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RESEARCH

Antioxidant Capacity and Angiotensin-I Converting Enzyme (ACE)-Inhibitory Activities of Peptide Fractions Obtained from Triggerfish (*Balistes capriscus)* Co-products

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Abstract

The fish industry can generate a significant amount of waste that has economic potential for use in the pharmaceutical and food industries. The aim of this study was to evaluate the antioxidant and ACE-inhibitory activities of peptide fractions from triggerfish (*Balistes capriscus*) processing coproducts. Protein fractions were extracted from fish viscera and hydrolyzed using papain (HP), bromelain (HB), and trypsin (HT) (3% p.p^{-1}, 6 h). The molecular mass distribution of soluble protein extract (SPE) and hydrolysate was determined by gel filtration chromatography. Samples were extracted and ultra-filtrated (> 100 MWCO, 30–100, 10–30 and < 10 MWCO). Antioxidant activity of fractions was evaluated, and fraction SPE4 (< 10 MWCO) showed the highest value of Trolox Equivalent Antioxidant Capacity—TEAC (10,157.7 µmol Trolox. g^{-1}) and Ferric Reducing Antioxidant Power—FRAP (1588.71 µmol $FeSO_4$. g^{-1}). SPE and hydrolysates (< 10 MWCO) were distributed into fractions by ion-exchange chromatography and subjected to antioxidant activity assays. F1 fraction showed the highest value for TEAC capacity (8839.04 µmol Trolox. g^{-1}) and FRAP (1749.94 µmol $FeSO_4$. g^{-1}). ACE-inhibitory activity was evaluated for SPE and hydrolysate and fractions F3, F5, and HP3 showed the lowest IC_{50} values (30.1, 42.7 e 37.7 µg, respectively). Amino acid sequencing of peptides indicated the presence of hydrophobic amino acids such as leucine (L), valine (V), phenylalanine (F), and alanine (A) in the C-terminal position, which contributed to antioxidant activity of peptides fractions. ACE inhibitory capacity was influenced by the presence of arginine (R) and lysine (K) positively charged in the C-terminal. Protein extracted from triggerfish viscera is a good source of bioactive peptides that can be used in the pharmaceutical and food industries.

Keywords Fish protein · Antioxidant · ACE-inhibitory peptides · Fish hydrolysate · Triggerfish

Introduction

Global fisheries and aquaculture production reached a record 223.2 million tonnes in 2022, and the world's consumption of aquatic foods has increased significantly in recent years. Proportionally, production of fish co-products tends to rise and maybe a lucrative activity in addition to reducing environmental pollution (Food and Agriculture Organization, 2024) Fish waste (including head, skin, scales, and viscera) can represent up to 60% of its volume (Klomklao & Benjakul, 2017), and it is a great source of bioactive molecules such as oils

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and proteins. Biotechnological processes applied to fish proteins and by-products can generate bioactive peptides with high nutritional, pharmacological, and technological interest. These processes consist of different steps, which include extraction, hydrolysis, fractionating and purification of peptides, sequencing, synthesis, studies of bioactive properties (Idowu et al., 2021), and subsequent industrial scaling. Bioproducts integrate the productive basis of several economic segments using safer, cleaner, and more efficient industrial processes (Fernandes et al., 2018). Enzymatic hydrolysis involves the targeted cleavage of structures using specific enzymes to catalyze the corresponding chemical reactions. Thus, it offers greater control over the hydrolysis process and the product, becoming the most efficient way to obtain biologically active protein hydrolysates, allowing greater use of the potential of proteins from fish co-products, with different industrial applications (Yuan et al., 2024). Bioactive peptides usually contain between 3 and 20 amino acid residues and remain inactive while the sequences are kept within the precursor protein sequence, but are active when released by enzymatic hydrolysis promoted by peptidases in biotechnological processes or during gastrointestinal digestion (Mora et al., 2019). These characteristics are mainly defined by the protein from which they were cleaved and the enzymes used during the process. Several proteinases can be used for hydrolysis, including alcalase®, papain, trypsin, bromelain, pepsin, pronase, and other commercial enzymes. Bioactive peptides from fish may present several properties such as antioxidant (Sierra et al., 2021), anti-hypertensive (Gouic et al., 2018; Mora et al., 2019), antimicrobial (Lima et al., 2018), anti-inflammatory (Kemp & Kwon, 2021), and hypoglycemic (Wu et al., 2022). Peptides can act through different mechanisms, such as inhibiting key enzymes or as electron capturers, and their different functionalities depend on their size, composition, and amino acid sequence, as well as the amino acid present at the amino or carboxyl terminals (Tacias-Pascacio et al., 2021). This relationship has been investigated by several authors in studies carried out with different species: *Gadus mohua* (Farvin et al., 2016), shrimp (*Heterocarpus reedi*) (Leiva-Portilla et al., 2023), fish protein hydrolysate from heads of *Navodon septentrionalis* (Chi et al., 2015), and peptides obtained by enzymatic hydrolysis of *Clupeonella cultriventris caspi* (Qara & Najafi, 2018). The large biodiversity of living organisms that inhabit marine habitats results in a wide range of structurally diverse and complex components, characterized by specific functionality and marked biological activities, but the biotechnological potential of many resources there is still underexploited (Caruso et al., 2020). Triggerfish (*Balistes capriscus),* also known as Peroa fish, stands out as a fishery resource widely exploited, being one of the most important species for fishing communities along the southeast-south coast of Brazil (Batista, 2022). A few studies have been reported about characteristics of *Balistes capriscus* co-products, such as skin extracts and intestinal enzymes. The vasorelaxant activity of the aqueous extract of *Balistes capriscus* fish skin in the mesenteric arterial bed of rats was studied by Cavalli et al. (2003), and it was observed that vasodilation is mediated by primary sensory fibers. Souissi et al. (2017) evaluated the development of gelatin-based biofilms prepared from *Balistes capriscus* skin, showing that hydrolysis with pepsin improved extraction yield of gelatin. Purified trypsin from the intestine *Balistes capriscus)* was characterized showing high homology with trypsin from other marine vertebrates. The enzyme showed high activity at low and moderate temperatures (around 40 °C) and had more than 80% of its maximum activity at 20 °C, stability in the presence of surfactants and oxidizing agents, indicating it could find application in detergents for clothes (Jellouli et al., 2011). However, there are no studies reporting about the bioactivity of peptides obtained from *Balistes capriscus* viscera. The aim of this study was to evaluate the extraction, hydrolysis, and fractionation processes of protein from triggerfish *(Balistes capriscus)* co-products and assay the antioxidant capacity and ACE-inhibitory activities of the peptide fraction obtained.

Materials and Methods

Materials

Fluorescence resonance energy transfer (FRET) substrates for ACE (Abz-FRK(Dnp)P-OH) were purchased from AminoTech (São Paulo, SP, Brazil). Captopril (Fluka). Leupeptin, AEBSF, bestatin, Pepstatin A, E-64, aprotinin, TPCK / TLCK, cocktail inhibitor, Trolox, ABTS, and potassium persulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade. Solvents used in HPLC were HPLC grade.

Biological Materials

Caught gray triggerfish were purchased from artisanal fishermen on the south coast of Espirito Santo State. It was packed in an isothermal box with ice and sent to processing room, where it was weighed to calculate the yield of the residues. Inedible parts (liver, stomach, intestine, kidney, gonad, swim bladder, heart, and gills) were extracted using knives and processed in a cutter to obtain a homogeneous paste. All the procedures were carried out keeping the fish temperature at 4 °C to inhibit autolytic action.

Yield and Centesimal Composition of Non-edible Parts

The yield was calculated as the percentage of the weight of the inedible part in relation to the total weight of the fish ($n = 10$). Centesimal composition of homogenized fish waste was carried out according to the methods of the International Association of Official Analytical Collaboration – AOAC, including analysis of moisture by drying oven, ash (AOAC 920.153), and total protein (AOAC 940.25) (Dr. Latimer, 2019). Lipid content analysis was performed according to the classic method described by Bligh and Dyer (Bligh & Dyer, 1959).

Soluble Protein Extraction

Soluble protein extract (SPE) was obtained by chemical method with alkaline and acid solubilization, adapted for fish meat (Arnesen & Gildberg, 2006). Sample was homogenized in distilled water with a solid/solvent ratio of 1:1 (w:v), and the mixture being homogenized and adjusted to alkaline value (pH 11,0) by adding 5 mol.L^{-1} NaOH aqueous solution, followed by stirring for 15 min and centrifugation (8000 × g, 4 °C, 15 min). Lipid fraction was removed, and the supernatant was stored at 4 °C. Remaining sediment was once more stirred in water at pH 11 for 15 min and then centrifugated (8000 × *g*, 4 °C, 15 min). Sediment was then added with distilled water and adjusted to acid value (pH 2) with 2 mol L^{-1} HCl aqueous solution, stirred for 15 min, and centrifugated (8000 × g, 4 °C, 15 min). Supernatant was finally collected and added to the previously extracted portions, which were mixed and adjusted to pH 7, stirred for 15 min, and centrifugated (8000 × g, 4 °C, 60 min). Supernatant was filtered and freeze-dried, resulting in soluble protein extract (SPE). The other fractions were weighed on a semi-analytical balance to calculate yield.

Enzymatic Hydrolysis

SPE was subjected to an enzymatic hydrolysis process, using commercial enzymes papain, bromelain, and bovine trypsin (Sigma-Aldrich, St. Louis, USA). Samples were dissolved in 0.1 mol L^{-1} phosphate buffer solution at 20 mg mL^{-1}. Solution was heated at 85 °C for 15 min to inactivate endogenous enzymes and then hydrolyzed for 6 h using 3% (w/v) enzyme (on the SPE mass). Hydrolysis conditions (temperature and optimum pH value) were defined based on known data (Villamil et al., 2017) for each enzyme, papain (37 °C, pH 6.0), bromelain (50 °C, pH 7.0) and trypsin (37 °C, pH 8.0). Enzymatic hydrolysis was stopped by heating for 15 min at 85 °C and hydrolysate fish protein (HFP) was centrifugated (8000 × *g*, 4 °C, 15 min). Supernatant was collected, freeze-dried, and stored at − 20 °C for further analysis.

Molecular Mass Distribution

The molecular mass distribution of the SPE and hydrolysate was evaluated by gel permeation chromatography using HPLC system Prominence (Shimadzu, Quioto, Japão). A Biosep Sec 3000 Column (300 × 7.8 mm) was equilibrated with Tris–HCl 100 mmol L^{-1} and NaCl 250 mmol L^{-1} (aqueous), at pH 7.0, at flow rate of 1 mL min^{-1} isocratic elution, at 30 °C. Eluate absorbance was monitored at 280 and 220 nm. Apoferritin (443 kDa), alcoholdehydrogenase (150 kDa), ovalbumin (42.3 kDa), carbonic anhydrase (29 kDa), cytochrome (12 kDa), and substance P (1.348 kDa) were used as protein molecular weight standards.

Soluble Protein Extraction (SPE) and Hydrolysate Fish Protein (HFP) Fractioning

SPE and HFP were resuspended and fractioned using Vivaspin® system (centrifugal filtration tubes) of 100, 30, 10, and 3 MWCO (molecular weight cut off), being centrifuged (6000 × g, 4 °C, 10 min) in a refrigerated Eppendorf centrifuge, model 5804R. Four fractions (including > 100 MWCO, 30–100, 10–30, and < 10 MWCO) were separated for further analysis.

Ion-Exchange Chromatography

Samples with molecular mass bellow 3 MWCO were submitted to ion exchange chromatographic separation in an HPLC system using TSKGel ® CM 25 W cationic column (Sigma-Aldrich, St. Louis, MO, USA), balanced with 50 mmol L^{-1} Glycine, 100 mmol L^{-1} NaCl, at pH 3.0, at flow rate of 0.8 mL min^{-1} and oven temperature at 30 °C. Detection was performed by a UV detector with selected wavelengths at 220 and 280 nm. Peaks observed in the chromatogram were collected at detector output. Waters OASIS® C-18 HLB 1 cc Cartridge filtration system was used to remove buffer and salt from peptide fractions. Fractions were eluted in acetonitrile and the solvent was evaporated in a vacuum concentrator miVac – Genevac and stored at refrigeration temperature for further analyses.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

TEAC assay is based on evaluating the reduction of the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) – $ABTS^{+}$. The $ABTS^{+}$ reagent reacts with a hydrogen atom donor, being converted into a colorless form of ABTS (Zheng et al., 2018). Reagent $ABTS^{+}$ was obtained by reaction of 1 mL of 7 mmol L^{-1} ABTS solution with 17.6 µL of 140 mmol L^{-1} potassium persulfate solution. Mixture was kept overnight (12–16 h) in the dark at room temperature. Before analyses, $ABTS^{+}$ radical was diluted in ethanol to get

an absorbance at 734 nm around 0.7 ± 0.05. An aliquot of 5 µL sample solution (at four different concentrations) was added to 250 µL of diluted $ABTS^+$ reagent and the reaction occurred for 6 min in dark room. Absorbance was measured at 734 nm in a microplate reader Synergy H1. Readings of blank (diluent—$ABTS^+$) and sample control (sample—diluent) were also performed. Fraction curves were plotted and related to Trolox standard curve. Antioxidant activity was expressed as Trolox equivalent antioxidant capacity.

Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP reagent was freshly prepared by mixing 25 mL of 0.3 mol L^{-1} acetate buffer, 2.5 mL of 10 mmol L^{-1} 2.4.6-tripyridyl-striazine (TPTZ) solution, and 2.5 mL of 20 mmol L^{-1} ferric chloride solution. An aliquot of 5 µL sample solution (at four different concentrations) was added to 250 µL of diluted FRAP reagent and incubated at 37 °C for 30 min. Absorbance was measured at 593 nm. Fraction curves were plotted and related to $FeSO_4$ standard curve, and antioxidant activity was expressed as µmol L^{-1} $FeSO^4$ g^{-1} (Choonpicharn et al., 2015).

ACE- Inhibition Assay

Tissue (kidney) of male Wistar rats, used as ACE source, was provided by the UFES vivarium (Animal Use Ethics Committee—Process CEUA/UFES n.48/2018). All procedures were performed on an ice bath or at 4 °C. Tissue sample was suspended in 5 volumes (w/v) of 50 mmol L^{-1} Tris–HCl buffer, pH 7.4, and 50 mmol L^{-1} NaCl and disrupted in a Potter Elvehjem homogenizer. After twelve strokes (2 × 6, with an interval of 30 s of the sample in the ice bath), the homogenate was centrifuged at 1000 × g for 10 min at 4 °C. The supernatant collected and designed as the crude extract was divided in aliquots and stored at − 20 °C during the time interval of the experiments, which did not exceed 1 month. The protein concentration was determined by the Bradford method. ACE activity was assayed with Abz-FRK(Dnp) P-OH to measure the somatic ACE activity (Carmona et al., 2006). This FRET peptide substrate contains Abz at the N-terminus and Dnp (2,4-dinitrophenyl) attached to Lys in the substrate. The activity on this Abz/Dnp peptide was determined in 0.1 mol L^{-1} Tris–HCl buffer, pH 7.0, which contained 0.05 mol L^{-1} NaCl, 10 µmol ZnCl2, 10 µg. mL^{-1} BSA, enzyme sample (5 µL), substrate (10 µmol) with 0.1 mol L^{-1} of TPCK, 0.1 mol L^{-1} of TLCK and a protease cocktail inhibitor (#P8340, Sigma) diluted to get a final concentration of 1 µmol L^{-1}AEBSF, 80 µmol L^{-1} aprotinin, 4 µmol L^{-1} bestatin, 1.4 µmol L^{-1} E-64, 2 µmol L^{-1} leupeptin and 1.5 µmol L^{-1} pepstatin). Incubation was carried out for 5 min at 37 °C and the fluorescence intensity was measured using spectrofluorometer Hitachi F-2000 (Hitachi Ltd., Tokyo, Japan), with excitation at $\lambda = 320$ nm and emission at $\lambda = 420$ nm. ACE activity values were reported as mol of substrate hydrolyzed per minute per mg protein (nmol min^{-1} mg^{-1}) (Sabatini et al., 2007). In parallel, specific inhibitor to ACE (30 nmol captopril) were added to each assay medium, immediately prior to the pre-incubation period. To evaluate ACE inhibition capacity of the peptides fraction, 50 µL of each fraction were added to the reaction, in concentrations ranging from 0 to 0.8 mg mL^{-1} and the decrease of ACE activity in the control was observed. IC_{50} was defined as the amount of peptide fraction (µg) necessary to inhibition of 50% ACE activity of control sample.

Purification of Peptide Fractions by Reversed-Phase Liquid Chromatography

Fractions obtained from ion exchange chromatography were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Kinetex® EVO C18 column (5 µm, 4.6 × 250 mm, Phenomenex®, Torrance, CA, USA). Mobile phase was composed by eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile). Elution occurred at a flow rate of 0.9 $mLmin^{-1}$, conducted with the following gradient: 0–5 min (100% A); 5–35 min (0 to 100% B); 35–40 min (100% B) and 40–50 min (0 to 100% A). Absorbance was monitored at 214 nm. Peaks were collected at the detector output and freeze-dried.

Peptide Sequence Identification by Mass Spectrometry

Samples of interest were resuspended in a total volume of 50 µL and applied to the Waters Nano Acquity system (Waters, Milford, MA), and 5 µL of the volume was used for injection. Samples were desalted using the Waters Symmetry C18 precolumn (180 µm X 20 mm, 5 µm), which was separated by liquid chromatography using the HSS T3 column, (100 µm, MA) eluting at 0.5 $µL.min^{-1}$, with a linear gradient of acetonitrile (3–40%) containing 0.1% formic acid, for 25 min. Sequential mass spectra were obtained using the Synapt HDMS spectrometer (Waters, Milford, MA) interfaced to the Nano Acquity capillary chromatography system. Electrospray ionization (ESI) was performed using 4000 V, with a source temperature of 80 °C and a cone voltage of 40 V. Data acquisition and instrument control were conducted using the MassLynx program (Version 4.1, Waters). Chromatographic runs were performed with an amplitude of 400–2000 mass/charge ratio (m/z), using 1-s interval applied throughout the chromatographic process. From each spectrum (MS), the most intense ion with an ion count greater than 500 was selected for subsequent fragmentation by MS/MS. Collision-induced dissociation (CID) was obtained through the use of argon gas at a

pressure of 1 bar and a collision energy ranging from 18 to 90 V depending on the charge and mass of the precursor, over a range of 50–2000 (m/z) with a range of 2 m/z. All MS/MS data were processed using the De Novo sequencing algorithm of PEAKS® Studio 11 software BioInformatics Solution Inc (trial version).

Statistical Analysis

All the experiments were performed in triplicate ($n = 3$), and results were presented as means and standard deviation (SD). ANOVA was applied to analyze ACE inhibition and antioxidant capacity parameters using Origin® Software (licensed for Federal University of Espirito Santo). Significant differences between the means of the sample parameters were determined by Tukey's test ($P < 0.05$).

Results and Discussion

Centesimal Composition of Raw Material and Extraction of SPE Yield

Fish waste yield ($n = 10$) obtained was 10% ± 1 (waste weight / total fish weight). There are no published data on the yield of the inedible parts of triggerfish, but generally fish processing can generate a percentage (% w/w) of 12–18% inedible parts containing viscera (12–18%), head (9–12%), skin (3%), scale (5%), and other by-products (Montoya & Sanchez, 2022). Centesimal composition of homogenized fish waste was evaluated in wet basis (Table 1) and expressed in percentage of weight per weight (% w/w). Homogenized viscera showed that the total protein content is 10.12% (w/w) in wet basis which represent 29.50% on dried basis. On the other hand, viscera of triggerfish fish showed a very high lipid content, 23.03% (w/w) of the total inedible parts, while a low ash content is due to material content without scales and bones. A previous study with *Oncorhynchus mykiss* viscera presented a total protein content of 64.8% (w/w) (Vásquez et al., 2022) which is higher than the triggerfish viscera in the present study. The centesimal composition of red tilapia viscera was reported to have 4.03% w/w of protein, 32.9% w/w of fat, 61.3% w/w of moisture, and 0.67% w/w of ash (Gaviria et al., 2021) while Atlantic salmon viscera was 8, 44, 6, and 1% w/w of these constituents, respectively. The centesimal composition of the inedible parts of fish depends on the species and part of waste. Extraction method used in the current study, also known as "pH shifting," is based on the increased protein solubility induced by changing the pH, which allows the separation of protein from lipids and insoluble material (Abdollahi & Undeland, 2019).

Table 1 Centesimal composition analysis of triggerfish non-edible parts (mean ± SD; $n = 3$)

Component	Composition on wet basis % (w/w)
Moisture	64.83 ± 1.03
Protein	10.12 ± 0.70
Fat	23.03 ± 1.60
Ash	2.04 ± 0.45

Protein extraction of triggerfish viscera showed that the soluble and insoluble protein obtained a mass yield of 8.8% and 1.2% w/w, respectively. This value is close to the value of extracted muscle proteins and gelatin obtained from cod heads, which showed that the two alkaline extracts yielded three times as much protein as the acid extract. The three-pooled extracts recovered 47.5% of the total protein. The major part (73%) of this protein was precipitated during neutralization, and the soluble fraction obtained was 12.8% (Arnesen & Gildberg, 2006).

In a study of squid protein extraction, the fraction obtained at low ionic strength was 20.59% (w/w), and at high ionic strength dissolution was 57.35% (w/w), while alkaline dissolution was 10.86% (w/w). Alkaline solubilization obtained a higher yield due to the solubility of myofibrillar protein at pH 10, and isoelectric precipitation (pH 5.5) determined the final yield of the process (Márquez-Alvarez et al., 2015). In the evaluation of fish protein extraction from *Clupea harengus membras* and *Rutilus rutilus*, there was a slightly higher mass yield using the pH shifting method compared to the enzymatic method, especially in the alkaline extraction step. The protein yield had no significant difference. According to the authors, the yields of protein and oil fractions separated from fish raw materials are the key factors in developing successful technology processes for industry (Nisov et al., 2022). This method, which uses pH 2 and 12, also contributes to the inactivation of microbes originating from the raw material (Freitas et al., 2016). The physicochemical properties of protein extracted from salmon, cod, and herring were studied, and the extraction method used was based on increased protein solubility induced by changing the pH, which allows the separation of protein from lipids and insoluble material. The alkaline process step resulted in higher protein solubility compared with the acid step in which protein yield ranged from 75.49% for salmon by-products and 90.10% for herring by-products. Despite the high solubility of the herring proteins at alkaline pH, they were not fully recoverable during the next steps of the pH-shift processing (Abdollahi & Undeland, 2019). Soluble protein extract (SPE) was then subjected to subsequent assays.

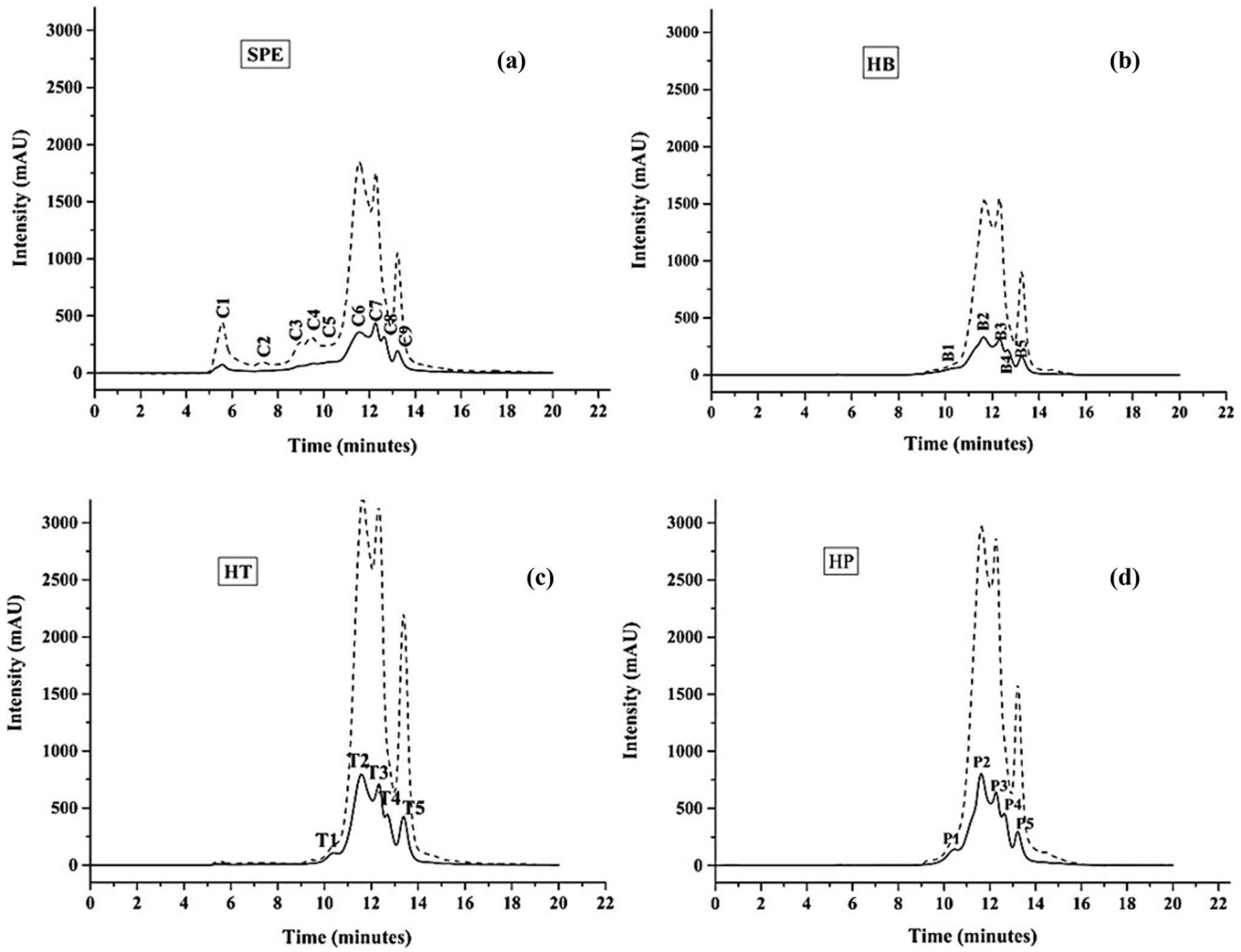

Fig. 1 Size-exclusion chromatogram of SPE and hydrolyzed samples (HB, HT, HP) in HPLC System, using Gel Filtration Biosep Sec 3000 Column (300 × 7.8 mm); mobile phase: Tris–HCl 100 mmol L^{-1} and NaCl 250 mmol L^{-1} (aqueous solution); pH 7.0; flow rate: 1 mL. $min.^{-1}$, isocratic elution, at 30 °C. Absorbance at 280 nm (straight line) and absorbance at 220 nm (dash line). Soluble protein extract - SPE (**a**) ; hydrolyzed by bromelain - HB (**b**); hydrolyzed by trypsin - HT (**c**); hydrolyzed by papain - HP (**d**)

Molecular Mass Distribution of SPE and Hydrolysate Samples

Characterization of the protein fraction by molecular mass distribution was performed by size exclusion chromatography using an HPLC system, using Gel Filtration Biosep Sec 3000 Column (300 × 7.8 mm) (Fig. 1). Figure 1 shows monitoring at 220 nm (straight line) and 280 nm (dash line), where peptide bonds are detected at 220 nm and at 280 nm tyrosine, tryptophan, and phenylalanine residues can be detected. As can be seen and expected, the absorption of peptide bonds at 220 nm is greater than that of aromatic residues at 280 nm. Thus, monitoring with two wavelengths allowed a broader and more precise observation of the results. Due to this being the first stage of chromatography of a complex mixture of proteins and peptides, the chromatographic profile did not present good resolution. The peaks were very close and asymmetrical, and the analysis was taken as an estimate of the molecular mass range. Mass distribution by peaks observed in the SPE chromatogram (Fig. 1a) showed that they ranged from low molecular mass (300 Da) to highest molecular mass proteins (> 440 kDa). Hydrolysates sample ranged from > 440 kDa (T1) to < 1300 Da (P5, B5, T5) (Table 2). It was observed that in the hydrolysates sample, the amount of large proteins (> 10,000 Da) decreased significantly, and before 10 min of running, the peaks in HB (Fig. 1b), HT (Fig. 1c), and HP (Fig. 1d) samples showed a low intensity (mAU), indicating very low concentration of proteins larger than 10,000 Da. Similar behavior was observed in a study with hydrolysates from defatted salmon backbones, in which hydrolysis process decrease the amount of large (> 10,000 Da) components, and increased the amount of smaller (200–500 Da) peptides (Slizyte et al., 2016). The hydrolysis conditions of

Table 2 Molecular mass range of SPE protein fractions and hydrolysates obtained by size-exclusion chromatography

Peak	Retention time (minutes)	Relative molecular mass estimated (kDa)
C1	5.6	> 440.0
C2	7.3	> 440.0
C3	9.0	145.8
C4	9.5	79.2
C5	10.3	29.8
C6	11.6	6.1
C7	12.3	2.6
C8	12.8	< 1.3
C9	13.3	< 1.3
B1, P1	10.5	23.3
B2, P2, T2	11.6	19.9
B3, T3, T3	12.3	5.8
B4, P4, T4	12.7	< 1.3
P5, B5, T5	13.2	< 1.3

specificity, enzyme type, pH, temperature time, temperature, enzyme-to-substrate ratio, solid–liquid ratio, and enzyme amount impact the amino acid sequences and size of the peptides and resulted in different biological activities because of the different peptides produced (Gao et al., 2021).

Bioactivity of stripped weakfish (*Cynoscion guatucupa*) hydrolysate was evaluated using Alcalase and Protamex (5% w/w) and peptides obtained were in the range of 300 to 3000 Da. Most of the peptides identified were in the range of 1000 to 2000 Da (Lima et al., 2019). Grass turtle muscle hydrolysates with papain were evaluated and the protein mass distribution showed the highest fraction (64%) of peptides between 180 and 1000 Da while in fresh muscle, 95% of the proteins showed > 10,000 Da (Islam et al., 2021). Hydrolysate protein from tilapia by-products was characterized and showed a molecular weight distribution ranging from 3600 to 26,600 Da. Amino acid composition of tilapia by-product hydrolysate powder was slightly different from non-hydrolysate tilapia by-product and glutamic acid was the largest component followed by glycine, aspartic acid, and alanine in hydrolysate (Roslan, et al., 2014). In a study of antioxidant activity of Klunzinger's mullet (*Liza klunzingeri*) muscle hydrolysate, the sample with the highest antioxidant had 95% of the peptides with molecular weight of less than 1000 Da, in which 30.56% of peptides in this sample had less than 180 Da, 47.26%, 180–500 Da, and 17.46% had molecular weight of 500–1000 Da. The most abundant amino acids in hydrolysate sample were serine (9.5%), tyrosine (8.4%), cysteine (7.1%), valine (6.6%), histidine (5.8%), and glutamine (4.9%) (Rabiei et al., 2019). Enzymatic hydrolysis of fish by-products is a favorable tool for reducing the pollution generated by the tonnes of fish waste produced, and raw material and enzyme type used during the hydrolysis process, as well as their interaction, affect hydrolysates physico-chemical and functional properties (Fuentes et al., 2024).

Antioxidant Activity of Protein Fractions

In antioxidant capacity equivalent to Trolox assays, SPE (< 10 MWCO) had a higher antioxidant capacity compared to others, as shown in Table 3. Samples showed significant difference by Tukey's test at the 5% significance level. A similar behavior was observed in the evaluation of the antioxidant capacity to reduce Fe2 +, in which SPE4 sample had the highest antioxidant power (1588.71 µmol FeSO4.g^{-1}). The SPE1 sample had the lowest value (881.56 µmol $FeSO_4$.g^{-1}). Except for the SPE and SPE2 samples, the other fractions showed a significant difference between them. Antioxidant capacity of SPE (4392.08 µmol Trolox. g^{-1}) was higher than the values reported in a study of *Oncorhynchus mykiss* viscera, which found values of 70 µmol TE g^{-1} (FRAP) and 544 µmol TE g^{-1} (TEAC) for non-hydrolyzed viscera sample, 269 µmol TE g^{-1} (TEAC) and 1469 µmol TE g^{-1} (FRAP) for hydrolyzed viscera sample (Arnesen & Gildberg, 2006). In another study with red tilapia viscera, TEAC values of 818 µmol TE g^{-1} were found for non-hydrolyzed viscera and 1530.8 for hydrolyzed viscera, while FRAP values of 141.4 and 548.9 µmol TE g^{-1} were reported. The study indicated that the hydrolysis favored an increased antioxidant activity (Nisov et al., 2022), which can be attributed to changes in the size and sequence of the amino acids of peptides obtained in the reaction, and to expose the terminal amino groups which capable of reacting with oxidizing agents (Freitas et al., 2016). Antioxidant activity of hydrolyzed tilapia skin gelatin was evaluated and the FRAP values were ranging from 2800 µmol TE g^{-1} to 4900 µmol TE g^{-1}, while TEAC values were between 10 and 1500 µg TE g^{-1}, depending on the enzyme used for the hydrolysis process. In a study on the use of by-products from shrimp processing, the highest antioxidant activity was obtained using

Table 3 TEAC (µmol. Trolox g^{-1}) and FRAP (µmol FeSO4. $g^{-1)}$ (µmol. Trolox.g^{-1}) of protein fraction obtained by size exclusion (mean ± SD; $n = 3$)

Protein fraction	TEAC µmol Trolox g^{-1}	FRAP µmol $FeSO_4$ g^{-1}
SPE	$4392.08^{a} \pm 75.53$	$1072.62^{a} \pm 17.74$
SPE 1 (> 100 MWCO)	$5029.28^{b} \pm 131.70$	$881.56^{b} \pm 18.60$
SPE 2 (> 30 < 100 MWCO)	$5900.60^{c} \pm 38.33$	$1066.49^{a} \pm 19.95$
SPE 3 (> 10 < 30 MWCO)	$7818.39^{d} \pm 164.19$	$1331.43^{c} \pm 73.14$
SPE 4 (< 10 MWCO)	$10{,}157.69^{e} \pm 353.76$	$1588.71^{d} \pm 23.44$

SPE soluble protein extract; SPE 1: > 100 MWCO; SPE 2: 30–100 MWCO; SPE 3: 10 and – 30 MWCO; SPE 4: < 10 MWCO

Different letters in the same column indicate significant differences by Tukey's test ($P < 0.05$).

Flavourzyme (31.7 μmol TE g^{-1}) (Slizyte et al., 2016). ABTS radical scavenging activity was evaluated in hydrolyzed European seabass showing values of 848.11 μmol. TE g^{-1}.

Separation by Ion-Exchange Chromatography

An additional fractioning by ion-exchange chromatography was applied in SPE and hydrolysate samples (MWCO < 10,000). Chromatogram obtained from non-hydrolysate (SPE) and hydrolysate (HT, HP, HB) samples (Fig. 2) showed a slight difference which can be attributed to previous filtration in 10,000 MWCO filter, that excluded the fraction of samples in which hydrolysis was more effective. SPE sample (Fig. 2a) obtained 5 peaks; HP (Fig. 2b) and HB (Fig. 2c), 3 peaks; and HT (Fig. 2d), 4 peaks. It was observed that the 3 last peaks eluted were similar in all samples. The chromatography performed, despite having satisfactory reproducibility, was not resolving enough, as can be seen by the width of the base of the peaks and their overlap during the elution time. In SPE 1 and HT (Fig. 2a, d), the first peak (monitored at 280 nm) is observed at the elution time of 2.8 min, indicating that it is a protein with more negative charge than the others, and it may be composed of higher mass proteins that were not hydrolyzed. It was not detected in hydrolyzed samples HP and HB (Fig. 2b, c). Peaks which eluted between 2.8 and 6.8 min were very close, showing an overlap. An isolated peak was observed in all samples after 7 min of elution, indicating that they are peptides more positively charged than other fractions. The peaks that eluted after 3.3 min showed greater intensity when monitored at 220 nm, compared to 280 nm, due to the presence of peptides bond which is detected at this wavelength.

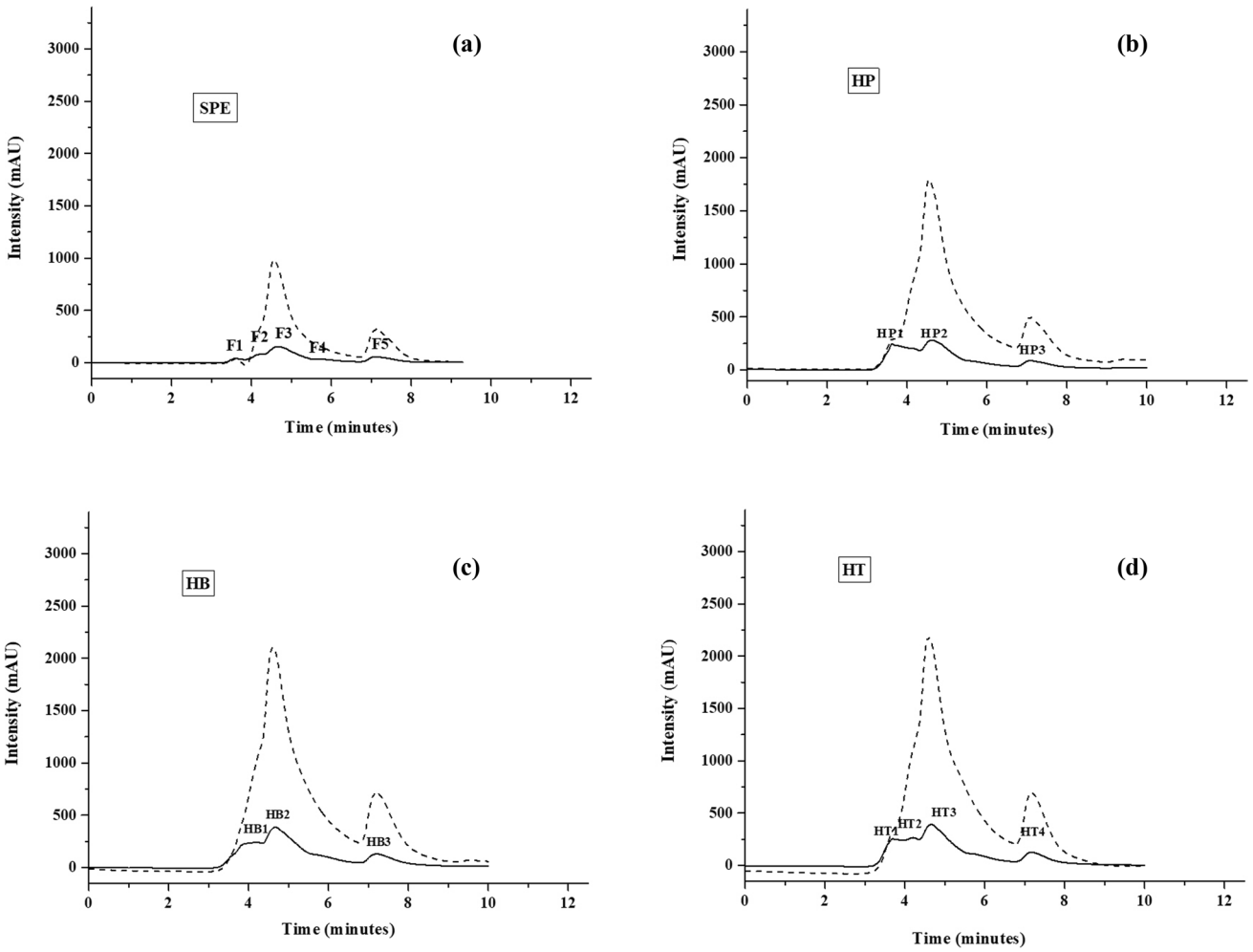

Fig. 2 Chromatogram of the SPE and hydrolysate samples conduced in an HPLC system using TSKGel CM 25 W cation exchange column (25 cm×4.6 mm) mobile phase: glycine 50 mmol. L^{-1}, NaCl 100 mmol. L^{-1}, pH 3.0, flow rate: 0.8 mL. $min.^{-1}$, isocratic mode, at 30 °C. Absorbance at 280 nm (straight line) and 220 nm (dash line) ($n=3$). Soluble protein extract - SPE (**a**); hydrolyzed by papain - HP (**b**); hydrolyzed by bromelain - HB (**c**); hydrolyzed by trypsin - HT (**d**)

Bioactive Activities of Peptides Fractions Obtained from Ion-Exchange Chromatography—TEAC and FRAP Assays

SPE was then subject to antioxidant activity assays by TEAC and FRAP mechanisms. For both mechanisms, FT (non-fractioned) and F1 showed the highest capacity. F1 fraction antioxidant activity was 8839.04 µmol Trolox. g^{-1} and 1749.94 µmol FeSO4.g^{-1}, in TEAC and FRAP, respectively (Table 4). The lowest observed value was in F5 fraction, which was the last peak eluted during ion exchange chromatography separation.

As observed in the present study, other studies have also shown that fractionation contributes to improving antioxidant activity. Peptides obtained from the fractionation of Chinese sturgeon (*Acipenser sinensis*) hydrolysates showed excellent results indicating that the fractionation and purification process significantly increased antioxidant activity, indicating excellent capabilities to scavenge free radicals (Noman et al., 2022). Similar results were also observed in research carried out with hydrolysates from pony fish (*Photopectoralis bindus*), showing that antioxidant activity increases according to the degree of hydrolysis and fractionation (Ramezani et al., 2020). Antioxidant activity was also evaluated in peptides obtained from the hydrolysis of proteins from Indian mackerel by-products, demonstrating DPPH antioxidant activity of 478 µmol Trolox. g^{-1} (Gaikwad et al., 2021).

ACE-Inhibitory Activity of Peptide Fractions

ACE inhibition of fractions was evaluated by IC_{50}, which was defined as the amount of peptide fraction (µg) necessary to inhibit of 50% ACE activity of control (Table 5). The activities which were inhibited by 30 nmol of specific ACE inhibitor captopril were established as control. Fractions F3, F5, and HP3 showed the lowest IC_{50} (30.1, 42.7, and 37.7 µg, respectively), which require a lower amount of peptide fraction to reach 50% of ACE inhibition. Relating the fractions with each corresponding retention time, it was observed that F3 corresponded to the time in which HP2, HB2, and HT2 eluted. Fraction F5 corresponded to HP3, HB3, and HT4. Among the hydrolysates fractions, hydrolysate with papain (HP) got the lowest IC_{50}. Analyzing all samples by ANOVA, there was a significant difference ($P < 0.05$). The IC_{50} values showed a significant difference between all samples by means comparison Tukey's test ($P < 0.05$). In studies with hydrolysates of tilapia processing by-products, peptide fractions with smaller size showed greater potential for ACE inhibition (Roslan et al., 2014). Investigation of ACE inhibitory activity of hydrolysates obtained from lizard fish showed an IC_{50} value of 175 µmol and the sequence of peptide RVCLP was identified (Wu et al., 2015). Another study showed percentages of ACE inhibition of 75, 40, and 38% for peptide fractions purified from lizard fish with using 0.16 mg mL^{-1} of the peptide solution. The fraction with the highest percentage of inhibition was identified as VYP, with an IC_{50} of 105 µmol (Wu et al., 2012). ACE inhibition activity of Mediterranean fish residues was investigated. For horse mackerel (*T. mediterraneus)*, the fraction with the size between 130 and 2350 Da exhibited greatest inhibitory activity, and for "small-spotted catshark" (*S. canicula*), the study presented greater inhibitory activity among those investigated samples ($IC_{50} = 85$ µg mL^{-1}). Fraction D (< 470 Da) had the lowest IC_{50} value (27 µg m L^{-1}) (García-Moreno et al., 2015).

ACE plays an important role in cardiovascular function by converting the decapeptide Angiotensin I to the octapeptide Angiotensin II (vasoconstrictor), which causes an increase in blood pressure. Furthermore, it inhibits the vasodilator bradykinin (Kim and Wijesekara, 2010). Industrially

Table 4 TEAC and FRAP assays of protein fraction obtained by ionic exchange chromatography (mean ± SD; $n = 3$)

Peptide fraction	TEAC µmol Trolox g^{-1}	FRAP µmol $FeSO_4$ g^{-1}
FT (SPE < 10 MWCO)	10,157.69^{a} ± 353.76	1782.35^{a} ± 83.46
F1	8839.04^{b} ± 149.64	1749.94^{ab} ± 113.52
F2	5579.10^{c} ± 161.82	656.83^{d} ± 22.71
F3	5360.95^{c} ± 353.35	1297.81^{c} ± 37.77
F4	7404.72^{d} ± 66.77	1655.53^{ab} ± 48.22
F5	3922.06^{e} ± 5.28	1576.90^{b} ± 49.74

Different letters in the same column indicate significant differences by Tukey's test ($P < 0.05$)

Table 5 ACE Inhibition – IC_{50} value of peptide fractions obtained by ionic exchange chromatography (mean ± SD; $n = 3$)

Inhibitor	ACE specific activity of control sample (nmol Abz. mg^{-1})	IC_{50} value (µg. mL^{-1})
F3	226.45 + 5.68	30.1^{a} ± 0.5
F5	226.45 + 5.68	42.7^{b} ± 0.4
HP1	244.37 + 0.32	91.8^{c} ± 0.8
HP2	244.37 + 0.32	69.1^{d} ± 0.3
HP3	244.37 + 0.32	37.7^{e} ± 0.6
HB1	265.30 + 10.90	102.9^{f} ± 1.1
HB2	265.30 + 10.90	134.6^{g} ± 1.9
HB3	265.30 + 10.90	108.8^{h} ± 0.4
HT2	257.18 + 1.81	130.1^{i} ± 2.5
HT3	257.18 + 1.81	98.9^{j} ± 1.1
HT4	257.18 + 1.81	95.3^{l} ± 7.8

Different letters in the same column indicate significant differences by Tukey's test ($P < 0.05$)

produced angiotensin I converting enzyme (ACE) inhibitors are very specific synthetic drugs, widely used to control hypertension. The most commercially used are Captopril®, Enalopril®, Lisinopril®, among others. Food-derived ACE inhibitory peptides could be a good alternative to synthetic drugs (Daskaya-Dikmen et al., 2017). Molecular mass, hydrophobic amino acid residues, charge, composition, and amino acid sequence of the peptides (especially at the C-terminus and N-terminus) have a significant influence on the ACE inhibitory activity (Li et al., 2012; Xiang et al., 2021). Greater ACE inhibitory activity was observed when the C-terminus was aromatic amino acids, proline, and hydroxyproline. Furthermore, peptides with hydrophobic or basic amino acids at the N-terminus exhibited greater ACE inhibitory activity (Zhang et al., 2013). Studies indicated that peptides of lower molecular mass, with just a few amino acids in their sequence, showed greater bio-efficacy and bioavailability compared to polypeptides of higher molecular mass, due to the greater resistance of these small peptides to digestive proteases. Another factor is that the presence of tyrosine, tryptophan, proline, phenylalanine, or positively charged amino acids close to hydrophobic amino acids would interact with ACE active sites (Abachi et al., 2019). Isolation and characterization of peptides extracted from fish hydrolysates and other aquatic organisms with potential to inhibit ACE activity were reported by several researchers, including hydrolysates from rainbow trout (*Oncorhynchus mykiss)* skin (Cheung & Li-Chan, 2017), hydrolyzed tilapia skin protein (Dong et al., 2024), hydrolyzed lizard fish protein (Lan et al., 2015), and hydrolysates from *Mustelus mustelus* (Abdelhedi et al. 2018). In vivo tests using naturally hypertensive rats revealed that the ingestion of peptides obtained from hydrolysates of the Asian fish *Misgurnus anguillicaudatus* promoted a significant reduction (25 mmHg) in systolic pressure (Li et al., 2012). Peptides obtained from Pacific Sauri (*Cololabis saira*) hydrolysates were also tested in "in vivo" assays, and the peptides showed a good source of bioactive ACE-inhibiting peptides (Wang et al., 2024). Several studies have already shown that these peptides can replace conventional synthetic drugs with similar potency and few or no adverse effects. However, some studies are necessary to increase productivity, improve the bioavailability of peptides, and evaluate their bioaccessibility and efficiency in reducing blood pressure to support the development and application of health products (Abachi et al., 2019). In a study with muscle protein from Indian catfish *Clarias magur*, digested by trypsin, 23 peptides were identified and showed bioactivity for ACEinhibition using bioinformatic approaches. Fish-derived ACE inhibitory bioactive peptides can prove to be a natural, less toxic therapeutic sources than traditionally synthetic compounds used for ACE inhibition that pose side effects (Jayant Singh et al., 2024).

Reverse-Phase HPLC Chromatography

Due to similarity of the SPE and hydrolyzed sample fractions obtained by ion exchange chromatographic separation and the results from ACE-inhibition activity, which showed better results to IC_{50} in F3- F5 fractions, only the SPE fractions followed additional purification steps in reversed-phase chromatography, in order to obtain better purification by eliminating interferents and peptide fragments that could harm mass spectrometry analysis. Preliminarily, samples F1 to F5 were filtered in a Waters OASIS® C-18 HLB 1 cc Cartridge system to remove salt and buffer and then separated in a reversed-phase HPLC system. Chromatograms showed the separation of peaks obtained from RP-HPLC chromatography (Fig. 3). Due to the characteristics of sample F1, it presented inappropriate behavior when injected into the system, mainly due to its greater molecular mass and characteristics that caused increase in system pressure. In the F2 sample (Fig. 3a), three initial peaks were detected with better resolution and significant intensity, which indicated the elution of more polar samples (100% water eluent) and a higher molecular mass. After 11 min of running, as the percentage of acetonitrile increased, several peptides were eluted, and the eluent had a higher proportion of acetonitrile. In this range are peptides with lower mass and polarity. The most intense peaks F2A, F2B, and F2P were collected separately. The same behavior was observed in F3 (Fig. 3b), and F4 (Fig. 3c), fractions and the most intense peaks were collected. It was observed similar peaks at 6.2 and 8 min of running, which can be attributed to low resolution in ion-chromatography step that promoted overlap between F2 and F3 peaks. F5 sample (Fig. 3d) showed one intense and symmetric peak. The peaks with the highest intensity and resolution were selected for mass spectrometry analysis.

Peptides Sequencing

Sequential mass spectra were obtained using the Synapt HDMS spectrometer (Waters). Analysis showed that most of their mass peaks were close to 300 Da. Samples F4 and F2 contained a reasonable variety of peptides and were subjected to analysis using the PEAKS software (trial version). "De Novo" sequencing generated an infinity of possible sequences, that fragmented as peptides. The average local confidence (ALC) score indicated the accuracy of the identified peptide structure and peptides with ALC value higher than 85% were selected (Table 6).

A total of 16 peptides were selected from the F2 fraction, and they presented very diverse sequences. The presence of hydrophobic amino acids such as leucine (L), valine (V), phenylalanine (F), and alanine (A) at the C-terminus position in most of these peptides could increase ACE-inhibiting ability, which is in agreement

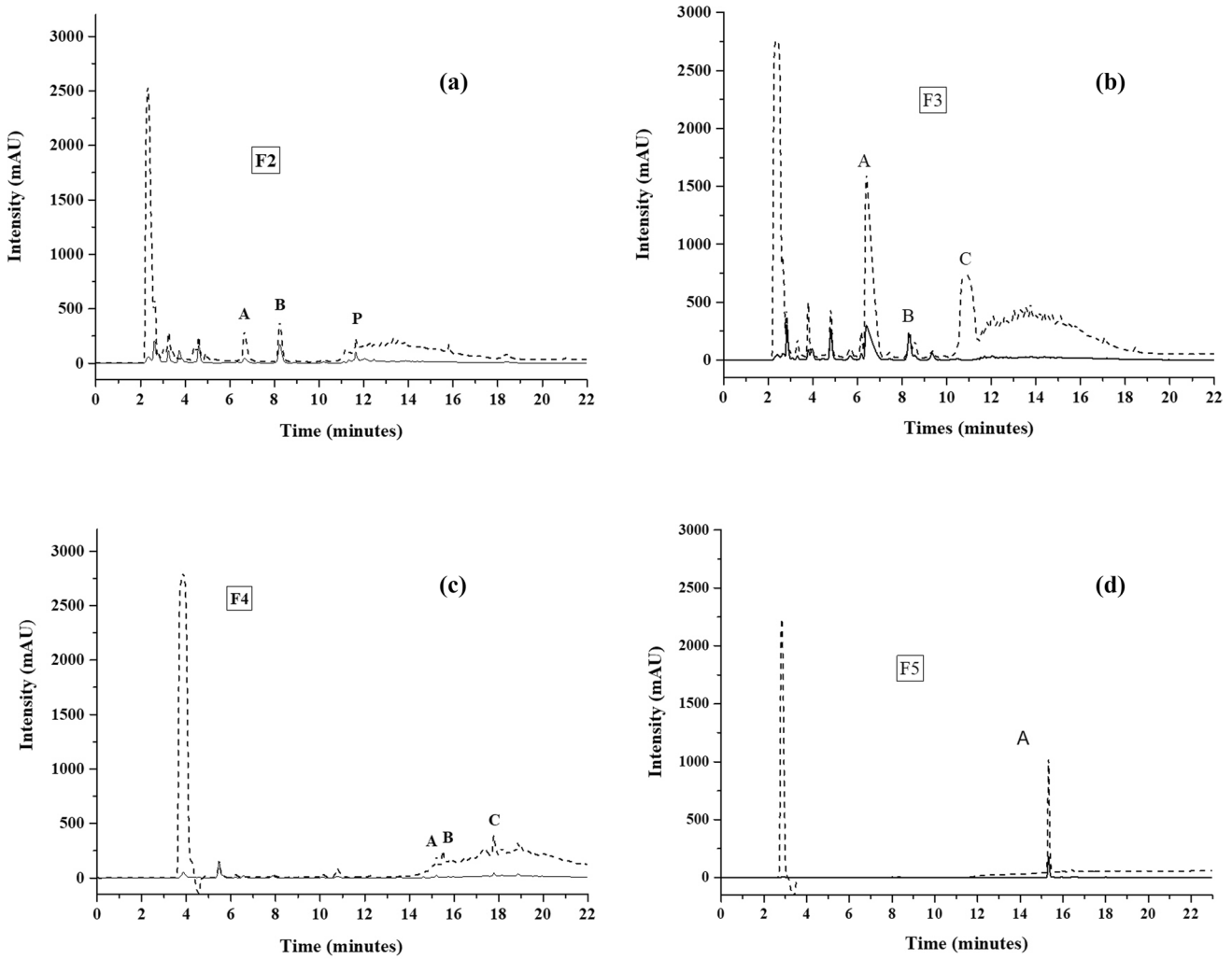

Fig. 3 Reverse-phase HPLC chromatography of fractions F2, F3, F4, and F5 carried out on a Prominence HPLC system (Shimadzu, Kyoto, Japan), Kinetex® EVO C18 column (5 µm, 4.6×250 mm, Phenomenex®, Torrance, CA, USA), eluent A (0.1% TFA in water), and eluent B (0.1% TFA in acetonitrile). Conducted with gradient: 0–5 min (100% A); 5–35 min (0 to 100% B); 35–40 min (100% B); and 40–50 min (0 to 100% A). Absorbance monitored at 214 nm (straight line) and 280 (dash line). Fraction F2 (**a**); Fraction F3 (**b**); Fraction F4 (**c**); Fraction F5 (**d**)

with previous studies. Aromatic amino acid at C-terminus, as shown in VDPSNF, also be related with an ACE inhibitory activity, and the same behavior was observed by Zhang et al. (2013). Furthermore, the N-terminal site also contains valine, which may improve the effect on ACE inhibitory activity, as shown in previous studies (Dong et al., 2024; Ling et al., 2018). The N-terminus of the FVPLDVLE peptide presented an aromatic amino acid (phenylalanine), which could contribute to ACE-inhibition. Peptides having cysteine (C) residue at any position had higher antioxidant activity than other peptides (Uno et al., 2020), which was observed only in CVLPDMQ sequence. The properties of N-terminal amino acids are weakly correlated with radical-scavenging potency for antioxidative peptides measured by the TEAC method, while the hydrophobicity of C-terminal amino acids contributes more to antioxidant activity, and it is important for predicting antioxidant activity (Li & Li, 2013). The sequences identified in fraction F2 were analysed by Uniprot Consortium database (Uniprot, 2023). CVLPDMQ was once reported as derived from ornithine decarboxylase protein from *Clupea gigantea* fish and showed molecular function of carbon–carbon lyase activity, also acting in polyamine biosynthesis. The AEGANGPTTPDADKL sequence was reported to be derived from the protein of spotted green pufferfish (*Tetraodon nigroviridis*), which presented oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as an acceptor, in addition to being part of the amino acid metabolic process. The MGALDSL sequence

Table 6 Amino acid sequence of identified peptides and their respective mass

Peptide fraction	Amino acid sequence	Mass (Da)
F2	SLLDSL	646.3
	GSGTTFYPGLADR	1340.6
	VDPSNF	677.3
	VLPELN	683.4
	RLVSVPLQEANR	1380.8
	STALDSL	705.3
	MGALDSL	705.3
	AEGANGPTTPDADKL	1455.7
	CVLPDMQ	804.3
	DVLLPVPA	822.5
	LENLTDGL	873.5
	FVPLDVLE	930.5
	EVNLTAGLQ	943.5
	VPRVPGGAPL	961.6
	RGGAGGLPAGGL	981.5
	RPGGVVPGTGV	994.5
F4	YPTEDVPR	975.5
	YADEELPR	991.5
	EDLGPK	657.3

was found in galanin peptides, present in several fish, as an extracellular component with hormone activity. Fraction F4 presented 3 peptide sequences: YPTEDVPR (975.46 Da); YADEELPR (991.46 Da); and EDLGPK (657.3 Da) in which the presence of amino acids such as lysine, valine, arginine, tyrosine, leucine, and proline can be observed. These amino acids are related to antioxidative activity for their ability to remove oxygen-free radicals (Wu et al., 2019). These peptides contain the positively charged amino acids arginine (R) and lysine (K), and it was reported previously that R and K in the C-terminal showed good ACE inhibitory ability (Abdelhed & Nasri, 2019). In previous studies, five peptides, VVLASLK, LTLK, LEPWR, ELPPK, and LPTEK were isolated and identified from Pacific saury fish, and the peptide LEPWR showed the best ACE inhibitory ability (Wang et al., 2024). Peptides WEGPK, GPP, and GVPLT, isolated from the protein hydrolysate of bluefin leatherjacket (*Navodon septentrionalis*) heads, demonstrated antioxidant activities, which could be attributed to their smaller molecular size and hydrophobic and/or aromatic amino acids in their sequences (Chi et al., 2015). Analysing the mechanism of ACE inhibition, substrates or inhibitors with hydrophobic amino acid residues in any of the three positions closely following the three positions of the C-terminus are preferred (Wang et al., 2024). In addition to the hydrophobicity of amino acids, the presence of aromatic rings, and charge, other factors such as hydrogen interactions and metal-acceptor interactions (due to one zinc-binding domain of ACE) have been reported as critical factors in ACE inhibitory activity (Chen et al., 2022). According to Nirmal et al. (2023), to determine the exact interaction of peptides derived from fish proteins with a given molecular target, structure–activity relationship (SAR) analyses using advanced techniques including QSAR, molecular docking studies, and simulation have been applied. However, there are few reported SAR studies of fish-derived antioxidant peptides, and further investigations into the mechanism of action of peptides against oxidative enzymes are needed. Fish-derived bioactive peptides promise enormous potential for various applications in the food and healthcare industries, but more research needs to be developed to overcome challenges in terms of regulation, structural stability, mass production, and technological and research developments to increase their utilization (Kurnianto et. al., 2024).

Conclusions

Peptide fractions obtained from inedible parts of *Balistes capriscus* seem to be a good source of proteins that can be hydrolyzed, fractionated, and purified for application as bioactive products. Although the enzymatic processes used were more effective in the hydrolysis of high molecular weight proteins and the other fractions showed slight differences in the chromatographic profiles, the conditions can be optimized to increase the yield of low molecular weight peptides. Ultrafiltration and fractionation methods followed by ion exchange chromatography on the *Balistes capriscus* peptides resulted in a series of peptide fractions that presented activities with significant differences between them. The antioxidant and ACE-inhibitory activities of the fractions were higher compared to the findings with other species carried out by previous studies, and those with low molecular mass were the ones that stood out. A total of 19 peptide sequences were identified, and the hydrophobicity, size, and amino acids present in the C- and N-terminals are the main factors correlated with biological activity. It is suggested that more steps be taken in the purification process to improve peptide sequencing and to identify new sequences with greater biological activity.

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Author Contribution M.L.R.: planning and experiments, conception, formal analysis, investigation, data analyses, data curation, writing – original draft; A.C.S.K. (undergraduated scholarship holder): experiments supporting and graphic editing; A.L.C.; A.V. M.; R. B. Z.; spectrometry analysis (execution and data analysis); M. A.C.: planning and supervising of ACE-inhibiting analyses; A. S.: Advising, conceptualization, data curation, writing – review and editing.

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Data Availability The data are available from the corresponding author upon suitable request.

Declarations

Competing Interest The authors declare no competing interests.

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