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Antioxidant, chelating, and angiotensin-converting enzyme inhibitory activities of peptide fractions from red lionfish (*Pterois volitans* L.) muscle protein hydrolysates

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Abstract

Peptide fractions from marine animal hydrolysates can have biological activity. Red lionfish (*Pterois volitans* L.) is an invasive fish species in the tropical Atlantic, and harvest is a proposed control mechanism. With the aim of identifying possible bioactivity in peptides from red lionfish, an evaluation was done for the antioxidant, Cu^{2+} and Fe^{2+}chelating, and angiotensin-converting enzyme inhibitory (ACE-I) activities of ultra-filtered peptide fractions derived from lionfish muscle enzymatically hydrolysed with the commercial enzyme Alcalase®. Hydrolysates were generated at 0, 30, 60, and 90 min, and the degree of hydrolysis (DH) were determined. The 30-min hydrolysate yielded the highest DH (30.78 ± 1.57%). This hydrolysate was ultra-filtered using four cut-offs (10, 5, 3, and 1 kDa), and the resulting polypeptides were analysed to generate their amino acids profile and estimated molecular weight (EMW). The F 5-3, F 3-1, and F < 1 kDa peptide fractions yielded the highest copper-chelating activity with values of approximately 88%. Fractions F > 10 and F 10-5 kDa yielded the highest iron-chelating activity with values of approximately 18.8%. The β-carotene bleaching test showed that the F 10-5, F 5-3, F 3-1, and F < 1 kDa fractions to have high antioxidant capacity, inhibiting more than 80% of β-carotene discoloration versus the control. The F 5-3 kDa fraction exhibited the highest ACE inhibition (34.57%), possibly due to the presence of amino acids such as Gly, Leu, Phe, Tyr, and Pro. Polypeptides with an EMW of 6.51 to 3.49 kDa were identified in F > 10, and 2.17 kDa in F 5-3. Peptide fractions from hydrolysed red lionfish muscle exhibit *in vitro* activities, and could serve as potential source of functional ingredients.

Introduction

Red lionfish (*Pterois volitans* L.) is a tropical marine fish native to the Indo-Pacific which was introduced to the south-eastern coast of the United States in the early 1980s. Thirty years later, it has invaded much of the tropical Atlantic Ocean, Caribbean Sea, and Gulf of Mexico. It is highly voracious, grows rapidly, and lacks natural enemies in these areas, thus making it as an ecosystem risk; it is considered one of the 15 major worldwide threats to biodiversity. As part of a control strategy, government agencies and conservation organizations suggest consuming lionfish, especially in high density regions. In Mexico, the National Commission of Protected Natural Areas (Consejo Nacional de Areas Naturales Protegidas - CONANP) has promoted its consumption through tastings at fishing tournaments in the states of Quintana Roo and Yucatan. The International Coral Reef Initiative (ICRI) has called for commercial fishing of the species, and proposed consumption, marketing, and import of lionfish meat (ICRI, 2010).

Fish are a rich source of protein-derived bioactive compounds. Antimicrobial peptides and antioxidants have been isolated from tuna protein. Peptides with antihypertensive and calcium-binding activity have been isolated from Alaska pollock, tuna muscle, and dab proteins; while anticoagulant activity has been documented in peptides extracted from starfish and mussel, among others. One of the benefits of peptides from marine sources is their ability to bind free radicals and their reactive oxygen species content, both of which prevent oxidative damage by interrupting the lipid peroxidation chain reaction (Kwon and Wijesekara, 2010).

Compared to metal salts, which have some limitations, chelating peptides are an excellent alternative for increasing mineral absorption and bioavailability.

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Chelating peptides have been isolated by enzymatic hydrolysis of vegetable and animal proteins (Jiang *et al.,* 2014). Iron has vital biochemical activities and is an essential element that participates in many biochemical processes in the human body. Iron deficiency in humans has been a nutritional problem for millennia. This metal can be supplied to the organism via salts, metal-chelating agents, and iron-chelating peptides (Guo *et al.,* 2013). Copper is a fundamental trace element, which plays vital role as a cofactor of many enzymes, but it also has oxidative activity. Copper-chelating peptides can prevent this oxidative activity by chelating this metal ion. Angiotensin-converting enzyme (ACE) is involved in cardiopathies which are often treated with pharmaceutical ACE inhibitors. These can have serious side effects. Peptides which can inhibit ACE are generally small; thus, easily, and quickly absorbed in the gut (García-Moreno *et al.,* 2015). They effectively reduce blood pressure and have no known adverse effects (Fitzgerald and Meisel, 2000).

Peptides from marine sources are promising potential functional food ingredients or nutraceuticals. The present work thus aimed to evaluate the antioxidant, copper- and iron-chelating activities, and ACE inhibitory capacity of ultra-filtered peptide fractions isolated from hydrolysed red lionfish muscle. Their presence would identify this species as a source of functional ingredients, thus broadening the possible uses of its meat and serving as an impetus for its harvest.

Materials and methods

Animal collection

Red lionfish specimens 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, and the skinless fillets were freeze-dried until use. The dried samples were pulverised, mixed until homogeneous, and stored at -20°C in a polyethylene bottle for later analysis. Fillet moisture and protein content were analysed using AOAC methods: moisture (method 934.01) and protein (method 954.01) (calculated as nitrogen × 6.25).

Protein hydrolysate preparation

Hydrolysates were isolated from a subsample of freeze-dried fillet in two replicates following the first step of the hydrolysis method described by Megías *et al.* (2007); using a hydrolysis reactor vessel equipped with a stirrer, thermometer, and pH electrode. Lyophilised fish fillet (5% protein w/v) was digested with Alcalase® (0.3 AU/g protein) for 90 min at 50°C and at pH 8. Aliquots were taken at five different times (0, 15, 30, 60, and 90 min) and hydrolysis stopped by heat inactivation of Alcalase® at 80°C for 20 min. The resulting hydrolysates were clarified by centrifugation at 11,227 *g* for 30 min in a Beckman Coulter Ultracentrifuge (LE-80K, Palo Alto, California), and then frozen at -20°C until use. Hydrolysate protein content was quantified following the method of Lowry *et al*. (1951), and the results 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 in the presence of dithiothreitol, which forms a coloured 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 as a standard, using Eq. 1:

$$DH = \left(\frac{h}{h_{tot}} \right) x100 \qquad \text{(Eq. 1)}$$

where, h_{tot} = total number of peptide bonds per protein equivalent, and h = number of hydrolysed bonds. All experiments were performed in triplicate.

Ultrafiltration of protein hydrolysate

Ultrafiltration was done following Cho *et al.* (2004) using ultrafiltration membranes (Millipore PLGC06210, Bedford, MA, US). Four membranes with different molecular weight cut-offs (10, 5, 3, and 1 kDa) were used in an ultrafiltration device (Model 2000, Millipore, Inc., Marlborough, MA, USA). Nitrogen (40 psi) was used as an inert gas. Ultrafiltration of the protein hydrolysate produced at 30 min resulted in five fractions: F > 10, F 10-5, F 5-3, F 3-1, and F < 1 kDa.

Quantifying peptide fraction antioxidant and chelating activity

β-carotene bleaching method

Antioxidant activity was measured with β-carotene bleaching method, with modifications as described by Del Ré and Jorge (2011). A mixture of 4 mg β-carotene (Sigma 22040) in 1 mL chloroform and 1 mL Tween 20 (P1379) was vigorously stirred by vortex. After removal of chloroform under a nitrogen stream, a clear solution was obtained by mixing in 50 mL 100 mM oxygen-sparged phosphate buffer at pH 7.4. Each peptide fraction (equivalent to 500 µg protein) was dissolved in 60 µL phosphate buffer and 200 µL 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 50 µM $FeCl_2$ (Sigma 44939). The negative control was β-carotene / Tween 20 solution + 10 µL 50 µM $FeCl_2$ mixed with 60 µL phosphate buffer containing no peptide fraction. The positive control was β-carotene / Tween 20 solution + 10 µL 50 µM $FeCl_2$ mixed with 10 µg butylated hydroxyanisole (BHA) (Sigma B1253). Peroxidative degradation of β-carotene was monitored by recording absorbance at 470 nm up to 120 min with a microplate reader. The percentage of inhibition of β-carotene discoloration was calculated using Eq. 2:

$$\text{β-carotene inhibition (\%)} = \left(\frac{\text{Abs C - Abs M}}{\text{Abs C}}\right) \times 100 \quad \text{(Eq. 2)}$$

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

Copper-chelating activity

Copper (Cu^{2+})-chelating activity was measured using pyrocatechol violet reagent according to Saiga *et al.* (2003). Peptide fractions (equivalent to 500 µg 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 following 1 min incubation at room temperature. Runs were done in triplicate. A calibration curve was constructed using different copper concentrations (2, 4, 6, 8, and 10 µ g/µL). Copper concentration was determined using a linear regression equation, and Cu^{2+}-chelating activity was calculated using Eq. 3:

$$\text{Chelating activity (\%)} = \left(\frac{[Cu]_i - [Cu]_f}{[Cu]_i}\right) \times 100 \quad \text{(Eq. 3)}$$

where, $[Cu]_i$ = initial Cu^{2+} concentration, and $[Cu]_f$ = final Cu^{2+} concentration.

Iron-chelating activity

Iron (Fe^{2+})-chelating activity was measured based on formation of the Fe^{2+}-ferrozine complex, according to Carter (1971). Peptide fractions (equivalent to 500 µg protein) were added to Eppendorf tubes containing 1 mL 100 mM sodium acetate buffer (pH 4.9) and 100 µL $FeCl_2 \cdot 4H_2O$ solution (0.01 mg Fe / mL water). Again, 50 µg EDTA was used as a positive control. Absorbance at 562 nm was measured after adding the ferrozine solution (50 µL, 40 mM in water) (Sigma P5338) and incubated for 30 min at room temperature. Runs were done in triplicate. A calibration curve was built using different iron concentrations (0.2, 0.4, 1, 1.5, and 2 µg/µL). Iron concentration was determined using a linear regression equation, and iron-chelating activity was calculated using Eq. 4:

$$\text{Chelating activity (\%)} = \left(\frac{[Fe]_i - [Fe]_f}{[Fe]_i}\right) \times 100 \quad \text{(Eq. 4)}$$

where, $[Fe]_i$ = initial Fe^{2+} concentration, and $[Fe]_f$ = final Fe^{2+} concentration.

Angiotensin-converting enzyme inhibition (ACE-I)

Inhibitory activity was quantified by peptide fraction, following a modified version of Cian *et al.* (2011). These modifications consisted of purifying the enzyme from the lung of a recently killed rabbit as follows: 1 *g* of lung was extracted with buffer containing 0.25 M sucrose and 0.1 M sodium anhydrous phosphate (pH 8.3; 1:5 p/v), to which 5 µL PMSF (phenylmethylsulphonyl fluoride) were added and the mixture centrifuged at 15,500 *g* for 10 min at 4°C. Later, this mixture was added with 20 µL sample, 20 µL ACE, 20 µL hippuryl-L-histidyl-L-leucine, 15 µL 5 M NaCl (0.3%), and 175 µL 0.1 M NaH_2PO_4 (pH 8.3). This was incubated at 37°C for 45 min, and the reactions inactivated using 665 µL 2.4.6-trichloride-triazine in 3% dioxane and 1.1 mL NaH_2PO_4 added. This mixture was centrifuged at 15,500 *g* for 10 min at 4°C and absorbance measured at 382 nm. The percentage of ACE inhibition was expressed as the ratio between the reactions with the sample and that of the control, and calculated using Eq 5:

$$\text{ACE inhibition}(\%) = 100 - \left[\left(\frac{AS - ABS}{AE - ABE}\right) \times 100\right] \quad \text{(Eq. 5)}$$

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

Amino acid analysis of peptide fractions

Amino acid analysis was carried out by acid hydrolysis and HPLC, following derivatisation with diethyl ethoxymethylenemalonate (Aldrich D94208), according to Alaiz *et al.* (1992), using D,L-α-aminobutyric acid (Aldrich D94208) as internal standard.

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

This analysis was done following the method of Schägger and Jagow (1987), using 18% acrylamide gel

and 4% stacking gel. Peptide fractions (5 - 6 µg/µL protein) were separately dissolved in a buffer (50 mM Tris-HCl [pH 6.8]; 10% glycerol [v/v], 1% SDS [w/v], and 0.01% bromophenol blue [w/v]), and heated to 100°C for 5 min. Runs were done at 40 mA for 1.5 h in a Miniprotein electrophoresis chamber (BIORAD, Hercules, California). The resulting gels were stained with 0.05% Coomassie Brilliant Blue G-250, and cleaned with an acetic acid:methanol:distilled water (1:4:5) solution. Wells were loaded with 10 µg protein or one of the hydrolysates. The low-range molecular weight standard (BIORAD, USA, Cat. #1610305) contained phosphorylase B (105.2 kDa), bovine serum albumin (84.2 kDa), ovalbumin (50.4 kDa), carbonic anhydrase (36.8 kDa), soybean trypsin inhibitor (29.0 kDa), and lysozyme (20.5 kDa). A polypeptide standard (BIORAD, USA, Cat. #1610326) was used which contained triosephosphate isomerase (26.625 kDa), myoglobin (16.950 kDa), α-lactalbumin (14.437 kDa), aprotinin (6.512 kDa), insulin b chain, oxidised (3.496 kDa), and bacitracin (1.423 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 hydrolysate DH values and peptide fraction *in vitro* activities.

Results and discussion

Moisture and protein

The lionfish muscle had 81.64 ± 0.12% (db) protein content and 11.46 ± 0.1% moisture content. These values are similar to the 88.6 ± 0.3% protein and 3.6 ± 1.9% moisture contents reported for northern Pacific hake (*Merluccius productus*) (Pacheco *et al.,* 2008).

Degree of hydrolysis (DH)

The lyophilised sample had 8.35 ± 0.96% DH. This level may be due to the presence of endogenous enzymes such as trypsin, pepsin, chymotrypsin, and visceral and digestive tract enzymes, which can contribute to protein breakdown by autolysis (Samaranayaka and Chan, 2011).

The highest DH values were in the 30-min hydrolysate (30.78 ± 1.57%) and 90-min hydrolysate (30.08 ± 0.25%); these did not differ significantly ($p > 0.05$), so the shortest hydrolysation time (30 min) was chosen for UF fractionation. The decrease in DH observed at 60 min (from 30.78 ± 1.57% at 30 min to 27.14 ± 1.20% at 60 min) may have occurred due to competition between non-hydrolysed protein and peptides that constantly formed during the hydrolysis process (Brownsell *et al.,* 2001). The high DH values are similar to the 34.73% reported for hydrolysates from the viscera and carcass of tilapia (*Oreochromis niloticus*) following 2 h hydrolysis with 0.5% Alcalase® (v/v) at 45°C (Silva *et al.,* 2014). Alcalase® is a broad specificity alkaline serine endoprotease, so it can easily produce peptides of different sizes. It is one of the most suitable microbial enzymes for producing fish protein hydrolysates for subsequent peptide fractionation (Saidi *et al.,* 2014a).

Antioxidant and chelating activity of lionfish peptide fractions

Inhibition of oxidative discoloration of β-carotene.

As the reaction time increased (30, 60, 90, and 120 min), absorbance decreased in the peptide fractions (F > 10, F 10-5, F 5-3, F 3-1, F < 1) and the BHA antioxidant, in the presence of Fe^{+2} as an oxidising metal (Figure 1). At 30 min, no significant difference ($p > 0.05$) was observed between the absorbance values of the F 5-3 and F < 1 fractions and the BHA. Again, no significant difference ($p > 0.05$) was observed between the absorbance values of the F 10-5, F 5-3, F 3-1, and F < 1 fractions, as well as BHA at 90 and 120 min reaction with the metallic ion and β-carotene. At 90 min, β-carotene discoloration was inhibited by 80.44% (F > 10), 73.88% (F 10-5), 79.78% (F 5-3), 78.22% (F 3-1), and 76.11% (F < 1). At 120 min, discoloration was inhibited by 81.79% (F > 10), 76.07% (F 10-5), 81.07% (F 5-3), 80.00% (F 3-1), and 79.38% (F < 1). The negative control (β-carotene with no sample) exhibited an exponential increase in absorbance values.

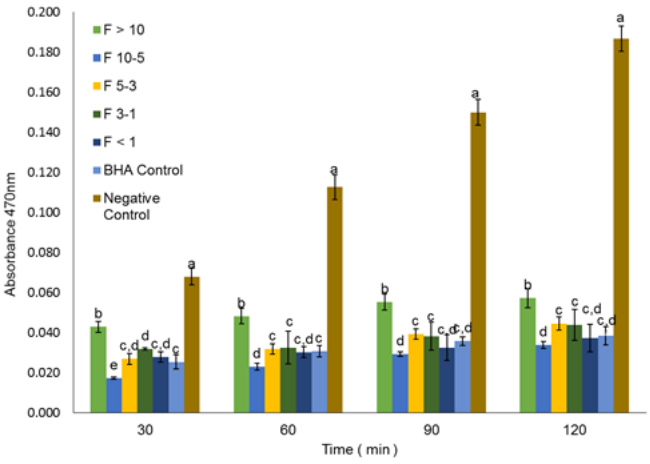

Figure 1. Oxidation of β-carotene in the presence of peptide fractions (equivalent to 500 µg protein) derived from red lionfish muscle subjected to enzymatic hydrolysis with Alcalase® for 30 min. Error bars indicate standard deviation. Different letters above bars in the same time period indicate statistically significant difference ($p < 0.05$). Positive control = 10 µg BHA.

Cu^{2+} chelation

Peptide fraction Cu^{2+} chelation values ranged from 86.05 ± 0.31 to 88.67 ± 0.43% (Figure 2a). Chelating capacity did not differ between the F 10-5 (87.64 ± 0.66%), F 5-3 (88.30 ± 0.53%) and F < 1 (88.67 ± 0.43%) fractions. These high Cu^{2+} chelation values may be attributed to the rupture of peptide bonds and increased concentrations of carboxylic (COO-) leading to greater Cu^{2+} binding, thus removing this prooxidative free metal ion (Kong and Xiong, 2006). The His amino acid is known to chelate copper (Kong and Xiong, 2006) and their amino acid profiles showed all the peptide fractions to contain His: 1.11 ± 0.08 (F > 10), 1.39 ± 0.02 (F 10-5), 1.23 ± 0.15 (F 5-3), 1.82 ± 0.02 (F 3-1), and 1.64 ± 0.12 g/100 g protein (F < 1).

Hydrophobic peptides are generally antioxidants and can also chelate metals (Ghribi *et al.,* 2015). This may partially explain the high copper chelation capacity of the fractions since their amino acid profiles (Table 1) showed them to have hydrophobic amino acids concentrations of 45.46 (F > 10), 36.73 (F 10-5), 47.43 (F 5-3), 37.45 (F 3-1), and 43.75 g/100 g protein (F < 1). High copper chelation levels are also associated with the presence of amino acids such as Glu and Asp, and α-amino acids such as Lys and Arg (Ghanbari *et al.,* 2015).

Hydrophobic amino acids (HHA) are known to possess antioxidant properties (Wijesekara *et al.,* 2011). The HHA content of F < 1 was higher than reported in tuna by-products, with higher levels of Ala, Val, Ile, Leu, Tyr, Phe, Trp, Pro, and Met (Saidi *et al.,* 2014b). However, aromatic amino acids (AAA), positively-charged amino acids (PCAA), and negatively-charged amino acids (NCAA) levels were also higher in F < 1 than in tuna by-products. These types of amino acids are characterised by their antioxidant and chelating properties which originate from their ability to donate or receive electrons to stabilise free radicals (Aluko, 2012) (Table 1).

Fe^{2+} chelation.

Peptide fraction iron chelation values ranged from 13.92 ± 0.14 to 18.84 ± 0.01%, substantially lower than the 98.4% standard (6 µg EDTA). The highest value was observed in the F > 10 (18.84 ± 0.01%) and F 10-5 (18.76 ± 0.14%) fractions; of note is that the chelation level decreased slightly with molecular weight (Figure 2b). This broadly coincides with the reductions in iron-chelating capacity observed in fractionated salmon muscle hydrolysates (Girgih *et al.,* 2013). In a study of peptides fractions from tuna by-products hydrolysed with Alcalase® at a 1% enzyme:substrate ratio (E/S), and 55°C for 60 min

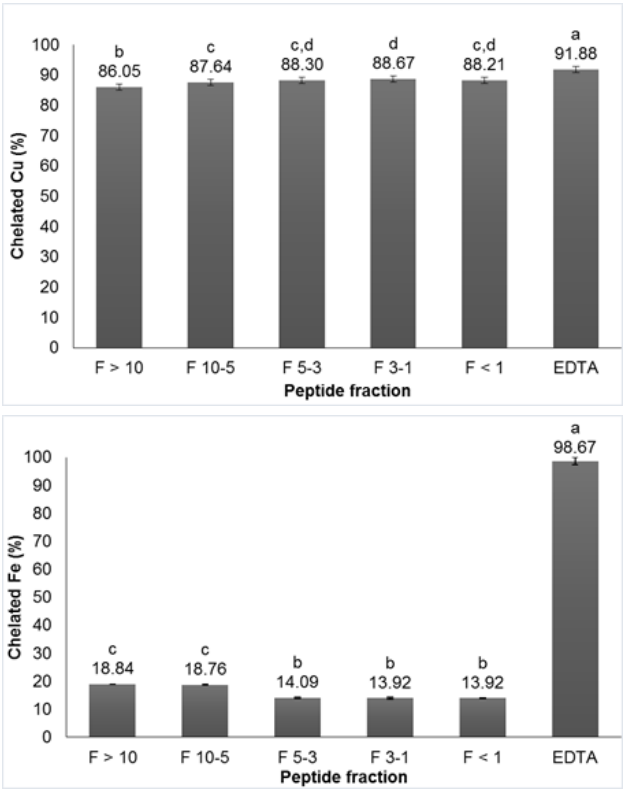

Figure 2. (a) Copper chelation in peptide fractions (equivalent to 500 µg protein); and (b) Iron chelation of peptide fractions (equivalent to 500 µg protein), derived from red lionfish muscle subjected to enzymatic hydrolysis with Alcalase® for 30 min. Error bars indicate standard deviation. Different letters above the bars indicate statistically significant difference ($p < 0.05$). Positive control : 50 g EDTA.

at pH 8.5 (Saidi *et al.,* 2014a), iron-chelation percentages were higher than those obtained in the present work: 35% in the < 4 kDa fraction, 40% in the 1-4 kDa fraction, and 20% in the > 1 kDa fraction.

Metal-chelating behaviour can be associated with amino acids structure, molecular weight, and composition; Gly and His have the highest reported iron-chelating activity (Lin *et al.,* 2014). The amino acid profiles of the *P. volitans* peptide fractions showed them to have relatively high Gly content: 4.47 ± 0.17 (F > 10), 5.60 ± 0.03 (F 10-5), (4.24 ± 0.19) F 5-3, (5.19 ± 0.16) F 3-1, and 4.48 ± 0.16 g/100 g protein (F < 1). These are higher than the Gly content in fractions from tuna by-product hydrolysates: 3.30 ± 0.12 (< 4), 4.8 ± 0.1 (1-4), and 3.0 ± 0.1 g/100 g (> 1 kDa) (Saidi *et al.,* 2014b). In contrast, His content was somewhat lower: 1.11 ± 0.08 (F > 10), 1.39 ± 0.02 (F 10-5), 1.23 ± 0.15 (F 5-3), 1.82 ± 0.02 (F 3-1), and 1.64 ± 0.12 g/100 g (F < 1). These levels are comparable to that of the F < 1 fraction of the tuna by-products hydrolysate (Table 1).

Angiotensin-converting enzyme inhibition (ACE-I)

Inhibition of ACE by the red lionfish protein

Table 1. Amino acid content (g/100 g protein) of peptide fractions of red lionfish muscle subjected to enzymatic hydrolysis with Alcalase® for 30 min.

AA	Peptide fraction					WHO	TPH[1]
	F > 10	F 10-5	F 5-3	F 3-1	F < 1		F < 1
Essential							
Ile	2.96 ± 0.01	3.28 ± 0.06	2.77 ± 0.11	3.27 ± 0.11	2.64 ± 0.04	3.0	2.4
Leu	6.88 ± 0.12	7.96 ± 0.15	7.04 ± 0.22	9.71 ± 0.34	8.68 ± 0.11	5.9	4.9
Lys	8.51 ± 0.24	9.76 ± 0.06	7.74 ± 0.18	8.24 ± 0.17	6.43 ± 0.09	4.5	4.5
Met	1.93 ± 0.13	nd	nd	3.08 ± 0.15	3.12 ± 0.34	1.6	1.7
Phe	3.01 ± 0.23	3.44 ± 0.03	3.11 ± 0.20	4.32 ± 0.19	4.19 ± 0.09	3.0[c]	2.2
Thr	3.56 ± 0.05	4.27 ± 0.02	3.57 ± 0.02	4.89 ± 0.12	4.58 ± 0.15	2.3	2.3
Val	9.15 ± 0.09	15.79 ± 0.10	14.86 ± 0.20	9.99 ± 1.36	13.27 ± 0.02	3.9	4.9
His	1.11 ± 0.08	1.39 ± 0.02	1.23 ± 0.15	1.82 ± 0.02	1.64 ± 0.12	1.5	3.4
Trp	nd	nd	nd	0.54 ± 0.05	0.51 ± 0.06	0.6	0.3
Non-essential							
Ala	2.74 ± 0.34	3.31 ± 0.12	2.43 ± 0.04	2.78 ± 0.05	2.21 ± 0.19		1.5
Arg	10.35 ± 0.12	12.97 ± 0.10	11.13 ± 0.12	15.57 ± 0.22	15.23 ± 0.40		2.4
ASX	10.68 ± 0.46	10.11 ± 0.23	8.44 ± 0.80	7.93 ± 0.33	8.80 ± 0.42		2.7
Cys	nd	nd	0.13 ± 0.18	nd	nd	2.2[d]	1.9
GLX	13.03 ± 0.34	15.71 ± 0.36	13.24 ± 0.54	14.69 ± 0.05	11.00 ± 0.41		4.9
Gly	4.47 ± 0.17	5.60 ± 0.03	4.24 ± 0.19	5.19 ± 0.16	4.48 ± 0.16		3.0
Ser	2.83 ± 0.23	3.47 ± 0.04	2.97 ± 0.07	4.22 ± 0.17	4.09 ± 0.12		1.9
Tyr	2.59 ± 0.07	2.96 ± 0.01	2.57 ± 0.05	3.76 ± 0.11	3.42 ± 0.05		1.7
Pro	16.19 ± 0.33	nd	14.52 ± 0.37	nd	5.71 ± 0.57		3.0
Group							
EAA	37.12	45.88	40.33	45.87	45.06		26.5
HAA	45.46	36.73	47.43	37.45	43.75		24.6
AAA	5.60	6.39	5.68	8.62	8.12		4.2
PCAA	19.97	24.12	20.10	25.63	23.30		10.2
NCAA	30.10	33.55	28.22	31.74	28.47		7.6

[1]Saidi *et al.* (2014a); [c] = Phe + Tyr; [d] = Met + Cys. ASX: aspartic acid and asparagine; GLX: glutamic acid and glutamine; EAA: essential amino acids; HAA: hydrophobic amino acids (Ala, Val, Ile, Leu, Tyr, Phe, Trp, Pro, Met, and Cys); AAA: aromatic amino acids (Phe, Trp, and Tyr); PCAA: positively-charged amino acids (Arg, His, and Lys); NCAA: negatively-charged amino acids (ASX, GLX, Thr, and Ser); nd: not detected.

hydrolysate peptide fractions ranged from 15.03 ± 1.71 to 34.57 ± 0.97% (Figure 3). The highest inhibition activity (34.57 ± 0.97%) was observed in the F 5-3 kDa fraction, which may be due to the presence of amino acids such as Gly, Leu, Phe, Tyr, and Pro (Lee *et al.,* 2014). In the amino acid profiles, all the peptide fractions had similar concentrations of Leu, Phe, Gly, and Tyr (Table 1). However, only the F 5-3, F < 1 and F > 10 fractions contained Pro, which may explain their higher ACE inhibition as compared to the other fractions. All the peptide fractions also contained high percentages of Arg, a precursor of nitric oxide, which is a potent vasodilator (Palmer *et al.,* 1988). The amino acids sequences of different peptides exhibiting ACE inhibition have been isolated and identified, and they all contain aromatic and branched amino acids in the C-terminal group (His-Leu, Phe-Arg, and Ala-Pro). Based on this arrangement, antihypertensive peptides are reported to owe their activity to the presence of Pro

(Balti *et al.,* 2015). Apparently, ACE inhibition in peptides is not associated with low molecular mass but rather with their amino acid composition (Abdelhedi *et al.,* 2016). For instance, in antihypertensive peptide sequences, this property is associated with amino acids such as Ala, Arg, Phe, Pro, Lys, His, and Leu (FitzGerald and Miesel, 2000). This may explain the inhibition values found in red lionfish peptide fractions F 5-3 and F < 1.

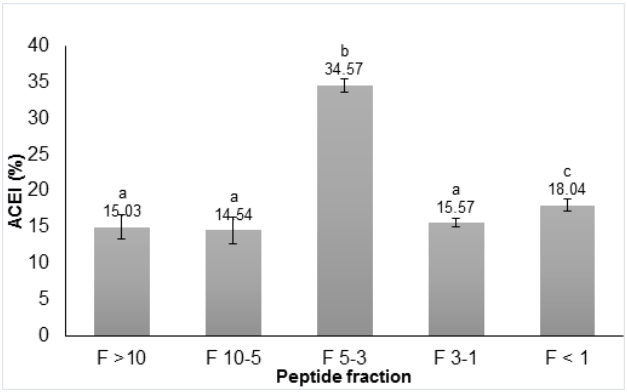

Figure 3. Angiotensin-converting enzyme inhibition (ACEI) of peptide fractions (equivalent to 500 µg protein) derived from red lionfish muscle subjected to enzymatic hydrolysis with Alcalase® for 30 min. Different letters above the bars indicate statistically significant difference ($p < 0.05$).

Inhibition was noticeably lower in the F > 10, F 10-5 and F 3-1 fractions than in F 5-3 and F < 1. This is generally analogous to the pattern observed in a study of hydrolysates from the herbivorous carp (*Ctenopharyngodon idella*) produced with Alcalase® at 50°C and at pH 9.0 in which the > 3 kDa peptide fraction exhibited higher ACE inhibition activity than the > 10 kDa fraction (Chen *et al.,* 2012).

Protein quality

The peptide fraction amino acid profiles also highlight the high-quality protein of red lionfish. Many of the EAA in the fractions occurred at levels above requirements for adults as established by the WHO (2007). For instance, they contained Ile (F > 10, F 10-5, and F 3-1), Leu (F > 10, F 10-5, F 5-3, F 3-1, and F < 1), Lys (F > 10, F 10-5, F 5-3, F 3-1, and F < 1), Phe and Tyr (F 10-5, F 3-1, and F < 1), Thr (F > 10, F 10-5, F 5-3, F 3-1, and F < 1), and Val (F > 10, F 10-5, F 5-3, F 3-1, and F < 1). Indeed, the red lionfish F < 1 fraction had a higher overall EAA content than the F < 1 fraction from black tuna muscle by-products hydrolysed with Alcalase® (TPH, Table 1) (Saidi *et al.,* 2014a). This high EAA content provides high nutritional value to red lionfish.

Electrophoretic profile

Fraction F > 10 contained a protein with an estimated molecular weight of 36.12 kDa, confirming that membrane fractionation was effective. Polypeptides were not detected in F 3-1 and F < 1, probably because their small size prevented their detection in the gel (Figure 4). However, free amino acids have been reported in fractions of < 3 kDa with molecular weight components smaller than the cut-off point for 3-5 kDa and > 5 kDa membranes (Farvin *et al.,* 2014). Analysis of molecular weight distribution, expressed as percentages of the area under the curve in fractions from cod, found that 83.8% in the 3-5 kDa fraction corresponded to molecules < 3 kDa. In a subsequent study (Farvin *et al.,* 2016), LC-MS/MS was used to identify amino acid sequences, mostly di-, tri, and tetra-peptides in the 3-5 kDa fraction. The lack of polypeptides in the present F 3-1 and F < 1 fractions may therefore be due to membrane fouling or the attraction of small molecules to larger oligopeptides with which they associate.

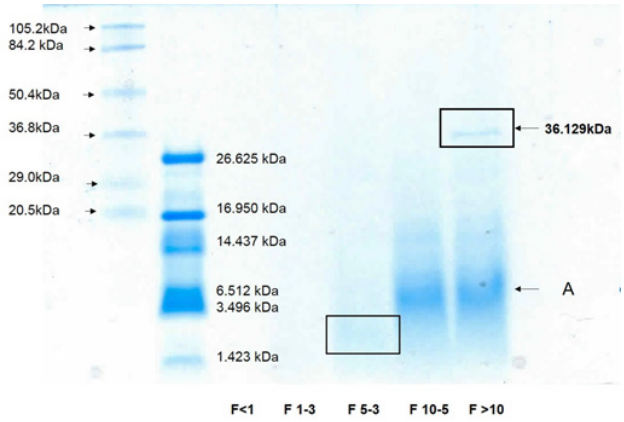

Figure 4. Electrophoretic profile of peptide fractions derived from red lionfish muscle subjected to enzymatic hydrolysis with Alcalase® for 30 min.

In the F > 10 and F 10-5 fractions, polypeptides were identified with estimated molecular weights ranging from 6.512 to 3.496 kDa (based on standard) (Figure 4, marked with the letter A). In addition, a band with an estimated molecular weight of 2.172 kDa was observed in the F 5-3 fraction. This coincides with the presence of polypeptides smaller than 3.5 kDa in hydrolysates of tilapia (*Oreochromis niloticus*) by-products produced with Alcalase® (Roslan *et al.,* 2014). Higher DH resulted in medium and small peptides.

To our knowledge, this is the first attempt at evaluating the biological activity of peptide fractions from red lionfish hydrolysates. It is a starting point for future studies characterising amino acids sequences with more sensitive techniques such as LC-MS.

Conclusion

Red lionfish muscle hydrolysates were produced using the commercial enzyme Alcalase®. The resulting peptide fractions exhibited high contents of amino acids such as Ile, Leu, Lys, Met, Thr, and Val. The proportions of these amino acids are probably linked to the observed inhibition of β-carotene discoloration and high copper-chelating activity. The F 5-3 and F < 1 kDa peptide fractions had the highest ACE inhibitory activity, probably due to the presence of hydrophobic and aromatic amino acids. These peptide fractions may have potential applications due to their high essential amino acids content, which would provide them nutritional value. Further research is needed on these peptide fractions to completely characterise their amino acids sequence, and *in vivo* studies are needed to assess their potential applications and add value to the meat of this invasive fish species.

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