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Antioxidant and angiotensin I-converting enzyme (ACE) inhibitory peptides of rainbow trout (*Oncorhynchus mykiss*) viscera hydrolysates subjected to simulated gastrointestinal digestion and intestinal absorption

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ABSTRACT

The objective of this work was to evaluate *in vitro* bioaccessibility, intestinal absorption, antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activities of peptides from rainbow trout viscera hydrolysate (H). Rainbow trout Viscera (V) was hydrolyzed by Alcalase® 2.4L and a degree of hydrolysis (DH) of 44.8 ± 2.5% was achieved. Viscera and its hydrolysate were subjected to simulated gastrointestinal digestion (SGID) and intestinal absorption across Caco-2/TC7 cell monolayers. After the hydrolysis with Alcalase® 2.4L and the SGID of V, the species between 60.6 kDa and 13.0 kDa were decreased, causing an increase in species less than 6.51 kDa. The SGID of H did not modify the oxygen radical absorbance capacity (ORAC) or ACE inhibitory values but caused a significant decrease in the hydroxyl radical antioxidant capacity (HORAC) (30.2%). It also produced an increase in ABTS radical cation (ABTS assay) scavenging activity and ferric reducing antioxidant power (FRAP) (9.46% and 20.2%, respectively). Bioactive peptides in H were stable after SGID and they were partially able to cross Caco-2/TC7 cell monolayer, which demonstrates their possible intestinal absorption and their potential to act inside the organism.

1. Introduction

In the last decades, global fish production and consumption have greatly grown (FAO, 2018), which has caused concern about their environmental impact since fish industry produces large quantities of by-products frequently discarded without any recovery attempt or underutilized in low value-added products (Arvanitoyannis & Tserkezou, 2014). By-products are rich sources of compounds, such as collagen, gelatin, polyunsaturated fatty acids (EPA and DHA) with variable content of proteins (8–51%) and lipids (1–44%) according to the species, age, nutritional and health status of the animal and type of by-product (Sierra, Sepúlveda, Vásquez, Figueroa, & Zapata, 2018). In rainbow trout (*Oncorhynchus mykiss*) production, between 60 and 70% of its fresh weight is discarded (Torres, Chen, Rodrigo-Garcia, & Jaczynski, 2007). The most representative by-product is the viscera which constitutes between 12 and 20% of the total weight. It is considered an important source of lipids and proteins as well as an interesting matrix to produce bioactive peptides (Villamil, Vaquiro, & Solanilla, 2017).

Bioactive peptides can be generated by chemical hydrolysis, enzymatic hydrolysis and microbial fermentation (Kim et al., 2012). They generally contain 2 to 20 amino acid residues which may have antihypertensive, antioxidant, antimicrobial, antithrombotic, anticancer properties depending on their composition, sequence, structure and physicochemical characteristics (Mora, Gallego, Aristoy, Reig, & Toldrá, 2019).

Living organisms produce reactive oxygen species (ROS) through normal processes like respiration. However, their overproduction leads to oxidative stress that produces cellular damage which, in turn, causes chronic diseases (Xiong, 2010). Fish by-product hydrolysates are a potential source of antioxidant peptides as reported by Sepúlveda &

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Zapata, 2020; Gómez et al., 2019 and Nikoo, Benjakul, Yasemi, Ahmadi Gavlighi, & Xu, 2019.

Hypertension is one of the major risk factors for cardiovascular diseases. Angiotensin I-converting enzyme (ACE) plays a crucial role in regulating blood pressure by converting angiotensin I to the vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin. Thus, ACE inhibition is considered one of the strategies for treating hypertension (Karami & Akbari-Adergani, 2019). Several studies have found ACE inhibitor peptides from fish by-product hydrolysates (Ngo, Vo, Ryu, & Kim, 2016; Roslan, Mustapa Kamal, Yunos, & Abdullah, 2017).

Nevertheless, gastrointestinal digestion effects (bioaccessibility) on bioactive peptides from fish by-products and their possible absorption in the intestinal epithelium have not been frequently evaluated. Furthermore, studies on these two fundamental aspects for bioavailability have not been reported on bioactive peptides from rainbow trout viscera so far, although this species is of great importance in aquaculture. In this sense, the objectives of this work were to evaluate the effects of simulated gastrointestinal digestion on the antioxidant and ACE inhibitory activities of peptides from both rainbow trout viscera (V) and its hydrolysate (H) and to evaluate their intestinal absorption through Caco-2/TC7 cells monolayers. This will set added value to rainbow trout viscera as a functional ingredient and generate the basis for a possible alternative that can help mitigate the environmental impact caused by their disposal.

2. Materials and methods

2.1. Raw material

Fresh rainbow trout viscera were obtained from a local fish farm (Piscícola de Occidente, Antioquia, Colombia). They were heated (90 °C, 20 min) to separate fat and to inactivate endogenous enzymes as described by Vásquez and Zapata (2018). The remained viscera mass (V) was minced, freeze-dried and stored at 4 °C until use.

2.2. Enzymes and chemicals

Alcalase® 2.4L (2.4 Anson Units (AU)/g) was acquired from Novo Nordisk Co. (Bagsvaerd, Denmark). Pepsin (1:15000 5X National Formulary standards) and porcine pancreatin (4X-100 United Stated Pharmacopeia units/mg) were from MP Biomedicals LLC (Solon, OH, USA). The chemicals, including 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azo-bis-(2-methylpropionamidine) dihydrochloride (AAPH), N-hippuryl-L-histidyl-L-leucine hydrate (HHL), 2,4,6-trinitrobencenesulfonic acid (TNBS) and bovine serum albumin were obtained from Sigma- Aldrich (St. Louis, MO, USA). Fluorescein sodium was acquired from Fluka (Steinheim, Germany). All the other reagents were of analytical grade.

2.3. Enzymatic protein hydrolysis

The hydrolysis was carried out in a 7.5 L reactor (BioFlo 110, New Brunswick Scientific, USA) with a substrate concentration of 5.45 g/100 mL and Alcalase® 2.4 L concentration of 0.72 AU/g protein. The hydrolysis was conducted at 60 °C and pH 8.50 for 6 h under constant agitation (102 rpm) and controlled using BioCommand software (New Brunswick Scientific, USA). The reaction was stopped by heating (85 °C, 20 min) and the hydrolysate (H) was centrifuged (6700×*g*, 10 °C, 20 min), freeze-dried and stored at 4 °C until use. The DH was measured using the TNBS method (Adler-Nissen, 1979) and calculated with equation (1).

$$DH = \frac{\left([-NH_2]_h - [-NH_2]_0\right)}{\left([-NH_2]_\infty - [-NH_2]_0\right)} \times 100 \quad (1)$$

where $[\text{-}NH_2]_h$ and $[\text{-}NH_2]_0$ indicate the concentration of free amino groups in the hydrolyzed or the nonhydrolyzed samples, respectively. $[\text{-}NH_2]_\infty$ was estimated according to equation (2).

$$[-NH_2]_\infty = \frac{1}{PM_{aa}} \times \left(1 + f_{Lys}\right) \times C_{prot} \quad (2)$$

where PM_{aa} (127.56 g/mol) is the average molecular weight (MW) of amino acids in V, f_{Lys} (0.064) is the proportion of lysine in these proteins, and C_{prot} is protein concentration.

2.4. Simulated gastrointestinal digestion (SGID)

Viscera and its hydrolysates were subjected to *in vitro* digestion according to Minekus et al. (2014) with some modifications (Rodríguez, García Fillería, & Tironi, 2020). *Oral phase:* samples (2.50 g) were homogenized with 6.0 mL of simulated salivary fluid (SSF, pH = 7); 0.5 mL of α-amylase (26.0 mg/mL, 57.4 U/mg), 25 μL of 0.3 mol/L $CaCl_2$ and 975 μL H_2O were added. The mix was incubated for 2 min at 37 °C with constant agitation (350 rpm, Thermo-Mixer Eppendorf, Sigma-Aldrich, USA). *Gastric phase*: oral solution was mixed with 7.5 mL of simulated gastric fluid (SGF), 1.6 mL of pepsin solution (47.8 mg/mL, 4530 AU/mg) and 5 μL of 0.3 mol/L $CaCl_2$, pH was adjusted to 3 (2 mol/L HCl). Then, water was added in order to complete 10 mL. The mix was incubated for 2 h at 37 °C with constant agitation (350 rpm). *Intestinal phase:* gastric solution (20 mL) was mixed with 11 mL of simulated intestinal fluid (SIF), 5.0 mL of pancreatin solution (15.3 mg/mL, 68 USP/mg), 2.5 mL of 150 mg/mL bovine bile salts, and 40 μL of 0.3 mol/L $CaCl_2$. pH was adjusted to 7 (1 mol/L NaOH) and water was added to complete 20 mL. The mixture was incubated for 2 h at 37 °C with constant agitation (350 rpm). Finally, enzymes were inactivated by heating (85 °C, 10 min) and digested viscera (VD) and digested hydrolysate (HD) were freeze-dried and stored at 4 °C. Electrolyte solutions (SSF, SGF, and SIF) were prepared according to Minekus et al. (2014). The DH of VD and HD was measured by the TNBS method.

2.5. Soluble and total protein content determination

The protein content of V, H, VD and HD was determined using the Micro-Kjeldahl method accompanied by the Berthelot colorimetric method (Nkonge & Ballance, 1982). Soluble fractions of the samples (10 mg/mL) in PBS buffer (pH = 7.4) were prepared (500 rpm, 37 °C, 1 h) and then centrifuged (10,000×*g*, 10 min, 20 °C). Protein concentration of soluble and fast protein liquid chromatography (FPLC) fractions was evaluated using the Lowry method (Lowry & Randall, 1951).

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

The samples were analyzed by SDS-PAGE according to Laemmli (1970), using a 4% stacking gel and a 12% resolving gel. Freeze-dried samples were solubilized in sample buffer (0.0625 M Tris, 2% SDS, 10% glycerol). Low molecular weight markers (GE HealthCare, USA) were used: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The gels were run at constant current (30 mA per gel, maximum voltage 200 V) at 20 °C. Subsequently, they were stained with Coomassie Brilliant Blue for 2 h and then decolored (30% ethanol, 10% acetic acid). Finally, silver staining was used to increase detection sensitivity (Blum, Beier, & Gross, 1987).

2.7. Fast protein liquid chromatography (FPLC)

Soluble fractions were analyzed by FPLC using an AKTA Purifier (GE Healthcare, Sweden). Two different molecular exclusion columns were used: Superdex 75 10/300 GL column (exclusion limit: 100 kDa, GE Healthcare) was calibrated with blue dextran for exclusion volume (V_0

= 7.3 mL), albumin (67 kDa), ovalbumin (44 kDa), chymotrypsin (25 kDa), ribonuclease (19 kDa) and aprotinin (6.5 kDa); and Superdex Peptide 10/300 GL column (exclusion limit: 10 kDa, GE Healthcare) was calibrated with blue dextran (V_0 = 7.6 mL), aprotinin (6500 Da), vitamin B_{12} (1855 Da) and hippuric acid (179 Da). For both columns, 500 μL of soluble samples were loaded and eluted with PBS buffer (pH = 7.4) at 0.4 mL/min. Polypeptides and peptides were detected by absorbance at 210 nm. Thirty-six fractions corresponding to MW < 10 kDa from digested samples (VD and HD) were automatically collected.

2.8. Antioxidant activity determination

2.8.1. Oxygen radical absorbance capacity (ORAC)

The ORAC was determined according to Ou, Hampsch-Woodill, and Prior (2001) with modifications (Orsini Delgado, Galleano, Añón, & Tironi, 2015). Fluorescein (53.3 nmol/L, 150 μL) was mixed with 25 μL of sample, PBS buffer (negative control) or Trolox and pre-incubated (37 °C, 15 min). Then, AAPH (160 mmol/L, 25 μL) was added and the reaction mixture was incubated (37 °C, 45 min). Fluorescence intensity (λ_{exc}: 485 nm; λ_{em}: 535 nm) was monitored every minute for 45 min by a microplate reader (Synergy HT–SIAFRT, Biotek Instruments, USA). The area under the fluorescence decay curves was obtained. Scavenging percentage was calculated according to equation (3).

$$ROO\bullet\ scavenging\ (\%) = \frac{AUC_S - AUC_{NC}}{AUC_B - AUC_{NC}} \times 100 \quad (3)$$

where AUC_S, AUC_B, and AUC_{NC} are the area under the curve of the sample, the blank (without AAPH) and the negative control, respectively. The *ROO•* scavenging percentage was plotted versus the protein concentration of soluble fractions to obtain mean scavenging concentrations (SC_{50}). A standard curve was prepared using Trolox (6.25–75 μmol/L). The results were expressed as μmol Trolox equivalents (TE)/g of protein and as SC_{50} values.

2.8.2. Hydroxyl radical antioxidant capacity (HORAC)

The HORAC activity was determined as described by Ou et al. (2002) and Moore, Yin, and Yu (2006) with slight modifications (Orsini et al., 2015). A 20 μL of sample, PBS buffer (negative control) or chlorogenic acid was mixed with fluorescein (60.3 mmol/L, 190 μL), H_2O_2 (0.75 mmol/L, 15 μL) and cobalt solution (0.2 g/L picolinic acid, 0.22 g/L $CoCl_2{\cdot}6H_2O$, 75 μL). The reaction was carried out at 37 °C. Fluorescence intensity (λ_{exc}: 485 nm, λ_{em}: 535 nm) was read every minute for 3 h. The *•OH* scavenging percentage was calculated according to equation (4).

$$\bullet OH\ scavenging\ (\%) = \frac{AUC_S - AUC_{NC}}{AUC_B - AUC_{NC}} \times 100 \quad (4)$$

where AUC_S, AUC_B, and AUC_{NC} are the area under the curve of the sample, the blank (without cobalt-hydrogen peroxide), and the negative control. The *•OH* scavenging percentage was plotted versus the protein concentration of soluble fractions to obtain SC_{50} values. Chlorogenic acid (50–500 μg/mL) was used as a reference compound. The HORAC value was expressed as μg chlorogenic acid equivalents (CAE)/g of protein and as SC_{50} values.

2.8.3. ABTS radical cation

This method was performed according to Re et al. (1999). For this, $ABTS^{\bullet+}$ stock solution was generated by mixing ABTS (7 mmol/L) and potassium persulphate (2.45 mmol/L) and incubated for 16 h in the dark at room temperature. The stock solution was diluted in PBS buffer (pH 7.4) to obtain an $ABTS^{\bullet+}$ solution with an absorbance of 0.70 ± 0.02 at 732 nm. Sample or Trolox (100 μL) was mixed with $ABTS^{\bullet+}$ solution (1 mL) for 1 h in the dark. The $ABTS^{\bullet+}$ scavenging activity was measured after reading absorbance at 732 nm. A standard curve was prepared using Trolox (0–250 μmol/L). The results were expressed as μmol TE/g of protein.

2.8.4. Ferric reducing antioxidant power (FRAP)

The FRAP activity was determined according to Pulido, Bravo, and Saura-Calixto (2000) with some modifications. The FRAP reagent was prepared freshly (containing TPTZ, FeCl3 and an acetate buffer) and stored at 37 °C. Sample or Trolox (30 μL) was mixed with FRAP reagent (900 μL) and H_2O (90 μL). The mixture was incubated in the absence of light at 37 °C for 30 min. Absorbance was measured at 595 nm. The results were expressed as μmol TE/g of protein based on a Trolox standard curve (0–350 μmol/L).

2.9. ACE inhibitory activity determination

The ACE inhibitory assay was performed as described by Terashima et al. (2010). Sample (10 μL) was pre-incubated with phosphate buffer (50 mmol/L, pH = 8.3, 25 μL) and ACE solution (100 mU/mL, 10 μL) for 10 min at 37 °C. A 25 μL of HHL solution (8.3 mmol/L HHL, 133 mmol/L KH_2PO_4 and 500 mmol/L NaCl) was added. The reaction was carried out at 37 °C for 30 min and terminated by adding HCl (1 mol/L, 70 μL). Then, 20 μL of the reaction mixture was injected into a HPLC system (Shimadzu, Japan) equipped with a hydrophobic column (Hypersil Gold Thermo 4.6 × 250 mm) by using an isocratic mobile phase (80:20 water-acetonitrile, 0.1% (*v/v*) trifluoroacetic acid) at a flow rate of 1.0 mL/min. Hippuric acid (HA) formed by ACE reaction and the unreacted HHL were detected at 228 nm. The ACE inhibition percentage was calculated according to equation (5).

$$ACE\ Inhibition\ (\%) = \frac{A - B}{A - C} \times 100 \quad (5)$$

where A, B, and C are the peak area of the blank (HA generated without ACE inhibitors), the sample (HA generated in the presence of ACE inhibitor component) and the control (HA generated without ACE, corresponding to HHL autolysis during the enzymatic assay), respectively. The results were expressed as IC_{50} values (indicating 50% ACE inhibition).

2.10. Cytotoxicity and absorption evaluation through Caco-2/TC7 cells (García Fillería & Tironi, 2021)

2.10.1. Cell culture

Caco-2/TC7 human colon adenocarcinoma cells (passages 39–40) from the American Type Culture Collection (ATCC, Maryland, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% (*v/v*) fetal bovine serum (FBS), 1% (*v/v*) of non-essential amino acids and 1% (*v/v*) of antibiotic solution (penicillin-streptomycin). The cells were incubated in a humidified incubator (37 °C, 5% CO_2) until they reached confluence (about 7 days). Then, cells were trypsinized and resuspended in DMEM with 15% of FBS. Total number of cells per mL was counted on an inverted microscope using a hemocytometer. Finally, according to the analysis to be performed, the cells were diluted to the desired concentration.

2.10.2. Cytotoxicity

Cell viability was evaluated by measuring lactate dehydrogenase (LDH) activity. A commercial kit (LDH-P UV unitest, Wiener Lab, Argentina) was used. Caco-2/TC7 cells were seeded in 96-well plates at a density of 2.5 × 10^4 cells/well. Cells were gently washed with PBS buffer, treated with 100 μL of samples (0.5–5 mg protein/mL) or DMEM with 3% (*v/v*) Triton X-100 (positive control for cell death) and incubated (30 °C, 3 h). Supernatant (2.5 μL) was mixed with 150 μL of Reagent A (0.6 mmol/L pyruvate, 0.18 mmol/L NADH). The absorbance (λ: 340 nm) was read every 30 s for 3 min. The activity of LDH (U/L) was expressed as the change of absorbance per min (ΔAbs/min) by calculating the average of the difference in absorbance per minute which was multiplied by the correction factor (f = 9.683). Cytotoxicity was determined as the ratio between LDH activity in sample-treated cells and LDH

activity in the positive control.

2.10.3. Caco-2/TC7 cell monolayers

Cells (200 μL) from confluent cultures were seeded in polycarbonate inserts (12 mm diameter, 0.4 μm pore size, Millipore, USA) at a density of 1×10^5 cells/cm^2 (apical compartments) and 600 μL of DMEM were added to the basolateral compartments. Cells were incubated (37 °C, 5% CO_2) and the culture medium of both compartments was changed every two days for 21 days. The transepithelial electrical resistance (TEER, Ω. cm^2) was measured at each change of medium, using a Millicell ERS-2 voltohmmeter (Millipore Co., MA, USA). Once monolayers were formed (constant TEER), absorption test was carried out.

2.10.4. Absorption assay across Caco-2/TC7 cell monolayers

For absorption test, PBS buffer (400 μL) was added to the apical and basolateral compartments and incubated (37 °C, 5% CO_2, 20 min). Then, PBS buffer was removed, 400 μL of the sample were seeded in the apical side and 400 μL of PBS was placed in the basolateral side to be incubated (37 °C, 5% CO_2, 3 h). Samples from both compartments were taken and analyzed in a HPLC system (Waters, USA) equipped with an analytical column (Phenosphere Next C18, 4.6 × 250 mm, 5 μm) and a diode array detector. The sample was eluted with a linear gradient where solvent A was water-acetonitrile (98:2) containing 0.065% (*v/v*) of trifluoroacetic acid and solvent B was water-acetonitrile (35:65) containing 0.065% (*v/v*) of trifluoroacetic acid at a flow rate of 1.1 mL/min (0–100% for 55 min). Elution profile was monitored at 210 and 280 nm.

2.11. Statistical analysis

Measurements were carried out in triplicate for each sample in all the experiments and results were expressed as mean ± standard deviation. The data were analyzed through an analysis of variance (ANOVA). Significant differences ($P < 0.05$) between mean values were evaluated by the Tukey's HSD test (Statgraphics® Centurion XVI, Statgraphics Technologies Inc., USA).

3. Results and discussion

3.1. Characterization of samples

Protein content of the samples is shown in Table 1. There were no significant differences between V and H protein percentages ($P > 0.05$) that were consistent with those reported for both fish viscera (21–65%) (Villamil et al., 2017) and fish viscera hydrolysates (42–88%) (Sepúlveda et al., 2021; Taheri, Anvar, Ahari, & Fogliano, 2013). A significant decrease in protein content ($P < 0.05$) was found after the SGID of V and H, which can be explained through the dilution effect exerted by the components of gastrointestinal fluids. After SGID, the results showed a significant increase ($P < 0.05$) in the DH of V and H (Table 1). The DH of VD showed a 2.7-fold increase compared to V ($P < 0.05$) reflected in the average peptide chain length (PCL = 100/DH, Adler-Nissen, 1986) varying from approximately 10 amino acids in V to a chain of 4 amino acids in VD (Table 1). Similarly, the DH of HD increased ($P < 0.05$) 1.2-fold compared to H. This is due to the action of pepsin (an endopeptidase) and pancreatin (multiple gastrointestinal enzymes including trypsin, elastase and chymotrypsin) which work together to increase the efficiency in cleavage of the peptide bonds (Ketnawa, Wickramathilaka, & Liceaga, 2018). However, the DH reached by VD was significantly lower than that reached by HD and H ($P < 0.05$). Thus, alcalase was more effective than digestive enzymes in hydrolyzing V, which produced a higher DH for H compared to VD. Furthermore, a prior alcalase hydrolysis led to greater availability of the resulting polypeptides for hydrolysis by digestive enzymes. This was shown by the greater DH of HD compared to VD.

Table 1
Protein content (%), degree of hydrolysis (DH) and average peptide chain length (PCL) of V, H, and their freeze-dried digests.

Sample	Protein (%)	DH (%)	PCL
V	64.8 ± 0.8 [a]	10.5 ± 1.8 [a]	9.7 ± 1.9 [a]
VD	56.8 ± 1.5 [b]	28.5 ± 4.3 [b]	3.9 ± 0.1 [b]
H	63.2 ± 1.7 [a]	44.8 ± 2.5 [c]	2.2 ± 0.1 [c]
HD	47.9 ± 0.8 [c]	55.5 ± 4.3 [d]	1.8 ± 0.1 [c,d]

Mean ± standard deviation (n = 3).
[a-d] Different letters in the same column indicate significant differences ($P < 0.05$).

3.2. Molecular weight (MW) distribution

Electrophoretic profiles of the samples are shown in Fig. 1. The profile of V (lane 1) showed bands of high MW (≥97 kDa) and low MW (<14 kDa). The reduction of V with β-mercaptoethanol (lane 2) showed bands with a MW of 93 kDa and 31 kDa as well as some faint bands between 25 and 20 kDa. In both profiles, it was possible to observe that some bands did not enter the gels, which indicates the presence of high MW proteins. Due to the diversity of organs and tissues (smooth muscle and connective tissue) that constitutes the viscera, different types of proteins can be found, such as collagen type III, elastin, glycoproteins, calmodulin, enzymes, myosin, myomesin (Gehring, Davenport, & Jaczynski, 2009; Khan et al., 2020). Moreover, after SGID of V, the profile without reducing agent showed a decrease in the intensity of all bands (VD, lane 3). No bands were detected in the case of H (lane 4) and HD (lane 5). Therefore, alcalase hydrolysis could probably generate peptides with MW lower than the detection limit of the gel (14.4 kDa) since this enzyme is an endopeptidase with broader specificity to break peptide bonds and so to produce short chain peptides (Abdelhedi & Nasri, 2019).

Molecular composition of soluble samples according to the chromatographic profiles (FPLC) are shown in Fig. 2. All the samples presented five fractions (Fig. 2A). Both alcalase hydrolysis and SGID of V decreased molecules between 60.6 kDa and 13.0 kDa (between fractions 1 and 2) but increased those lower than 6.51 kDa (fraction 2, 3 and 4). However, the increase was more significant in H and HD than in VD. As mentioned above, this is due to the tendency of alcalase to produce smaller peptides. On the contrary, digestive enzymes (pepsin, trypsin and chymotrypsin) have narrower specificity for amino acid residues (Wang et al., 2021). No substantial differences were observed between the chromatograms of H and HD, although a slight increase in species between 2.41 kDa and 0.93 kDa (fractions 3 and 4) was found. Fig. 2B shows that alcalase hydrolysis and SGID produced an increase in the molecules smaller than 1.86 kDa. There were no significant differences between H and HD; however, there was a shift in the range of fraction 3 from 0.47 to 0.16 kDa in H to 0.36–0.18 kDa in HD. Some studies have reported that small peptides can have better biological activities and a greater probability of crossing the gastrointestinal membrane (Abdelhedi & Nasri, 2019).

3.3. Antioxidant activity

Antioxidant activity of the samples are presented in Table 2. The results of ABTS and FRAP assays increased ($P < 0.05$) after alcalase hydrolysis (H versus V). Both were also increased after SGID whose effect was more significant for V than for H, since the results of ABTS and FRAP assays of VD were 152% and 197% higher than V activities, respectively. However, the antioxidant capacity increased approximately 9.46% and 20.2% when comparing the activities of HD with respect to those of H. Nonetheless, the highest values of ABTS and FRAP assays were found in HD peptides ($P < 0.05$). These values are slightly higher than those previously obtained for rainbow trout by-product hydrolysates for ABTS and FRAP assays (1200 μmol TE/g and 200 μmol TE/g, respectively) (Nikoo et al., 2019).

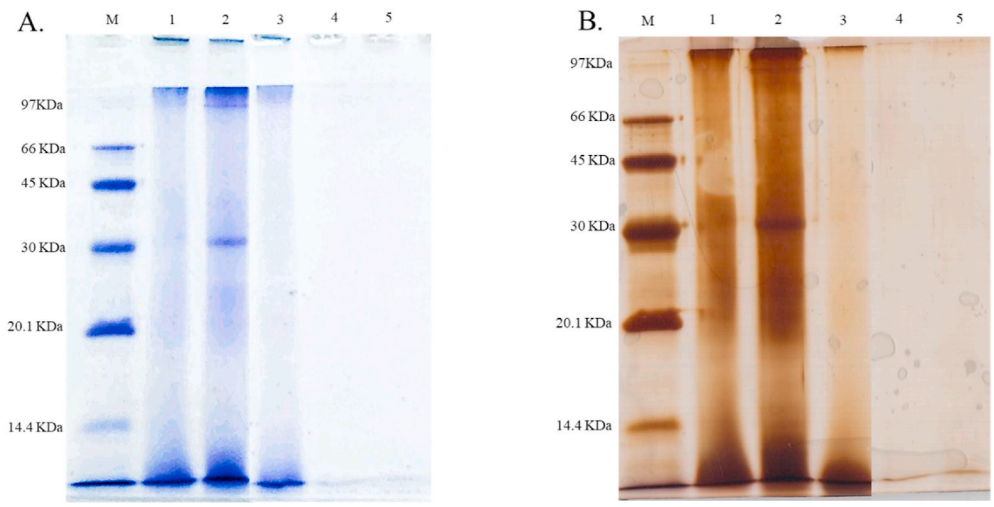

Fig. 1. SDS-PAGE Electrophoresis stained with: A) Coomassie Brilliant Blue; B) Silver. (1). V; (2). V reduced with β-mercaptoethanol; (3). VD; (4). H; (5). HD; (M) PM marker. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

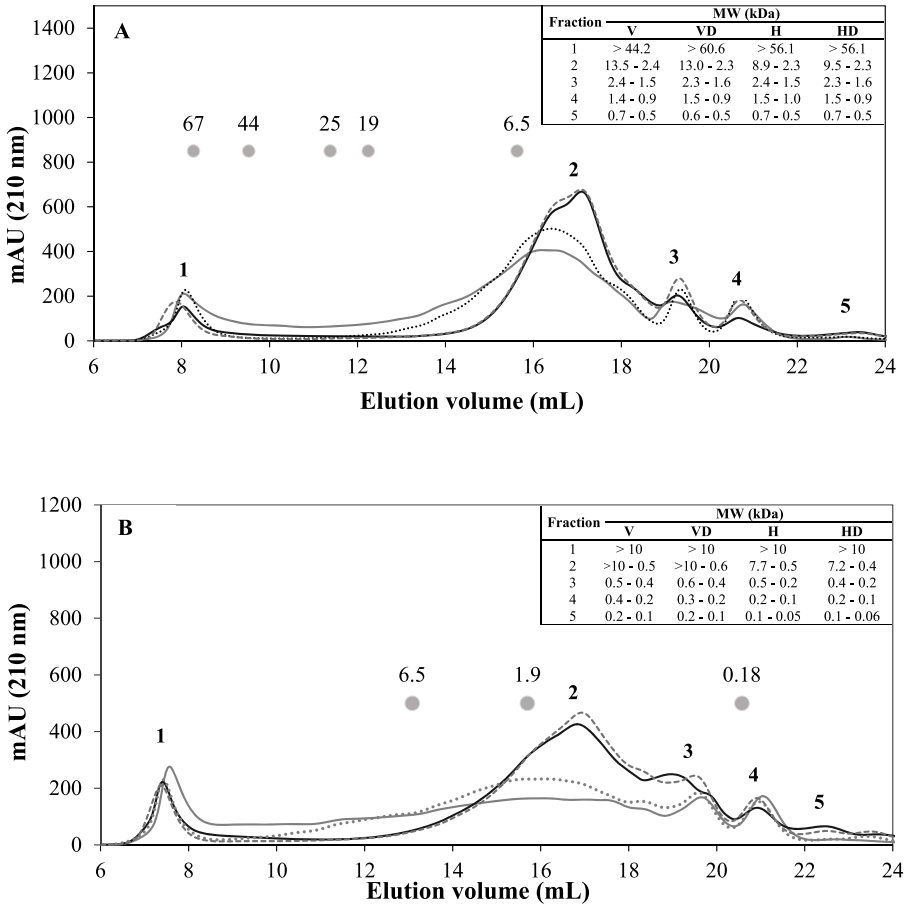

Fig. 2. FPLC chromatograms of V (—), VD (···), H (—) and HD (——) at 1 mg/mL: A) on Superdex 75 10/300 GL column; B) on Superdex peptide 10/300 GL column. MW of markers in kDa (•).

The ORAC activity of V (antioxidant capacity against *ROO•*) significantly increased ($P < 0.05$) after alcalase hydrolysis. Even peptides in H were not affected by SGID since there were no significant differences between H and HD ($P > 0.05$). However, SGID of V caused an increase in ORAC activity ($P < 0.05$) since SC_{50} value of VD was about 35.1% lower than that of V. The ORAC values of VD, H and HD are slightly lower than those of red tilapia viscera hydrolysate (1554 ± 40 μmol TE/g protein) (Gómez et al., 2019). The HORAC assay, which evaluates the capacity to prevent the formation of •OH mainly by metal chelation, showed that HD exhibited the highest activity ($P < 0.05$). Therefore, the SGID of H increased its HORAC activity. Regarding V, neither alcalase hydrolysis nor SGID increased this activity.

These results show that SGID resulted in the release of V and H peptides capable of neutralizing $ABTS^{\bullet+}$, scavenging *ROO•* and

Table 2
Antioxidant and angiotensin I-converting enzyme (ACE) inhibitory activities of rainbow trout viscera and its hydrolysates (digested and undigested).

Sample	Antioxidant activity					ACE inhibitory IC_{50} (mg/mL)
	FRAP (μmol TE/g)	ABTS (μmol TE/g)	HORAC SC_{50} (mg/mL)	ORAC (μmol TE/g)	ORAC SC_{50} (mg/mL)	
V	70 ± 2[a]	544 ± 38[a]	5.21 ± 0.42[a,b]	671 ± 45[a]	0.057 ± 0.003[a]	5.29 ± 0.06 [a]
VD	207 ± 2[b]	1374 ± 8[b]	5.28 ± 0.05[a]	1395 ± 11[b]	0.037 ± 0.001[b]	2.50 ± 0.06 [b]
H	269 ± 4[c]	1469 ± 66[b]	4.60 ± 0.23[b]	1513 ± 12[c]	0.026 ± 0.002[c]	1.38 ± 0.03 [c]
HD	323 ± 3[d]	1608 ± 27[c]	3.21 ± 0.15[c]	1464 ± 37[b,c]	0.031 ± 0.003[b,c]	1.49 ± 0.05 [c]

Values are given as mean ± SD from triplicate measures.
[a-d] Different letters in the column indicate significant differences ($P < 0.05$).

reducing ferric ions. However, only peptides in HD had a higher capacity to prevent •OH formation. This could be related to the release of new smaller peptides as indicated by DH values (Tables 1 and 24.0% higher for HD compared to H) and polypeptide population (Fig. 2). The results also demonstrate a great resistance of antioxidant peptides in H to SGID and an improved antioxidant capacity of V through prior alcalase hydrolysis. These observations are consistent with several investigations carried out on proteins from fish by-products (Ketnawa et al., 2018; Oliveira et al., 2019).

3.4. Angiotensin I-converting enzyme (ACE) inhibitory activity

The ACE inhibitory activity of the samples are presented in Table 2. Viscera (V) showed the highest IC_{50} value ($P < 0.05$) and therefore the lowest ACE inhibitory activity. The IC_{50} value of VD was significantly ($P < 0.05$) lower (about 53.7%) than that of V, indicating that potential antihypertensive peptides can be generated during gastrointestinal digestion of V. However, the IC_{50} value of VD was higher than the values found for H and HD ($P < 0.05$). These suggest that previous alcalase hydrolysis could have improved ACE inhibitory activity of V. Although no significant differences were found between IC_{50} values of H and HD, it can be inferred that ACE inhibitory peptides of H are resistant to SGID or that other peptides with similar activity could have been generated during digestion. A similar effect of SGID has been reported on ACE inhibitory peptides from hydrolysates of fish by-products (Martínez-Alvarez, Batista, Ramos, & Montero, 2016; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017). The IC_{50} values of H and HD are comparable to those reported for hydrolysates from basa skin (Zhang, Ma, & Otte, 2016), carp muscle (Elavarasan, Shamasundar, Badii, & Howell, 2016) and sardine heads (Martínez-Alvarez et al., 2016) with IC_{50} values of 1.42; 1.53 and 1.16 mg/mL, respectively.

3.5. Fractionation of VD and HD

Fig. 3A shows molecular weights of VD and HD fractions according to the chromatographic profiles (FPLC) and Fig. 3B shows FPLC fractions with their corresponding peptide concentrations. The VD fractions with MW between 7.14 and 0.55 kDa (fractions 14–24) and 0.17–0.09 kDa (fractions 30 and 32) showed a higher peptide concentration while HD fractions showed a higher concentration between 2.81 and 0.34 kDa (fractions 18–26) and 0.20–0.11 kDa (fractions 29 and 31).

The ORAC and HORAC activities of each fraction was evaluated to determine in which MW range the most active peptides were found. The highest ORAC activity of VD and HD fractions (Fig. 3C) corresponded to molecules with MW of 1.89–0.11 kDa (fractions 20 to 31) ranging between 53.1 ± 1.9 and 68.3 ± 0.9 μmol TE/L for VD, and between 59.0 ± 1.1 and 76.8 ± 1.4 μmol TE/L for HD. However, HD fractions showed slightly higher activities compared to VD fractions. Higher activities found in fractions 25 to 28 of both digests could not be associated with higher peptide concentrations. Their activities may be related to the presence of more potent molecules. Nevertheless, identifying peptides of the fractions would be necessary to confirm this. Based on HORAC assay results (Fig. 3D), it was possible to observe that HD fractions between 1 and 22 showed a higher activity than the corresponding VD fractions; however, activity values tended to be similar for both digests after fraction 23. Considering the above results, two fractions with high antioxidant activity were selected from VD (fractions 28 and 31) and HD (fractions 23 and 28) to be subsequently evaluated through intestinal absorption tests with Caco-2/TC7 cells.

3.6. Cytotoxicity analysis

A cytotoxicity test was carried out on differentiated cells that were treated at different concentrations of VD and HD (0.50–5.00 mg/mL). Both VD and HD showed concentration-dependent effects on membrane integrity with percentages of LDH released between 61.3 – 8.4% and 59.8–9.4%, respectively. Both digests exerted a minimal damaging effect on cell membrane at concentrations below 0.50 mg/mL. Likewise, cytotoxicity was evaluated at a single concentration of VD-28 (0.13 mg/mL), VD-31 (0.56 mg/mL), HD-23 (0.88 mg/mL) and HD-28 (0.23 mg/mL). However, VD and HD fractions were not cytotoxic in the analyzed concentrations with LDH percentages lower than 5%. Similarly, Gómez et al. (2019) found that hydrolysates of red tilapia (*Oreochromis* spp.) viscera did not have cytotoxic effects on Caco-2 cells at concentrations between 0.025 and 0.5 mg/mL. Correspondingly, Wiriyaphan, Xiao, Decker, and Yongsawatdigul (2015) reported that hydrolysates of threadfin bream (*Nemipterus* spp.) by-products did not show any cytotoxic effects on Caco-2 cells at concentrations below 0.20 mg/mL.

3.7. Simulation of intestinal absorption across Caco-2/TC7 cell monolayers

Peptide profiles obtained for each sample before and after simulated intestinal absorption are presented in Fig. 4. The VD profile (Fig. 4A) showed six peaks: three at t_R less than 5 min (more hydrophilic molecules), one at $t_R = 13.5$ min and finally two with the most hydrophobic molecules, which was identical to the apical compartment profile. Such similarity indicated that the molecules in VD resisted the action of intestinal peptidases. However, in the basolateral compartment profile, it was observed that only the most hydrophilic molecules were able to penetrate the monolayer, suggesting that only some peptides crossed the membrane in a detectable amount. In VD-28 profiles (Fig. 4B), the original fraction and the apical compartment were similar with several peaks with different hydrophilic/hydrophobic characteristics. In addition, these peaks had less intensity in the basolateral compartment profile, suggesting that molecules in VD-28 resisted the action of intestinal peptidases and were able to cross the monolayer in a considerable proportion. In the case of VD-31 (Fig. 4C), the molecules underwent modifications by brush-border peptidases since changes in the apical compartment profile were detected and a proportion of the resulting molecules managed to cross the monolayer (basolateral compartment).

The HD profile (Fig. 4D) had six well-resolved peaks. The first three represented the most hydrophilic molecules. The fourth peak occurred with a t_R of 10.9 min and the last two (hydrophobic molecules) with t_R greater than 19.1 min. The profiles of the apical and basolateral compartments were like that of HD and so they had the same number of peaks with their corresponding t_R but showed less intensity in the basolateral compartment profile. This suggests that molecules with different hydrophilic/hydrophobic characteristics in HD resisted the action of intestinal peptidases and were able to cross the monolayer in significant amounts. In HD-23 profiles (Fig. 4E), original sample had only two peaks with t_R less than 5 min that were preserved in the apical side. However, several small peaks with t_R greater than 20 min appeared

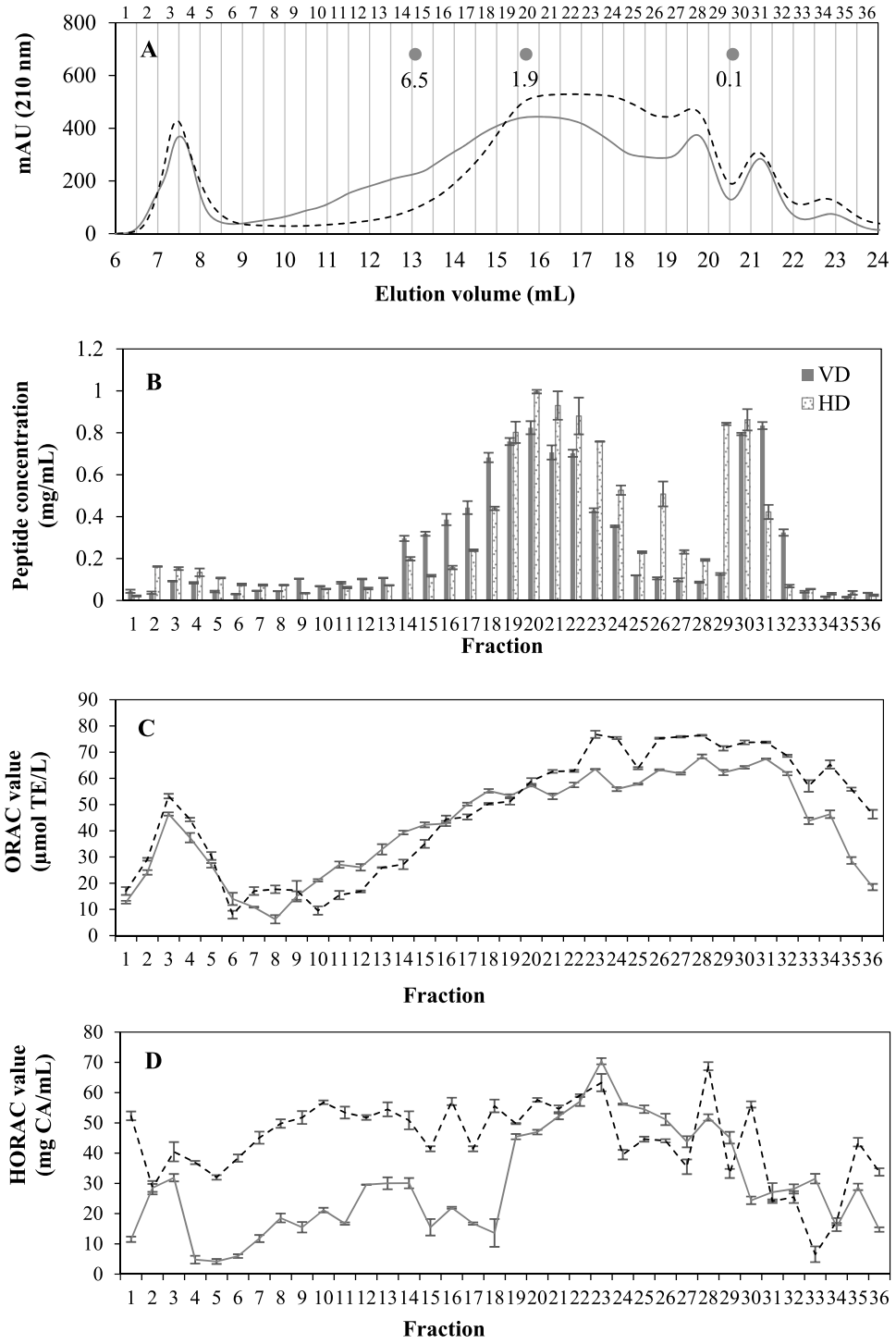

Fig. 3. A) FPLC chromatograms of VD (—) and HD (——) at 1 mg/mL, on Superdex peptide 10/300 GL column. Molecular weight of markers in kDa (•); B) Peptide concentration of fractions from VD and HD; C) ORAC activities of VD (—) and HD (——) fractions; D) HORAC activities of VD (—) and HD (——) fractions. Bars represent standard deviation.

in the same compartment and they were repeated in the basolateral compartment but with less intensity. Similarly, HD-28 profile (Fig. 4F) showed two peaks at t_R less than 5 min. Nevertheless, the apical compartment profile showed new peaks with molecules that had different hydrophilic/hydrophobic characteristics, most of which are repeated in the basolateral compartment. These results suggest that the molecules initially present in both HD fractions underwent the action of brush-border peptidases and a proportion of the resulting molecules managed to cross the monolayer into the basolateral compartment.

The results of VD and HD show that the previous alcalase hydrolysis of V could have improved intestinal absorption of SGID products since a greater passage through Caco-2/TC7 monolayers of the original components in HD was observed. However, it cannot be assured that no further modifications occurred during passage without identifying the peptides present in each compartment. Toopcham, Mes, Wichers, Roytrakul, and Yongsawatdigul (2017) found that SGID decreased ACE

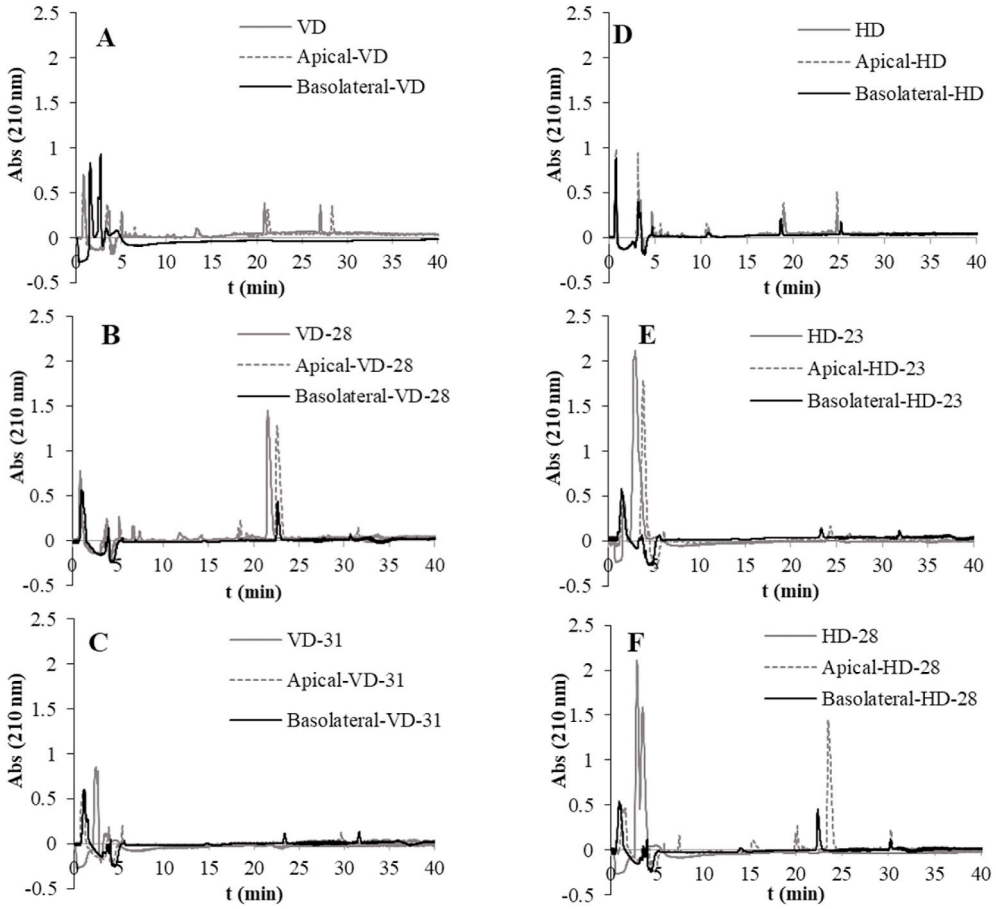

Fig. 4. RP-HPLC chromatograms corresponding to the absorption test: A) VD (0.25 mg/mL); B) VD-28 (0.13 mg/mL); C) VD-31 (0.56 mg/mL); D) HD (0.25 mg/mL); E) HD-23 (0.88 mg/mL); F) HD-28 (0.23 mg/mL).

inhibitory activity of peptides from tilapia muscle hydrolysate but improved the permeability of the peptides through Caco-2 cell monolayers. Similarly, Samaranayaka, Kitts, and Li-Chan (2010) reported that, after SGID of pacific hake muscle hydrolysate, peptide antioxidant capacity increased and the passage through Caco-2 cells improved.

4. Conclusions

This study provides valuable information about the potential of rainbow trout viscera as a source of bioactive peptides. The alcalase hydrolysis and SGID of V released peptides with antioxidant and ACE inhibitory activity. However, the hydrolysis of V with Alcalase® 2.4L before gastrointestinal digestion can improve the bioactivity and bioavailability of its proteins due to the possible release of new peptides with stronger biological effects and greater intestinal absorption. Likewise, bioactive peptides in H were stable after SGDI as well as able to penetrate at least partially Caco-2/TC7 cell monolayers, which indicates its possible intestinal absorption. Finally, these results suggest that rainbow trout viscera and their hydrolysates are a promising alternative as a source of compounds with antioxidant and ACE inhibitory activities. Therefore, they could be used as ingredients in functional food development and offer an alternative for reducing contamination associated with the disposal of such by-products.

CRediT authorship contribution statement

Priscilla Vásquez: Investigation, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **José E. Zapata:** Conceptualization, Methodology, Supervision, Formal analysis. **Verónica C. Chamorro:** Conceptualization, Methodology, Supervision, Formal analysis. **Susan F. García Fillería:** Investigation, Formal analysis. **Valeria A. Tironi:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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