE-I and sample (enzyme-sample), AE = optical density of ACE-I with substrate (enzyme-substrate), and ABE = optical density of substrate without ACE-I or sample (substrate).

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

Antioxidant activity in the hydrolysates was measured according to Pukalskas *et al*. (2002). The protein hydrolysates from red lionfish at a protein concentration of 1 mg/mL, and from commercial antioxidant butylated hydroxytoluene (Sigma 47168) at the same concentration were analyzed. $ABTS^+$ radical cation was produced by reacting $ABTS^+$ with potassium persulphate. 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 of antioxidant compound or Trolox standard (final concentration 0.5 - 3.5 mM) in PBS, absorbance was read at room temperature 6 min after initial mixing. All the tests were performed in triplicate.

β-carotene bleaching method

Antioxidant activity was measured with the β-carotene bleaching method with modifications, as described by Del Ré and Jorge (2011). A mixture of 4 mg of β-carotene (Sigma 22040) in 1 mL of chloroform and 1 mL of Tween 20 (P1379) was vigorously vortexed. After removing the chloroform under a nitrogen stream, a clear solution was obtained by mixing in 50 mL of 100 mM oxygen-sparged phosphate buffer at pH 7.4. Each peptide fraction (equivalent to 500 µg protein) was dissolved in 60 µL of phosphate buffer and 200 µL of β-carotene / Tween 20 solution, added to wells in a 96-well plate, and incubated at 50°C in the dark. The oxidant agent was 10 µL of 50 µM $FeCl_2$ (Sigma 44939). The negative control was β-carotene / Tween 20 solution + 10 µL of 50 µM $FeCl_2$ mixed with 60 µL of phosphate buffer containing no peptide fraction. The positive control was β-carotene / Tween 20 solution + 10 µL of 50 µM $FeCl_2$ mixed with 10 µg of butylated hydroxytoluene (BHT) (Sigma 47168). Peroxidative degradation of β-carotene was monitored by recording the absorbance at 470 nm after 120 min with a microplate reader. The percentage of inhibition of β-carotene bleaching was calculated using Eq. 3:

$$\text{β-carotene inhibition (\%)} = \frac{(Abs\ C - Abs\ S)}{Abs\ C}\, x\, 100 \quad \text{(Eq. 3)}$$

where, Abs C = absorbance in the negative control, and Abs S = absorbance in the sample; both readings were taken at the determined measurement times.

Evaluation of reducing power

Reducing power was analysed according to Oyaizu (1986). The red lionfish protein hydrolysates with the highest scavenging of $ABTS^+$ radical were evaluated considering 5, 10, 15, 20, 25, and 30 µg of protein. The samples were incubated with potassium ferricyanide 1% (w/v) in 0.2 M phosphate buffer with pH 6.6 at 50°C for 20 min. Then, 2.5% (w/v) TCA was added. Afterward, the solution was incubated with 0.01% (w/v) ferric chloride at 50°C for 10 min. Finally, absorbance was read at 700 nm. Blank sample included neither sample nor ferric chloride, and positive control included the synthetic antioxidant butylated hydroxytoluene (BHT). The assay was done in duplicate.

Statistical analysis

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

Results and discussion

Red lionfish muscle hydrolysates and their degree of hydrolysis

The sample (H_0) showed significant DH (8.35 ± 0.96%) which could have been due to the action of endogenous enzymes present in lionfish muscle such as cathepsin D or other cathepsin enzymes that induce autolysis (Lan *et al*., 2022) or degradation of proteins to polypeptides or peptides. No significant differences ($p > 0.05$) were found between H_{30} (18.38 ± 0.24%) and H_{60} (16.85 ± 0.48%) hydrolysates (Figure 1). When pancreatin was added to the enzyme system, there was an increase at H_{90} (34.87 ± 0.96%) and H_{120} (66.15 ± 0.96%), the latter with the highest value. This behaviour might have been attributed to a broader spectrum of action on red lionfish proteins, employing both endo- and exo-proteases. Subsequently, there was a decrease in DH at H_{150} (49.83 ± 1.92%) and H_{180} (48.81 ± 0.48%) over time, and no significant difference ($p > 0.05$) was found between the two reaction times; this effect could have been due to a transpeptidation generated during the enzymatic process. During protein hydrolysis, a transfer of one or more amino acids between peptides and/or the ligation of peptides by reverse proteolysis could occur through a series of reactions, possibly starting with the proteolysis itself, and continuing with a re-synthesis of peptide bonds hydrolysed by enzymes (Chibuike-Chinedu and Subin-Raj, 2016). Red lionfish hydrolysates presented a DH > 10%, which classifies them as extensive, and they may have a peptide composition that could favour ACE-I and antioxidant bioactivities, as mentioned by Khantaphanta *et al*. (2011), agreeing with what was presented by the marine source in the present work.

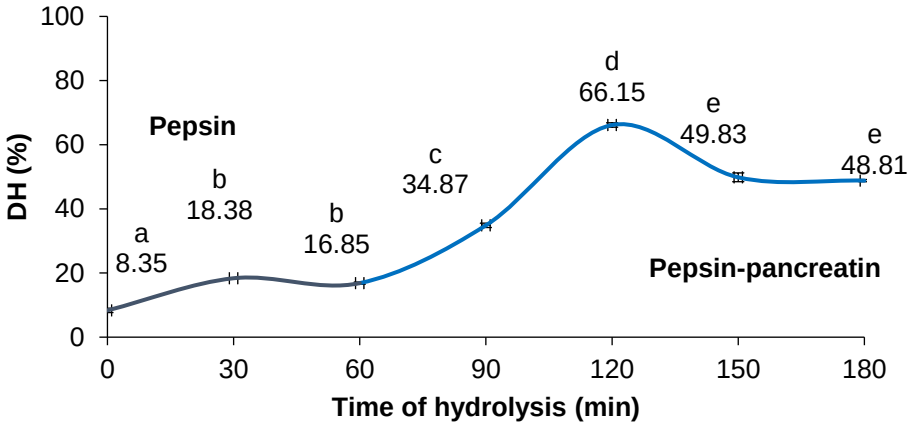

Figure 1. Kinetics of sequential hydrolysis with gastrointestinal enzymes of lionfish muscle proteins. Different lowercase letters indicate statistically significant difference ($p < 0.05$).

SDS-PAGE electrophoretic profile of red lionfish muscle proteins and their protein hydrolysates

The profile of the hydrolysates showed the action of the enzymes on the muscle proteins, demonstrating that they contained polypeptides of lower molecular mass. The estimated molecular weight of the proteins present in H_0 was 92, 63, 50, 45, 43, 43, 40, 40, 37, 37, 32, 29, 25, and < 20 kDa. For H_{30} and H_{60} hydrolysates, proteins and polypeptides with estimated molecular weights of 75.4, 55.3, 38, 29, 21.8, and < 20 KDa were observed (Figures 2A and 2B). Regarding the H_{90}, H_{120}, H_{150}, and H_{180} hydrolysates, the estimated molecular weights had values between 41 and < 20 kDa, slightly denoting the presence of bands and evidencing the degradation of the proteins into polypeptides; this is indicated in the gel with square brackets.

The digestion of muscle proteins using pepsin modified the original electrophoretic profile. Likewise, the following digestion with pancreatin showed an increase in low-molecular-weight polypeptides. Therefore, the action of the enzymes and the hydrolysis time affected the composition of the hydrolysates, which was also reflected by the increase in DH when adding pancreatin to the enzymatic system. This effect was also reported by

Nasri *et al.* (2013) in goby (*Zosterissessor ophiocephalus*) hydrolysates, where the molecular weights detected in the gel decreased in relation to the increase in DH; SDS-PAGE electrophoresis being the most widely used to characterise bioactive hydrolysates and proteins from marine sources (He *et al*., 2013).

Previous studies have shown that the bioactivities of fish hydrolysates are closely linked to their molecular weights. For example, croceine croaker and cod hydrolysates exhibited the most potent antioxidant activity when the hydrolysates had molecular weights below 3 and 10 kDa, respectively (Gao *et al*., 2021).

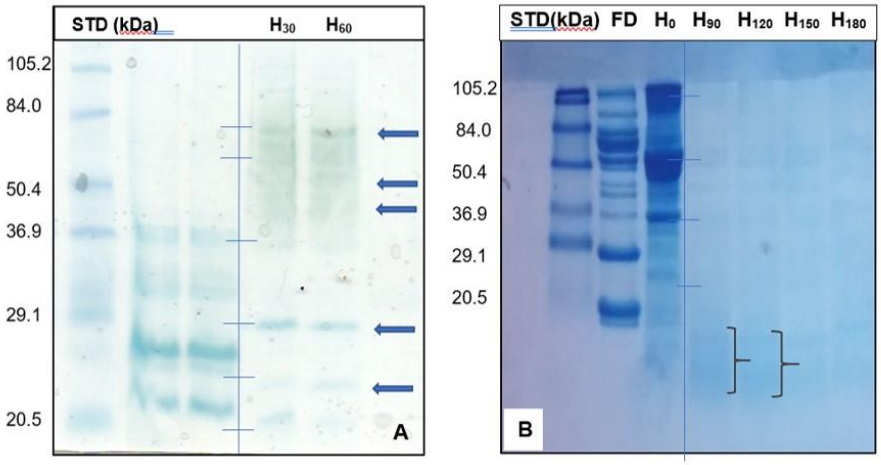

Figure 2. (A) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of H_{30} and H_{60} protein hydrolysates obtained with pepsin. **(B)** Electrophoretic pattern of freeze-dried lionfish muscle proteins (FD) and H_0, as well as H_{90}, H_{120}, H_{150}, and H_{180} hydrolysates obtained with pepsin-pancreatin. STD: Standard for the molecular weight marker; kDa: kiloDalton.

Amino acid composition of lionfish protein hydrolysates

The amino acid composition of lionfish protein hydrolysates is shown in Table 1. In the six protein hydrolysates, as well as in the non-hydrolysed protein (H_0), the amino acids Asx (Asp+Asn) and Glx (Glu+Gln) represented almost 20% of the total amino acid content by weight. However, the proportion was lower in relation to muscle (25.30%). These amino acids have also been reported in many other protein hydrolysates from other marine species such as capelin (*Mallotus villosus*) with 9.89% Asx and 13.4% Glx, and in north Pacific hake (*Merluccius productus*) with 10.10 and 13.80%, respectively (Chalamaiah *et al*., 2012).

For the six hydrolysates in the red lionfish study and H_0, the amino acids with antihypertensive properties of Arg, Leu, and Lys showed higher concentrations than the others. Among the hydrolysates, a similar proportion of amino acids such as His, Phe, Lys, and Leu were found, while others, such as Pro and Trp, varied between the hydrolysates obtained at different times (Dong *et al.*, 2024). Dong *et al*. (2008) also reported this differentiation on silver carp (*Hypophthalmichthys molitrix*) hydrolysates generated at 0, 15, 90, and 240 min of hydrolysis, also employing a sequential enzymatic system. Alcalase enzyme was used in ratio with the protein amount of 0.5% (w/w), and the Pro content was 5.09, 7.37, 7.60, and 8.01%, respectively, for each hydrolysis time. With Flavourzyme enzyme, considering the same enzyme/substrate ratio, the contents were 5.09, 4.85, 4.76, and 5.20%, comparatively.

The difference observed in the amino acid composition of the red lionfish protein hydrolysates could have been attributed to the fact that in the hydrolysis process, the insoluble part was discarded, which could contain, besides lipids and salts, other components like peptides or amino acids, which were also generated during the enzymatic process (Dong *et al.*, 2008). Likewise, it could be related to the DH since it influences the solubility of components in marine protein hydrolysates (Morales *et al.*, 2016), and pH variations during processing might have influenced the amino acid content (Nasri *et al*., 2013).

Low levels of Cys are common in fish protein hydrolysates (Morales *et al*., 2016). Red lionfish

Table 1. Amino acid composition of red lionfish protein hydrolysates.

Amino acid	Red lionfish protein hydrolysate (g of AA/100 g of protein)					
	Pepsin H_{30}	Pepsin H_{60}	Pepsin-pancreatin H_{90}	Pepsin-pancreatin H_{120}	Pepsin-pancreatin H_{150}	Pepsin-pancreatin H_{180}
Asx[1]	9.26 ± 0.15	7.29 ± 0.08	6.85 ± 0.31	9.98 ± 0.56	7.80 ± 1.41	7.89 ± 0.25
Glx[2]	13.40 ± 0.44	12.25 ± 0.78	11.23 ± 0.62	11.42 ± 0.28	12.14 ± 1.01	11.84 ± 1.36
Ser	1.80 ± 0.19	2.02 ± 0.24	1.89 ± 0.24	2.18 ± 0.10	1.99 ± 0.30	1.85 ± 0.29
His	1.55 ± 0.06	1.52 ± 0.03	1.65 ± 0.07	1.76 ± 0.08	1.99 ± 0.01	1.66 ± 0.10
Gly	4.70 ± 0.54	4.77 ± 0.09	4.79 ± 0.40	4.52 ± 0.25	5.12 ± 0.17	4.53 ± 0.42
Thr	3.60 ± 0.15	3.81 ± 0.03	3.89 ± 0.03	3.97 ± 0.09	4.08 ± 0.05	3.80 ± 0.06
Arg	11.81 ± 0.09	12.53 ± 0.18	12.87 ± 0.11	13.68 ± 0.18	13.52 ± 0.75	13.10 ± 0.09
Ala	2.80 ± 0.07	2.81 ± 0.03	2.88 ± 0.04	2.96 ± 0.08	3.22 ± 0.01	2.89 ± 0.17
Pro	4.29 ± 0.24	5.29 ± 0.06	5.28 ± 0.09	1.64 ± 0.15	5.44 ± 1.95	7.83 ± 3.17
Tyr	2.37 ± 0.01	2.29 ± 0.12	2.45 ± 0.32	2.51 ± 0.12	2.38 ± 0.41	2.14 ± 0.33
Val	12.44 ± 0.14	13.37 ± 0.17	16.34 ± 0.08	14.61 ± 0.08	10.41 ± 0.51	13.13 ± 0.38
Met	2.05 ± 0.13	1.89 ± 0.08	1.90 ± 0.04	2.29 ± 0.03	2.64 ± 0.02	2.21 ± 0.17
Cys	1.37 ± 0.79	1.30 ± 0.70	n.d.	n.d.	0.08 ± 0.17	0.03 ± 0.01
Ile	4.14 ± 0.26	4.19 ± 0.09	4.35 ± 0.37	4.57 ± 0.04	4.89 ± 0.02	4.36 ± 0.07
Leu	8.08 ± 0.35	8.22 ± 0.20	8.30 ± 0.36	8.52 ± 0.13	8.92 ± 0.15	8.12 ± 0.18
Phe	3.29 ± 0.18	3.29 ± 0.01	3.57 ± 0.26	3.64 ± 0.11	3.96 ± 0.29	3.47 ± 0.18
Lys	9.39 ± 0.16	9.30 ± 0.40	9.24 ± 0.12	9.16 ± 0.21	9.72 ± 0.07	8.83 ± 0.56
Trp	3.65 ± 0.11	3.84 ± 0.26	2.50 ± 0.16	2.59 ± 0.06	1.70 ± 0.21	2.30 ± 0.92
Amino acid distribution						
Hydrophobic	40.74	42.91	45.14	40.81	41.18	44.31
Neutral	13.84	14.20	13.02	13.18	13.65	12.36
Hydrophilic	45.42	42.89	41.84	46.00	45.17	43.33

Hydrophobic amino acids: Ala, Val, Met, Phe, Leu, Ile, Pro, Trp; and neutral amino acids: Ser, Gly, Thr, Tyr, Cys. [1]Asx: Asp+Asn; [2]Glx: Glu+Gln; and n.d.: not detected.

hydrolysates showed a low amount of this amino acid, and even in H_{90} and H_{120}, it was not detected in the analysis, being lesser compared to other hydrolysates. For example, the silver carp hydrolysate (from the above-mentioned study) obtained at 90 min of hydrolysis with the Alcalase enzyme presented a Cys composition of 2.38%, and with the Flavourzyme enzyme and the same hydrolysis time, the content of this amino acid was higher, with 5.06%. It is significant to highlight the importance of the total hydrophobic amino acid composition since it is related to the ability of peptides to interact with lipids, favouring antioxidant activity (He *et al*., 2013) and antihypertensive activity.

In the case of red lionfish hydrolysates, approximately 40% of hydrophobic amino acids were present, and the sum of amino acids with antioxidant properties was reported to be close to 20%. In this regard, there are reports on salmon (*Salmo salar*) and cod (*Gadus morhua*) hydrolysates with antioxidant properties, obtained with pepsin, trypsin, and α-chymotrypsin, in which the Cys contents were 0.41 and 0.38%, respectively. Additionally, the hydrophobic amino acid composition was 40.75 and 39.60%, respectively. The determination of amino acids in fish protein hydrolysates is important to deduce both of their functional properties and nutritional value (Gao *et al.*, 2021). In this sense, lionfish hydrolysates can be an important source of essential amino acids, being approximately 40%. The composition of amino acids Leu (6.6 g/100 g protein) and Phe (6.3 g/100 g protein) in the hydrolysates was

close to that established by the FAO and WHO (2007) for this type of food. Except for the H_{180} hydrolysate, the values in the present work for amino acids Val (3.5 g/100 g protein), Trp (1.1 g/100 g protein), and Lys (5.8 g/100 g protein) were higher.

Angiotensin converting enzyme inhibitory activity from red lionfish protein hydrolysates

The H_{30} hydrolysate presented a lower percentage of inhibition compared to H_{60} (Figure 3). It was observed that in the hydrolysates obtained with pepsin-pancreatin, a decrease in inhibition occurred. No significant differences ($p < 0.05$) were found between the values obtained for H_{90}, H_{120}, and H_{180}, which presented a similar inhibitory percentage. It was observed that the highest percentage of inhibition was obtained with the H_{60} hydrolysate.

The current trend is to substitute drugs used for the treatment of hypertension with products of natural origin, such as fish or its derivatives, thus diversifying their use. Therefore, red lionfish protein hydrolysates could be an effective derivative as ACE-I inhibitors; adding *in vivo* assays and cytotoxicity tests to their study, red lionfish hydrolysates could be a promising source for hypertension treatments with possible advantages over synthetic drugs without producing side effects (Ramírez *et al*., 2013).

Nasri *et al*. (2013) demonstrated that with gastrointestinal proteases from *Zosterissessor ophiocephalus* fish at higher DH, the hydrolysates exhibited higher ACEI-1. Moreover, Balti *et al*. (2010) in their study with squid (*Sepia officinalis*) muscle protein hydrolysates obtained with pepsin, chymotrypsin, or trypsin enzymes reported that DH and ACE-I inhibitory activity increased when hydrolysis time increased, but this was not a general rule. The ACE-I capacity of H_{60} might have been due to the specificity of the pepsin towards amino acid residues from proteins, producing peptides with ACE-I inhibitory structures capable of showing a higher inhibitory effect (Alemán *et al*., 2013). Similarly, the specificity of the enzymes used (pepsin-pancreatin) could have led to lower inhibitory effect since there was more cleavage action on amino acid residues, thus producing peptides with lower ACE inhibitory activity.

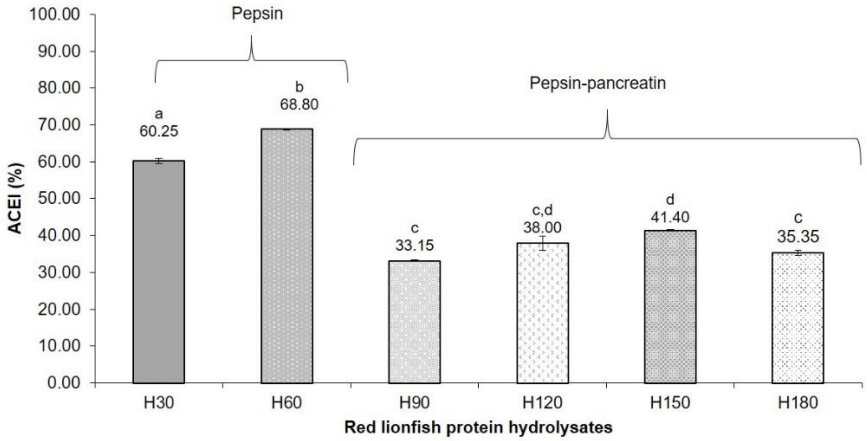

Figure 3. Percentage of angiotensin converting enzyme inhibitory activity (ACE-I) from lionfish hydrolysates obtained with pepsin-pancreatin (0.5 mg of protein tested). Different lowercase letters indicate statistically significant differences ($p < 0.05$).

ACE inhibitory activity is not associated with low molecular mass peptides, but affected by the composition of peptides. It is also attributed to differences in chain length and amino acid sequence in peptides, as well as their hydrophobicity (Alemán *et al*., 2013; Halim *et al.*, 2016). Peptide size may play an important role in the bio-functionality of H_{60}, as reported in protein hydrolysates with antihypertensive properties from fish species such as bonito (*Thunnus alalunga*) and yellowfin sole (Toopcham *et al*., 2015). In the present work, the amino acid composition and sequence likely influenced the bioactivity of H_{60} and H_{120} hydrolysates. In these hydrolysates, the content of the amino acid Pro, which has been reported as an ACE inhibitor (Halim *et al*., 2016), was 5.29 ± 0.02 and 1.64 ± 0.15%, respectively. This could have been responsible for the higher ACE-I of H_{60} since its presence in H_{120} was reported for the last three residues at the C-terminal group for the peptides reported as inhibitors (Alemán *et al*., 2013). A high proportion of aliphatic amino acids, such as Ile, Leu,

and Met, which play a role in ACE inhibitory activity, were identified in the hydrolysate of goby protein. The presence of aromatic (Trp, Tyr, Phe) and aliphatic (Ile, Ala, Leu, Met) residues suggests optimal sites that enhance peptide ACE inhibitor activity (Halim *et al*., 2016)

No significant differences ($p > 0.05$) were found in the total hydrophobic amino acid content between H_{60} (42.91%) and H_{120} (40.81%). Hydrolysates with higher DH containing larger numbers of smaller peptides have less exposed hydrophobic binding sites compared to larger peptides (Li *et al.*, 2012). This could explain the difference in bioactivity between H_{30} and H_{120}, knowing that ACE-I prefers as substrates or competitive inhibitors the peptides containing hydrophobic (aromatic and branched) amino acids (Lassoued *et al*., 2015). Other studies performed by different authors about hydrolysates from marine sources such as Atlantic salmon (*Salmo salar)*, coho salmon (*Oncorhynchus kisutch*), Alaska pollock (*Theragra chalcogramma*), and southern blue whiting (*Micromesistius australis*) using pepsin enzymes, pepsin-pancreatin, and thermolysin, showed that ACE-I changes depending on the method of hydrolysis, assuming that the hydrolysis time and enzyme/substrate ratio affect the production of ACE inhibitory peptides (Khantaphanta *et al*., 2011; Gao *et al*., 2021).

Trolox equivalent antioxidant capacity (TEAC)

The $ABTS^+$ radical is relatively stable but can be easily reduced in the presence of antioxidants, leading to its discoloration (Ilyasov *et al*., 2020). Based on the results obtained, it was observed (Figure 4) that H_{30} had the highest TEAC activity (mM/mg protein) presenting statistically significant differences ($p < 0.05$) compared to the other hydrolysates, with the exception of H_{150}. This higher effect indicated the presence of peptides with higher proton transfer capacity or positively charged hydrogens, which reacted with the unpaired electrons of the radicals.

He *et al*. (2013) indicated that antioxidant peptides could match or exceed the capacity of commercial antioxidants. In this sense, if the TEAC of lionfish hydrolysates obtained in the present work were compared with the antioxidant BHT (TEAC of 379.49, 1 mg/mL), we can observe values seven times lower than the reference antioxidant.

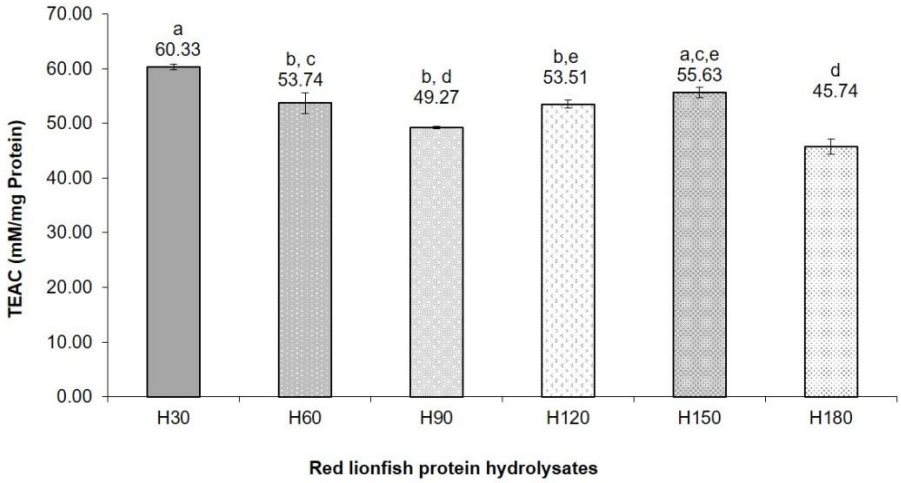

Figure 4. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) radical scavenging activity of red lionfish protein hydrolysates (1 mg of protein evaluated). Different lowercase letters indicate statistically significant differences ($p < 0.05$). TEAC: Trolox equivalent antioxidant capacity.

According to Li *et al*. (2012), DH influences antioxidant activity, since generally, a high activity is exhibited when the hydrolysate has values higher than 10%; however, this does not always happen since, as can be observed in the present work, the DH value of red lionfish muscle protein hydrolysates did not present a correlation with the bioactivity values obtained. The results showed that a higher scavenging activity for the $ABTS^+$ radical was not observed compared to the H_{30} hydrolysate (66.15 ± 0.96%). This behaviour was observed in sea bream (*Pagellus bogaraveo*) protein hydrolysates generated with pepsin enzyme coming from skipjack tuna (*Katsuwonus pelamis*) (Nalinanon *et al*., 2011), which resulted in TEAC of 150, 159, and 151 mM/mg with DH of 10, 20, and 30%, respectively. This

agreed with Najafian and Babji (2012) who stated that the molecular mass of peptides generated during hydrolysis influenced the bioactivity of the products. In a study on the determination of the Trolox equivalent antioxidant capacity, Zheng *et al*. (2016) reported the amino acids Tyr, Trp, Cys, Met, Lys, and Phe as $ABTS^+$ radical scavengers, presenting a higher scavenging activity in the order: Tyr > Trp > Cys, while the amino acids Met, Lys, and Phe presented a lower scavenging activity of this radical. The presence of these six amino acids could be favouring the radical scavenging activity in the H_{30} hydrolysate, the sum of these amino acids being 22.12%.

Based on what was established by Zheng *et al*. (2016) regarding the $ABTS^+$ radical scavenging activity, the sum of the amino acids with the highest and lowest effect for each hydrolysate can be seen in Table 2. Antioxidant peptides found in hydrolysates can act through different mechanisms in addition to hydrogens, and can donate electrons to convert radicals into more stable compounds (Halim *et al*., 2016). Likewise, it was reported that at different concentrations, peptides could vary their bio-functionality (Gao *et al*., 2021). Due to this, the hydrolysate that presented the highest $ABTS^+$ radical scavenging activity (H_{30}), as well as the one that presented the highest DH (H_{120}), were selected to compare the antioxidant responses in the present work.

Table 2. Percentage composition of amino acids with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) radical scavenging properties in red lionfish protein hydrolysates.

Red lionfish protein hydrolysate	Amino acid with higher $ABTS^+$ radical scavenging activity (Tyr+Trp+Cys)	Amino acid with lower $ABTS^+$ radical scavenging activity (Met+Lys+Phe)	Total (g/100 g protein)
H_{30}	7.39	14.73	22.12
H_{60}	7.44	14.49	21.93
H_{90}*	4.95	14.71	19.66
H_{120}*	5.10	15.10	20.19
H_{150}	4.16	16.32	20.49
H_{180}	4.47	14.51	18.99

*Cys was not identified in the analysis.

Bleaching of β-carotene by H_{30} and H_{120} hydrolysates

Statistical analysis indicated that from the factors evaluated, hydrolysates (H_{30} and H_{120}) and the amount of protein (50, 100, and 150 µg) had an effect ($p < 0.05$) on the bleaching of β-carotene, with values of this activity ranging from 52 to 66%. Likewise, H_{120} presented the highest activity compared to H_{30} when evaluating 100 and 150 µg ($p < 0.05$), being approximately 33% lower than BHT (Figure 5A). In this case, it was observed that DH influenced the antioxidant response, meaning that H_{120} with higher DH presented higher β-carotene bleaching than H_{30}.

It can be established that the peptides that make up these protein hydrolysates could have antioxidant properties in the inhibition of linoleic acid peroxidation. Some papers (Gao *et al*., 2021) mentioned that antioxidant peptides could also bind free radicals as well as ROS, preventing oxidative damage by interrupting the chain reaction of lipid peroxidation. It is important to emphasise that the inhibition of β-carotene is the result of the combination of copper chelating properties. Therefore, amino acids with chelating properties in peptides, like acidic amino acids (Asp and Glu), could explain their inhibition properties due to their carboxyl group possessing antioxidant properties (Chen *et al*., 2023).

Reducing power of H_{30} and H_{120} protein hydrolysates

The DH value had no relation with the antioxidant activity presented. A study working with brownstripe snapper (*Lutjanus vitta*) muscle using the Alcalase enzyme (Khantaphanta *et al*., 2011) reported that the percentage of DPPH radical scavenging was not directly related to the increase in DH value; the FRS % could likely be independent of the molecular weight of the peptides contained in the hydrolysates. Another research conducted by Girgih *et al*. (2016),

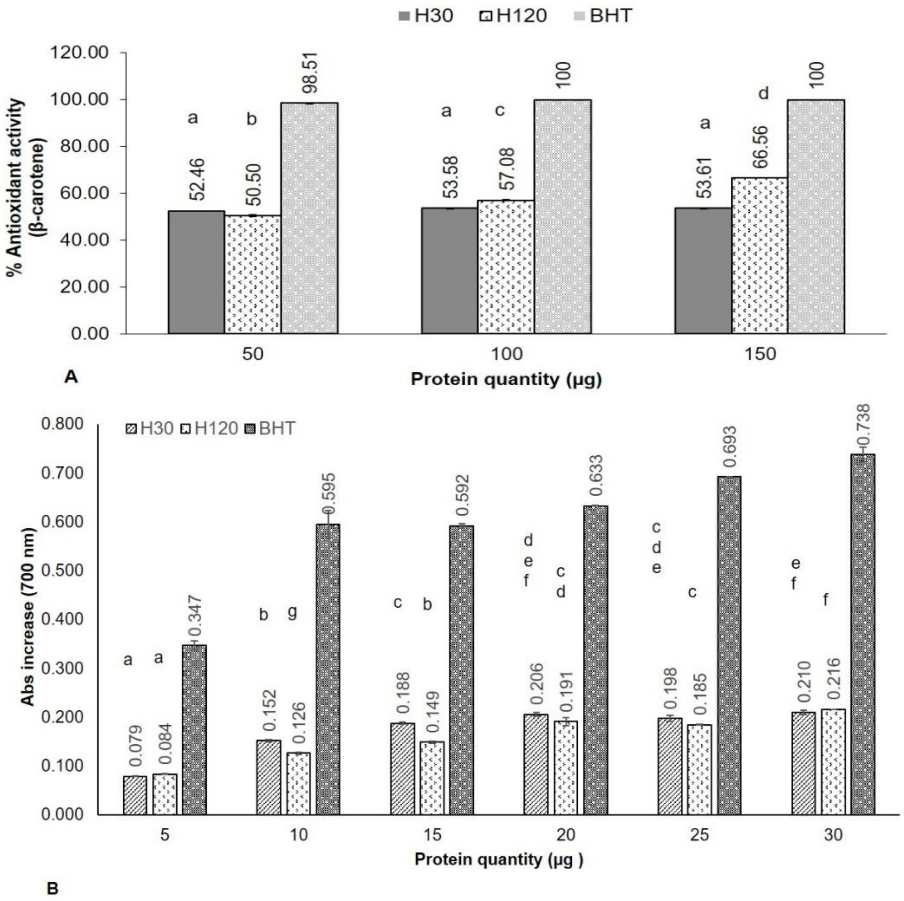

Figure 5. (A) Antioxidant activity of H_{30} and H_{120} hydrolysates determined by β-carotene bleaching method for 60 min evaluated at different amounts of protein. BHT: Butylated hydroxytoluene (10 µg). **(B)** Reducing power of red lionfish muscle protein hydrolysates H_{30} and H_{120} evaluated at different protein amounts and BHT antioxidant (5, 7.5, and 10 µg). Increase in absorbance compared to the control at 700 nm. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

where giant west African snail (*Archachatina marginata*) meat was hydrolysed, showed that the size of the peptides present in the hydrolysates did not intervene in the electron donation for the DPPH radical. Presumably, the composition of hydrophobic amino acids may be related to the bioactivity found in the present work for the hydrolysates evaluated, where the content of these amino acids in H_{30} was 40.74%, while for H_{120}, it was 40.81%, as earlier mentioned. Similarly, a high content of hydrophobic amino acids, which are the main ones responsible for the potent scavenging activity of the radical (DPPH), has been shown in studies with giant squid skin and muscle (Sabeena *et al.,* 2016). Other researchers (Morales *et al*., 2016) have reported that this activity was caused by the presence of amino acids and peptides containing Val, Leu, Ile, Ala, Phe, Cys, or Lys residues at the N-terminus. In the present work, the sum of these amino acids in the protein hydrolysates evaluated corresponded to 41.51 and 43.46% for H_{30} and H_{120}, respectively.

Statistically significant differences ($p < 0.05$) were found between the values obtained from the hydrolysates analysed compared to the commercial antioxidant BTH. H_{30} and H_{120} hydrolysates showed an increase in absorbance compared to the control at 700 nm, demonstrating their reducing power (Figure 5B). The results indicated that the samples analyzed could reduce Fe^{3+} to Fe^{2+}; they could also donate an electron to the free radicals to convert them into more stable compounds, attributing the antioxidant capacity to the availability of hydrogen ions (protons and electrons) of the peptides that comprise them. Statistical analysis showed that both factors, type of hydrolysate (H_{30} and H_{120}), and the amount of protein evaluated (5, 10, 15, 20, 25, and 30 µg), had statistically significant effect ($p < 0.05$) on the reducing power. Nazeer and Kulandai (2012)

reported that the reducing power increased with the concentration of protein evaluated (1, 2, 3, 3, 4, and 5 mg/mL) for hydrolysates from muscle and skin from giant trevally (*Caranx ignobilis*), presenting a higher response with the hydrolysate produced with pepsin, obtaining values of 0.23, 0.25, 0.26, 0.28, and 0.29. The values found were lower in comparison with the commercial antioxidant BHT and tocopherol. Also, several antioxidant hydrolysates and peptides derived from fish sources like longtail cod, cod, and mackerel exhibited better antioxidant activity compared to typical synthetic antioxidants like α-tocopherol and butylated hydroxyanisole (BHA) (Gao *et al*., 2021).

The higher electron-donating power leads to higher reducing power, which can be attributed to high DH, lower molecular weight peptides, and the high solubility of the protein (Pei-Teng *et al.*, 2021). The reducing power presented by red lionfish muscle protein hydrolysates might have been related to the hydrophobic residue composition of amino acids such as Ala, Val, and Leu due to their radical scavenging capacity. Likewise, some amino acids such as Gly, Ala, and Pro found in peptides could help to their solubility and facilitate accessibility towards hydrophobic species and hydrophobic polyunsaturated fatty acids (Ketnawa *et al*., 2016).

Conclusion

In vitro hydrolysis of red lionfish muscle proteins using the sequential pepsin-pancreatin system produced the highest DH at 120 min of the process (H_{120}); this was confirmed with the electrophoretic profile which indicated the presence of proteins and polypeptides of lower molecular weight. Electrophoretic analysis by SDS-PAGE allowed us to observe the action of the enzymes used on the fillet proteins. In the evaluation of ACE-I and antioxidant activity of red lionfish muscle protein hydrolysates, higher values were observed with the protein hydrolysates obtained with the enzyme pepsin; H_{30} presented the highest TEAC value, and H_{60} the highest ACEI value. *In vitro* evaluation of protein hydrolysates with higher TEAC (H_{30}) and higher DH (H_{120}) showed that the peptides may act with other antioxidant mechanisms. Comparison of the protein hydrolysate with higher TEAC (H_{30}) in relation to that with higher DH (H_{120}) showed that high DH did not produce a greater response in $ABTS^{+}$ radical scavenging, DPPH scavenging, reducing power, and copper chelation; with the exception of β-carotene bleaching. The responses obtained with the different tests performed showed that lionfish protein hydrolysates had remarkable antioxidant activities. Red lionfish fillets can thus be considered a source of bioactive peptides.

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References

Alaiz, M., Navarro, J., Girón, C. and Vioque, E. 1992. Amino acid analysis of high-performance liquid chromatography after derivatization with diethylethoxymethylenemalonate. Journal Chromatography 591(1-2): 181-186.

Alemán, A., Gómez, M. and Montero, P. 2013. Identification of ace-inhibitory peptides from squid skin collagen after *in vitro* gastrointestinal digestion. Food Research International 54(1): 790-795.

Balti, R., Nedjar, N., Bougatef, A., Guillochon, D. and Nasri, M. 2010. Three novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) using digestive proteases. Food Research International 43(4): 1136-1143.

Carreño Montoya, O., Cabrera Durán, E., Cabrera Durán, E., Bado Navarro, B. and Codina, G. 2016. Technological alternatives to face lionfish (*Pterois volitans*, Linnaeus 1758) invasion in the Colombian Caribbean Coast. Revista Ciencias Agropecuarias 2: 28-34.

Chalamaiah, M., Dinesh, B., Hemalatha R. and Jyothirmayi, J. 2012. Fish protein hydrolysates: Proximate composition, amino acid composition, antioxidant activities and applications: A review. Food Chemistry 135(4): 3020-3038.

Chel-Guerrero, L., Estrella-Millán, Y., Betancur-Ancona, D., Aranda-González, I., Castellanos-Ruelas, A. and Gallegos-Tintoré, S. 2020. Antioxidant, chelating, and angiotensin-converting enzyme inhibitory activities of peptide fractions from red lionfish (*Pterois*

volitans L.) muscle protein hydrolysates. International Food Research Journal 27(2): 224-233.

Chen, Y., Jing, H., Xiong, S., Manyande, A. and Du, H. 2023. Comparative study on hydrolysis, physicochemical and antioxidant properties in simulated digestion system between cooked pork and fish meat. Foods 12(9): 1757.

Chibuike-Chinedu, U. and Subin-Raj C. K. R. 2016. Old products, new applications? Considering the multiple bioactivities of plastein in peptide-based functional food design. Current Opinion in Food Science 8: 8-13.

Cian, R. E., Luggren, P. and Drago, S. R. 2011. Effect of extrusion process on antioxidant and ACE inhibition properties from bovine haemoglobin concentrate hydrolysates incorporated into expanded maize products. International Journal of Food Sciences and Nutrition 62(7): 774-780.

Del Ré, P. V. and Jorge, N. 2011. Antioxidant potential of oregano (*Oreganum vulgare* L.), basil (*Ocimum basilicum* L.) and thyme (*Thymus vulgaris* L.): Application of oleoresins in vegetable oil. Food Science and Technology 31(4): 955-959.

Dong, S., Zeng, M., Wang, D., Liu, Z. Zhao, Y. and Yang, H. 2008. Antioxidant and biochemical properties of protein hydrolysates prepared from silver carp (*Hypophthalmichthys molitrix*). Food Chemistry 107(4): 1485-1493.

Dong, Y., Yan, E., Zhang, Y. and Dai, Z. 2024. A novel angiotensin-converting enzyme (ACE) inhibitory peptide from tilapia skin: Preparation, identification and its potential antihypertensive mechanism. Food Chemistry 430: 137074.

Food Agriculture Organizations (FAO) and World Health Organization (WHO). 2007. Protein and amino acid requirements in human nutrition. Geneva: FAO and WHO.

Gao, R., Yu, Q., Shen, Y., Chu, Q., Chen, G., Fen, S., ... and Sun, Q. 2021. Production, bioactive properties, and potential applications of fish protein hydrolysates: Developments and challenges. Trends in Food Science and Technology 110: 687-699.

Girgih, A., Nwachukwu, I., Iwar, M., Fagbemi, T. and Aluko, R. 2016. Effect of peptide size on the antioxidant properties of giant African land snail (*Archachatina marginata*) meat protein hydrolysates. Current Topics in Nutraceutical Research 14(2): 109-118.

Halim, N., Yusof, H. and Sarbon, N. 2016. Functional and bioactive properties of fish protein hydrolysates and peptides: A comprehensive review. Trends in Food Science and Technology 51: 24-33.

He, Z., Franco, C. and Zhang, W. 2013. Applications and production of protein hydrolysates from fish processing co-products (FPCP). Food Research International 50(1): 289-297.

Ilyasov, I. R., Beloborodov, V. L., Selivanova, I. A. and Terekhov, R. P. 2020. ABTS/PP decolorization assay of antioxidant capacity reaction pathways. International Journal of Molecular Sciences 21(3): 1131.

Ketnawa, S., Martínez, O., Benjakul, S. and Rawdkuen, S. 2016. Gelatin hydrolysates from farmed giant catfish skin using alkaline proteases and its antioxidative function of simulated gastro-intestinal digestion. Food Chemistry 192: 34-42.

Khantaphanta, S., Benjakula, S. and Kishimurab, H. 2011. Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases. Process Biochemistry 46(1): 318-327.

Lan, W., Zhao, J., Liu, L. and Xie, J. 2022. Relevance of cathepsins activity and texture in slightly acidic electrolyzed water-slurry iced mackerel (*Pneumatophorus japonicus*). Food Bioscience 49: 101924.

Lassoued, I., Mora, L., Nasri, R., Aydi, M., Toldrá, F., Aristoy, M., … and Nasri, M. 2015. Characterization, antioxidative and ACE inhibitory properties of hydrolysates obtained from thornback ray (*Raja clavata*) muscle. Journal of Proteomics 128: 458-468.

Li, X., Luo, Y., Shen, H. and You, J. 2012. Antioxidant activities and functional properties of grass carp (*Ctenopharyngodon idellus*) protein hydrolysates. Journal of the Science of Food and Agriculture 92(2): 292-298.

Lowry, O, Rosebrough, N., Farr, L. and Randall, R. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 267-275.

Megías, C., Pedroche, J., Yust, M., Giron, J., Alaiz, M., Millan, F. and Vioque, J. 2008. Production of cooper-chelating peptides after hydrolysis of

sunflower protein with pepsin and pancreatin. Food Science and Technology 41(10): 1973-1977.

Morales, R., Tammb, F., Guadix, A., Guadix, E. and Drusch, S. 2016. Functional and antioxidant properties of hydrolysates of sardine (*S. pilchardus*) and horse mackerel (*T. mediterraneus*) for the microencapsulation of fish oil by spray-drying. Food Chemistry 194: 1208-1216.

Najafian, L. and Babji, A. 2012. A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. Peptides 33(1): 178-185.

Nalinanon, S., Benjakul, S., Kishimura, H. and Shahidi, F. 2011. Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. Food Chemistry 124(4): 1354-1362.

Nasri, R., Younes, I., Jridi, M., Trigui, M., Bougatef, A., Nedjar, N., … and Karra, M. 2013. ACE inhibitory and antioxidative activities of goby (*Zosterissessor ophiocephalus*) fish protein hydrolysates: Effect on meat lipid oxidation. Food Research International 54(1): 552-561.

Nazeer, R. and Kulandai, A. 2012. Evaluation of antioxidant activity of muscle and skin protein hydrolysates from giant kingfish, *Caranx ignobilis* (Forsskål, 1775). International Journal of Food Science and Technology 47(2): 274-281.

Nielsen, P., Petersen, D. and Dambmann, C. 2001. Improved method for determining food protein degree of hydrolysis. Journal of Food Science 66(5): 642-646.

Oyaizu, M. 1986. Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. Japanese Journal of Nutrition 44(6): 307-315.

Pei-Teng, C., Matanjun, P., Budiman, C., Shapawi, R. and Lee, J. S. 2021. Production of ACE-inhibitory and antioxidant hydrolysates from the fillet of hybrid grouper. Journal of Sustainability Science and Management 16(7): 5-19.

Pukalskas, A., Van Beek, T., Venskutonis, R., Linssen, J., Van Veldhuizen, A. and Groot, A. 2002. Identification of radical scavengers in sweet grass (*Hierochloe odorata*). Journal Agricultural Food Chemistry 50: 2914-2919.

Ramírez, H., Ramírez, J. and Mazorra, M. 2013. Biological properties of collagen-derived peptides from marine resources. Biotecnia 15(3): 34-45.

Sabeena, K., Lystbæk, L., Otte, J., Hauch, H., Jessen, F. and Jacobsen, C. 2016. Antioxidant activity of cod (*Gadus morhua*) protein hydrolysates: Fractionation and characterization of peptide fractions. Food Chemistry 204: 409-419.

Schägger, H. and Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel eletrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry 166: 368-379.

Toopcham, T., Roytrakul, S. and Yongsawatdigul, J. 2015. Characterization and identification of angiotensin I-converting enzyme (ACE) inhibitory peptides derived from tilapia using *Virgibacillus halodenitrificans* SK1-3-7 proteinases. Journal of Functional Foods 14: 435-444.

Yandle, T., Tookes, J., Bennett-Martin, P., Larkin, S. and Page, M. 2022. An assessment of the viability of a commercial lionfish fishery in the US Virgin Islands. Marine Police 143: 105103.

Zheng, L., Zhao, M., Xiao, C., Zhao, Q. and Su, G. 2016. Practical problems when using ABTS assay to assess the radical-scavenging activity of peptides: Importance of controlling reaction pH and time. Food Chemistry 192: 288-294.