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Hydrolyzates from *Pyropia columbina* seaweed have antiplatelet aggregation, antioxidant and ACE I inhibitory peptides which maintain bioactivity after simulated gastrointestinal digestion

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ABSTRACT

The aim of this work was to evaluate the bio-accessibility of bioactive peptides with ACE I inhibition, antioxidant and antiplatelet aggregation activity obtained by enzymatic hydrolysis of *Pyropia columbina* proteins. Two hydrolyzates were produced (A and AF). Bio-accesibility was determined using a gastrointestinal digestion (pepsin and pancreatin) and membrane dialysis system. Hydrolyzates had peptides with medium molecular weight (2300 Da), and Asp, Glu and Ala were the most abundant amino acids. Additionally, AF presented small peptides with 287 Da. Peptides from A showed the highest angiotensin-converting enzyme activity (ACE I) inhibition by uncompetitive mechanism (IC50%, 1.2 ± 0.1 g L^{-1}), and β-carotene bleaching inhibition. Peptides from AF presented the lower IC50% value for ABTS+• and DPPH radical inhibition, the highest copper-chelating activity (CCA), and antiplatelet aggregation activity. *In vitro* gastrointestinal digestion increased ABTS+• and DPPH scavenging and CCA of both hydrolyzates. Antiplatelet aggregation activity of A peptides was increased after simulated digestion process (≈157%). Peptides from both hydrolyzates were potentially bio-accessible, maintaining overall the bioactivity after gastrointestinal digestion.

1. Introduction

Macroalgae are harvested and used globally for many different applications in foods and as ingredients in cosmetic formulations and have considerable ecological and economic importance. Red, brown and green seaweeds are source of biologically active phytochemicals, which include carotenoids, phycobilins, fatty acids, sulphate polysaccharides, phenolic compounds and bioactive peptides among others (Mohamed, Hashim, & Rahman, 2012).

Bioactive peptides range in size from 2 to 20 amino acid residues. They are encrypted within the sequence of the parent protein and only become active when released either through (i) enzymatic hydrolysis by digestive enzymes, such as pepsin and trypsin, (ii) hydrolysis by microbial proteinases and peptidases during fermentation, or (iii) proteolysis by enzymes derived from microorganisms or plants or a combination of all the above (Harnedy & Fitzgerald, 2011). In addition to be a source of nitrogen and amino acids, bioactive peptides from algae have numerous potential physiological effects within the body, such as antihypertensive and immune-modulatory, among others. Regarding that, Suetsuna, Maekawa, and Chen (2004) characterized 10 kinds of dipeptides from *Undaria pinnatifida*; among them, four di-peptides (Tyr–His, Lys–Tyr, Phe–Tyr, and Ile–Tyr) significantly decreased blood pressure in spontaneously hypertensive rats. Similarly, oral administration of Val-Glu-Gly-Tyr peptide purified from *Chlorella ellipsoidea* hydrolyzates significantly decreased systolic blood pressure in spontaneously hypertensive rats (Ko et al., 2012). Morris et al. (2007) reported that oral administration of protein hydrolyzate from *Chlorella vulgaris* activates both, innate and specific immune responses, including a marked increase of

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lymphocyte pool, production of T cell-dependent antibody responses, and reconstitution of delayed-type hypersensitivity (DTH) responses in undernourished Balb/c mice.

Potential biological effect of peptides depends largely on its ability to remain intact after digestive process and reach the target organ. During digestion, peptides can be further digested by peptidases present in the stomach producing amino acids that can be absorbed in the intestinal mucosa (Segura-Campos, Chel-Guerrero, Betancur-Ancona, & Hernández-Escalante, 2011). The peptides that resist the digestive process and arrive intact in the intestine can have a local function or may be able to cross the epithelium, enter the blood stream, and have a systemic effect. Absorption of peptides by epithelial cells is achieved by receptor-mediated process. Apart from the peptide transporter route, peptides can be absorbed intact across the intestinal mucosa via other mechanisms, such as paracellular and transcellular transport, known as non-receptor-mediated process (Vermeirssen, Van Camp, & Verstraete, 2004). The paracellular route is found along the intestinal wall and is used as alternative pathway for peptide absorption (Stevenson et al., 1998). Transcellular route functions by absorbing peptides through the apical membrane brush border and moving them through the enterocyte to the basolateral membrane (Pappenheimer and Michel, 2013).

Simulation of physiological digestion is a very useful tool in evaluating *in vitro* bioactive peptide stability against digestive enzymes. However, there is no research about bioactive peptides bioaccessibility after *in vitro* gastrointestinal digestion. As far as we know, there is no literature related to bio-accessibility of *Pyropia columbina* bioactive peptides, resembling non-receptor-mediated process. Therefore, the aim of this work was to evaluate the bioaccessibility of bioactive peptides with ACE inhibition, antioxidant and antiplatelet aggregation activity obtained by enzymatic hydrolysis of *P. columbina* proteins using a gastrointestinal digestion and membrane dialysis system.

2. Materials and methods

2.1. Reagents

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The Alkaline protease (A) was provided by Danisco S.A. (Arroyito, Córdoba, República Argentina) and Flavourzyme (F) was obtained from Sigma Chemical Co. (USA).

2.2. Raw materials

One kilogram of different specimens of *P. columbina* was handpicked in Punta Maqueda (Comodoro Rivadavia, Argentina). The seaweed was processed according to Cian, Salgado, Drago, González, and Mauri (2014).

2.3. Preparation of protein extract (PE) and protein extract hydrolyzates (A and AF) from P. columbina

The red marine algae *P. columbina* was dispersed at 50 g kg^{-1} in distilled water for 2 h and filtered through a 50-mesh sieve (0.297 mm). The filtrate was then centrifuged at 3000 $\times g$ for 30 min at 4 °C and the supernatant was concentrated using Buchii RII rotary evaporator at low pressure and 40 °C. The concentrated (protein extract, PE) was used as substrate for enzymatic hydrolysis.

Hydrolyzates were obtained using 800 mL batch thermostatic reactor. The reaction pH was continuously measured using pH-meter IQ Scientific Instruments, and adjusted by adding base (NaOH, 2 mol L^{-1}) or acid (HCl, 2 mol L^{-1}) with a burette. Substrate concentration was 1.4 g 100 g^{-1} dispersion. Working conditions were: temperature 55 °C, pH 9.5, enzyme/substrate (E/S) ratio 0.4 g 100 g^{-1} and temperature 55 °C, pH 7.0, enzyme/substrate (E/S) ratio 5 g 100 g^{-1}, for A and F respectively. Once the hydrolysis was finished, the enzymes were inactivated by thermal treatment following the manufacturer guidelines and the hydrolyzates were centrifuged at 2000 $\times g$ for 15 min at 4 °C. The supernatant obtained were lyophilized. PE hydrolyzates were prepared using the following systems:

- Simple hydrolysis system: Hydrolysate A (hydrolysis with A enzyme during 2 h).
- Sequential hydrolysis system: Hydrolysate AF (hydrolysis with A enzyme 2 h + hydrolysis with F enzyme during 4 h; total reaction time, 6 h).

Free amino groups were measured using o-phthaldialdehyde, according to Nielsen, Petersen, and Dambmann (2001), and the degree of hydrolysis (DH) was calculated as:

$$DH = [(h - h_0)/h_{tot}] \times 100$$

where, h_{tot} is the total number of peptide bonds in the protein substrate (8.6 mEq g^{-1} protein); h is the number of peptide bonds cleaved during hydrolysis, and h_0 is the content of free amino groups of substrate.

2.4. Characterization of PE and PE hydrolyzates

2.4.1. Chemical composition

The chemical composition was determined using the AOAC (1995) procedures. Proteins were determined by the Kjeldahl method (N × 6.25). The total carbohydrates were determined according to Dubois, Gillis, Hamilton, Rebers, and Smith (1956). A standard curve with glucose solutions (0–100 mg L^{-1}), was used for calibration. Results were expressed as mg glucose equivalent 100 g^{-1} solid.

Free phenolic compound content (FPC) was quantified according to Schanderl (1970) with modifications, using Folin–Ciocalteu reagent. A standard curve with gallic acid solutions (0–100 mg L^{-1}), was used for calibration. Total phenolic compound content (TPC) was quantified according to Tarola, Van de Velde, Salvagni, and Preti (2013). In both cases, the results were expressed as mg galic acid equivalent g^{-1} solid.

Free amino groups content was determined as described above. Results were expressed as mEq L-Serine 100 g^{-1} solids.

2.4.2. Fast protein liquid chromatography (FPLC)

Gel filtration chromatography was carried out in an AKTA Prime system equipped with a Superdex 75 column (GE Life Sciences, Piscataway, NJ, USA). Injection volume was 100 μL (2.8 mg protein mL^{-1}) and elution was carried out using 50 mmol L^{-1} Potasium Phosphate Buffer pH 7.0 plus 150 mmol L^{-1} NaCl at 1 mL min^{-1}. Elution was monitored at 280 nm and molecular mass was estimated using molecular weight (MW) standards from Pharmacia: ferritine (440,000 Da), conalbumin (75,000 Da), carbonic anhydrase (29,000 Da), aprotinin (6500 Da) and glycine (75 Da).

2.4.3. Amino acid analysis

Samples (15 mg) were hydrolyzed with 1.5 mL of 6 mol L^{-1} HCl. The solutions were sealed in tubes under nitrogen and incubated in an oven at 110 °C for 24 h. Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by high-performance liquid chromatography (HPLC), according to the method of Alaiz, Navarro, Giron, and Vioque (1992), using D,L-α-

aminobutyric acid as internal standard. The HPLC system consisted of a Model Agilent 1100 Series with a VWD detector (Santa Clara, CA, United States) equipped with a 300 × 3.9 mm i.d. reversed-phase column (Novapack C18, 4 m; Waters). A binary gradient was used for elution with a flow of 0.9 ml min^{-1}. The solvents used were (A) sodium acetate (25 mmol L^{-1}) containing sodium azide (0.02 g 100 mL^{-1}) pH 6.0 and (B) acetonitrile. Elution was as follows: time 0.0–3.0 min, linear gradient from A/B (91/9) to A/B (86/14); 3.0–13.0 min, elution with A/B (86/14); 13.0–30.0 min, linear gradient from A/B (86/14) to A/B (69/31); 30.0–35.0 min, elution with A/B (69/31). Eluted amino acids are detected at 280 nm. The column was maintained at 18 °C. Tryptophan was determined by HPLC-RP chromatography after basic hydrolysis according to Yust et al. (2004).

2.5. Bioactive properties

2.5.1. Angiotensin-converting enzyme inhibition and inhibition mechanism

Angiotensin-converting enzyme (ACE I) activity inhibition was determined according to Hayakari, Kondo, and Izumi (1978). To determine the concentration causing an inhibition of 50% (IC50%) serial dilutions of A and AF from 0 to 13 g L^{-1} protein were made. To determine inhibition mechanism various substrate (HHL; N-Hippuryl-His-Leu) concentrations (3 mmol L^{-1}–9 mmol L^{-1}) were incubated with the ACE I solution with and without hydrolyzates at 13 g L^{-1} of protein. The kinetics of ACE in the presence of the inhibitory peptides from hydrolyzates extracts was determined using Lineweaver-Burk plots according to Ni, Li, Liu, and Hu (2012).

2.5.2. Antioxidant properties

2.5.2.1. Trolox equivalent antioxidant capacity (TEAC) and IC_{50}. To estimate the TEAC, a concentration–response curve for the absorbance at 734 nm for ABTS•+ (2,2′-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid)) as a function of concentration of standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solution (0–2.5 mmol L^{-1}) in 0.01 mmol L^{-1} (PBS, pH 7.4) was performed according to Cian, Martínez-Augustin, and Drago (2012). The absorbance reading was taken at 6 min after initial mixing and results were expressed as mmol Trolox g^{-1} of protein (TEAC). To determine the concentration causing an inhibition of 50% (IC50%) serial dilutions of PE and its hydrolyzates from 0 to 13 g L^{-1} protein were made.

2.5.2.2. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay and IC_{50}. DPPH radical scavenging activity assay was measured according to Brand-Williams, Cuvelier, and Berset (1995). DPPH scavenging activity from PE and its hydrolyzates was expressed as mg BHT equivalent g^{-1} protein, using a BHT (butylhydroxytoluene) standard solution (20–100 mg L^{-1}) in methanol. The absorbance at 517 nm was taken 90 min after initial mixing. To determine the concentration causing an inhibition of 50% (IC50%) serial dilutions of PE and its hydrolyzates from 0 to 13 g L^{-1} protein were made.

2.5.2.3. Copper-chelating activity by assay of β -carotene oxidation. Copper-chelating activity was determined by the assay of β-carotene oxidation according to Megías et al. (2008). The copper chelating activity (CCA) was calculated as follow and expressed as mg EDTA equivalent g^{-1} protein, using a concentration–response curve for the absorbance at 470 nm for CCA as a function of concentration of EDTA (ethylenediaminetetraacetic acid) standard solution (20–100 mg L^{-1}) in phosphate buffer.

$$\mathrm{CCA}(\%) = 100 - [(\mathrm{Abs\ control}(-) - \mathrm{Abs})/(\mathrm{Abs\ control}(-) - \mathrm{Abs\ control}(+)) \times 100]$$

where, Abs control (−) is the absorbance of negative control (200 μL β-carotene + 20 μL buffer phosphate); Abs control (+) is the absorbance of positive control (200 μL β-carotene + 10 μL $CuSO_4$ + 10 μL buffer phosphate); and Abs is the absorbance of sample (200 μL β-carotene + 10 μL $CuSO_4$ + 10 μL sample).

2.5.2.4. Reducing power activity assay. The reducing power activity (RP) of PE and its hydrolyzates was determined according to Oyaizu (1986). The absorbance was measured at 700 nm. The RP from PE and its hydrolyzates was expressed as μg ascorbic acid equivalent g^{-1} protein, using a standard curve with ascorbic acid solution (0–0.0564 μg mL^{-1}) in phosphate buffer.

2.5.2.5. β-carotene bleaching inhibition. The β-carotene bleaching inhibition of PE and its hydrolyzates were determined according to Yust, Millán, Alcaide, Millán and Pedroche (2012). One milliliter of 0.2 g L^{-1} β-carotene solution in chloroform was added to a mixture of 20 mg of linoleic acid and 200 mg of Tween 20. Subsequently, the chloroform was eliminated by evaporation and 50 mL of water rich in oxygen obtained by bubbling oxygen during 15 min was added. Aliquots of 2.5 mL of this mixture were added to Pirex tubes containing 100 μL of the sample. Samples were incubated at 50 °C, during 120 min and the absorbance at 470 nm was determined. The results were expressed as β-carotene bleaching inhibition (BCBI) as follows:

$$\mathrm{BCBI}(\%) = (\mathrm{Abs}_{120\mathrm{min}}/\mathrm{Abs}_{0\mathrm{min}}) \times 100$$

where, Abs_{120min} is the absorbance at 120 min of reaction and Abs_{0min} is the absorbance at 0 min of reaction.

2.5.3. Antiplatelet aggregation activity

Antiplatelet aggregation activity was determined according to Miyashita et al. (1999). This assay measures the peptide inhibition of human platelet aggregation. Blood from human volunteers (50 mL) was collected in Vacutainer® tubes with 4.5 ml of sodium citrate to prevent coagulation and then centrifuged at 1000 ×*g* for 15 min at room temperature to give platelet-rich plasma (PRP) as the supernatant. The platelet content from PRP was measured by blood cytometer (Sysmex KX-21) and adjusted with saline solution (154 mmol L^{-1} NaCl, pH 6.4) to 300 × 10^3 platelet μL^{-1}. Adjusted PRP (450 μL) was incubated with 10 μL of sample (60 g L^{-1} protein), which was dissolved in saline for 15 min at 37 °C. Platelet aggregation was induced by adding 10 μL of adenosine diphosphate (10 μmol L^{-1}) and results taken at 6 min of reaction. The increase in light transmission through PRP adjusted at 660 nm was obtained by aggregometer. A positive control, 450 μL PRP adjusted +10 μL Tirofiban® (4.6 μg mL^{-1}) + 10 μL of adenosine diphosphate, and a negative control, 450 μL adjusted PRP + 10 μL saline + 10 μL of adenosine diphosphate, were run. Antiplatelet aggregation activity was calculated as follow:

$$\text{Antiplatelet aggregation activity}(\%) = \left[\left(\mathrm{PAC}_{(-)} - \mathrm{PAS}\right)\Big/\left(\mathrm{PAC}_{(-)}\right)\right] \times 100$$

were, $PAC_{(-)}$ is platelet aggregation in negative control and PAS is platelet aggregation in sample system.

2.6. Bio-accessibility of bioactive peptides from hydrolyzates

Bioactive peptide dializability from A and AF was assayed using pepsin and pancreatin digestion. The hydrolyzates were dispersed at 8 g L^{-1} of protein. Aliquots (25 mL) of hydrolyzates dispersions were adjusted to pH 2.0 with 4 mol L^{-1} of HCl and after addition of 0.8 mL pepsin digestion mixture (16 g 100 mL^{-1} pepsin solution in 0.1 mol L^{-1} HCl), were incubated at 37 °C for 2 h in a shaking water bath. At the end of pepsin digestion, dialysis bags (cut off: 6–8 kDa) containing 20 mL 0.15 mol L^{-1} PIPES buffer were placed in each flask and were incubated for 50 min in a shaking water bath at 37 °C. Then, 6.25 mL of pancreatin solution (0.4 g 100 mL^{-1} pancreatin in 0.1 mol L^{-1} $NaHCO_3$) were added to each flask and the incubation continued for another 2 h. Then, dialysates from A and AF were weighed and named A_D and AF_D, respectively.

Free phenolic compounds (FPC) were determined as described above and dialyzability was calculated as follow: FPC dialyzability (%) = [(mg FPC dialysate/mg FPC sample) x 100]. Antioxidant, antihypertensive and antiplatelet aggregation properties of dialysates were evaluated as described above. TEAC, DPPH radical scavenging, CC y RP were expressed as: μmol Trolox, mg BHT, mg EDTA and μg ascorbic acid, respectively. BCBI, ACE inhibition and antiplatelet aggregation properties of dialysates were evaluated at the same protein concentration than PE, A and AF, and expressed as percentage.

2.7. Statistical analysis

All results were expressed as mean ± SD. The data were analyzed by one-way analysis of variance, using the software Statgraphics Plus 3.0. The statistical differences between samples were determined using the LSD (least significant difference) test. The significance was established at $P < 0.05$.

3. Results and discussion

3.1. Characterization of PE and its hydrolyzates

The protein content of PE was 36.4 ± 0.1 g 100 g^{-1} solid. It is well known that red seaweed have high protein level (Galland-Irmouli et al., 1999). In this sense, the crude protein content of *Porphyra* sp. is comparable with that of high-protein plant foods such as soybean (Norziah & Ching, 2000). On the other hand, the total carbohydrates content of PE was 20.9 ± 0.2 g 100 g^{-1} solid. This value was similar to that reported by Jiménez-Escrig, Gómez-Ordóñez, and Rupérez (2012) for the water extract of red seaweed *Nemalion helminthoides* (≈23 g 100 g^{-1} dw).

In the present study, PE was hydrolyzed by alkaline protease (A) or sequentially hydrolyzed by alkaline protease + Flavourzyme (AF), resulting in 13.7 ± 0.1% and 19.7 ± 1.2% degree hydrolysis (DH) for A and AF respectively. The higher DH of AF system is attributed to the addition of Flavourzyme enzyme, which is a mixture of endo- and exopeptidases. Both enzymes (endo- and exopeptidases) are able to hydrolyze proteins in a more efficient way than an endo-protease alone (Chang, Wu, & Chiang, 2007).

With respect to free amino groups (Fig. 1A), the content of PE was far from negligible and can be attributed to mycosporine-like amino acids (MAAs) naturally present in red seaweeds (Harnedy & Fitzgerald, 2011). As expected, A and AF had higher free amino group content than PE. This increase is due to proteolysis action of alkaline protease or/and Flavourzyme. Also, AF free amino group content was higher than that obtained for A. As mentioned before, Flavourzyme has exopeptidase activity, favoring the rapid release of free amino acids and di- and tri-peptides that contributes to increase free amino group (Chang et al., 2007).

Regarding free phenolic compound (FPC) (Fig. 1B), PE had lower content than A and AF (≈47%), indicating that proteolysis release phenolic compounds. As a result of protease treatment, peptides–polyphenol interactions decrease, and hence, polyphenol are easier extracted. In agreement with this result, no significant differences in total phenolic compounds (TPC) content from PE and its hydrolyzates were found (Fig. 1C), suggesting that PE has phenolic species conjugated with proteins are released after the proteolysis with both enzymatic system.

Table 1 shows amino acid profile of PE and its hydrolyzates. Glutamic acid, aspartic acid and alanine were the most abundant amino acids. For most seaweed, acidic amino acids constitute a large part of the amino acid fraction, representing between 22% and 44% of the total amino acids (Fleurence, 1999). The sum of acidic amino acids for PE, A and AF was 35.1, 32.4 and 32.5 g 100 g^{-1}, respectively. Similar results (31.4 g 100 g^{-1} of total amino acid), were obtained for other red seaweeds such as *Palmaria palmata*; (Galland-Irmouli et al., 1999). Alanine was present in high levels in PE, A and AF. Also, PE and its hydrolyzates have a relatively high content of proline, histidine and threonine.

Tyrosine, phenylalanine, valine, methionine, cysteine, isoleucine, tryptophan and leucine content of A was higher than that obtained for PE (ratio of each amino acid of A to PE: 2.2, 1.6, 1.5, 1.3, 1.4, 2.1 and 1.2, respectively). The ratio of AF to PE for the content of each amino acid is approximately 1.0. Therefore, AF amino acids profile was similar to that obtained for PE, except for Phe, Ile and Trp (1.9, 1.6 and 1.3, respectively).

FPLC gel filtration PE profile shows two main peaks (Fig. 2), having molecular weight (MW) higher than 175 kDa (*phycobiliproteins*) and 52.1 kDa. The first one could be attributed to R-phycoerythrin naturally presents in red seaweeds, which has a MW around 240 kDa (Denis, Massé, Fleurence, & Jaouen, 2009). The second peak corresponds to other proteins different than *phycobiliproteins*.

A and AF profiles shows effective protein degradation by enzymatic hydrolysis process of >175 and 52.1 kDa compounds present in PE generating intermediate MW species. This is evidenced in both hydrolyzates as a peak around 2300 Da. The second peak in AF hydrolyzate profile near to 287 Da probably corresponds to small peptides generated by Flavourzyme. Considering 120 Da the average MW of amino acids, this fraction could be form by di-peptides.

3.2. Bioactive properties

Table 2 shows the ACE inhibition results corresponding to PE and its hydrolyzates (A and AF). Hydrolyzates showed a relatively good antihypertensive activity (≈40%), which was higher than that obtained for PE. This increase respect to substrate (PE) may be due to peptides and phenolic compounds release during proteolysis. In this way, several studies have shown that peptides and phenolic compounds from seaweed have a potent ACE inhibitory activity (Sheih, Fang, & Wu, 2009).

As mentioned before, FPC of A and AF were higher than that obtained for PE (Fig. 1B), but no significant difference between A and AF was found. Therefore, the higher ACE inhibition activity of A can be attributed to the nature of peptides generated by alkaline protease. This result are in agreement with that reported by Sheih et al. (2009), who worked with different enzyme systems (pepsin, papain, Alcalase and Flavourzyme) and the green alga *Chlorella vulgaris* as substrate. In that work, the authors observed that the ACE inhibitory activity of peptides obtained by Alcalase was higher than that obtained with Flavourzyme.

As shown in Table 2, the IC50% value for ACE inhibition of A was lower than that obtained for AF. Therefore, this hydrolyzate exhibits

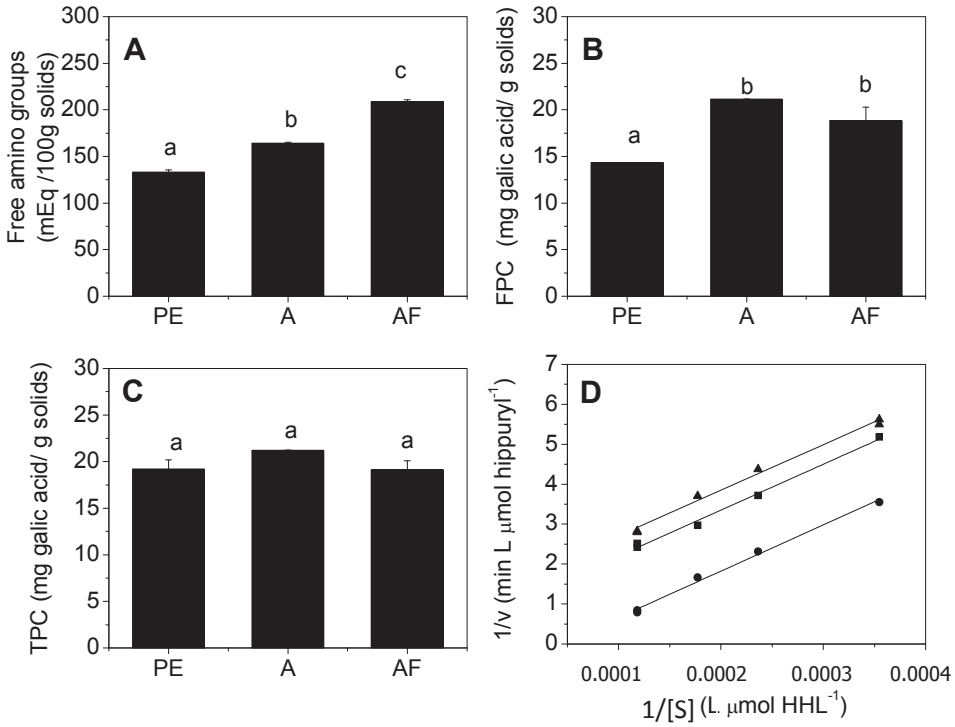

Fig. 1. Free amino group content (A), free phenolic compound content (FPC) (B), total phenolic compound content (TPC) (C), and Lineweaver-Burk plots inhibition of Angiotensin-converting enzyme (ACE I) in absence (●) or presence of A hydrolyzate (▲) or presence of AF hydrolyzate (■) (D). [S] is the substrate concentrations (N-Hippuryl-His-Leu or HHL; 3 mmol L^{-1}–9 mmol L^{-1}).

Table 1
Amino acid (AA) profile of protein extract (PE), protein extract hydrolyzates (A and AF) and the ratio of A and AF with PE profile.

Amino acids	Total amino acids (g 100 g^{-1} protein dw)			Ratio AA of A/PE	Ratio AA of AF/PE
	PE	A	AF		
Asp + Asn	15.5 ± 0.0[a]	14.8 ± 1.1[a]	15.3 ± 0.1[a]	1.0	1.0
Glu + Gln	17.6 ± 0.0[a]	17.6 ± 1.5[a]	17.2 ± 0.9[a]	1.0	1.0
Ser	2.5 ± 0.0[a]	2.3 ± 0.0[a]	2.2 ± 0.2[a]	0.9	0.9
His	9.7 ± 0.1[b]	7.2 ± 0.4[a]	9.1 ± 0.6[b]	0.7	0.9
Gly	2.6 ± 0.0[a]	2.2 ± 0.2[a]	2.2 ± 0.0[a]	0.9	1.0
Thr	7.7 ± 0.1[a]	7.0 ± 0.2[a]	7.1 ± 0.7[a]	0.9	0.9
Arg	4.7 ± 0.3[a]	4.2 ± 0.5[a]	4.3 ± 0.7[a]	0.9	0.9
Ala	15.3 ± 0.1[a]	15.1 ± 0.1[a]	16.0 ± 0.6[a]	1.0	1.0
Pro	8.6 ± 1.0[a]	8.5 ± 0.2[a]	8.0 ± 0.7[a]	1.0	0.9
Tyr	1.2 ± 0.0[a]	2.7 ± 0.2[b]	1.0 ± 0.1[a]	2.2	0.9
Phe	0.8 ± 0.0[a]	1.3 ± 0.1[b]	1.5 ± 0.1[b]	1.6	1.9
Val	2.6 ± 0.4[a]	4.0 ± 0.3[b]	3.2 ± 0.3[a]	1.5	1.2
Met	0.2 ± 0.0[a]	0.3 ± 0.0[b]	0.2 ± 0.0[a]	1.3	1.0
Cys	0.2 ± 0.0[a]	0.3 ± 0.0[b]	0.2 ± 0.0[a]	1.4	1.0
Ile	1.1 ± 0.0[a]	2.3 ± 0.1[c]	1.7 ± 0.0[b]	2.1	1.6
Trp	2.0 ± 0.0[a]	1.8 ± 0.2[a]	2.7 ± 0.11[b]	0.9	1.3
Leu	3.5 ± 0.1[a]	4.1 ± 0.0[b]	3.2 ± 0.1[a]	1.2	0.9
Lys	4.1 ± 0.2[a]	4.3 ± 0.3[a]	4.4 ± 0.3[a]	1.0	1.0

Mean ± SD (n = 3). dw (dry weight). Different letters in the same row mean significant differences between samples ($p < 0.05$).

the greatest potency (a lower amount is required to reach the 50% of ACE inhibition). These results match with those mentioned before and can be due to sequence of peptides generated by alkaline protease. It is important to note that serine proteases (such as alkaline protease) produce peptides whose C-terminals are amino acids with large side chains and no charge (aromatic and aliphatic amino acids), such as Ile, Leu, Vale, Met, Phe, Tyr and Trp (Segura Campos, Peralta González, Chel Guerrero, & Betancur Ancona, 2013). These amino acid residues at C-terminal position generally show high ACE inhibitory activity (Wijesekara, Qian, Ryu, Ngo, & Kim, 2011). Also, it was observed that ACE prefers substrates containing branched amino acid residues at the N-terminal position (Sato et al., 2002). Taking into account that the Ile, Leu, Val, Met and Tyr content of A was higher than that obtained for AF (Table 1), the high ACE inhibitory activity of this hydrolyzate could due to the presence of these amino acids at C and N-terminal position. Also, Flavourzyme action could hydrolyze active amino acids at C and N-terminal position from inhibitory peptides generated by alkaline protease, decreasing ACE inhibition activity and increasing AF IC50% value. In this sense, Segura-Campos, Chel-Guerrero, and Betancur-Ancona (2010) found that peptides obtained with Alcalase had lower IC50% than those generated with Flavourzyme.

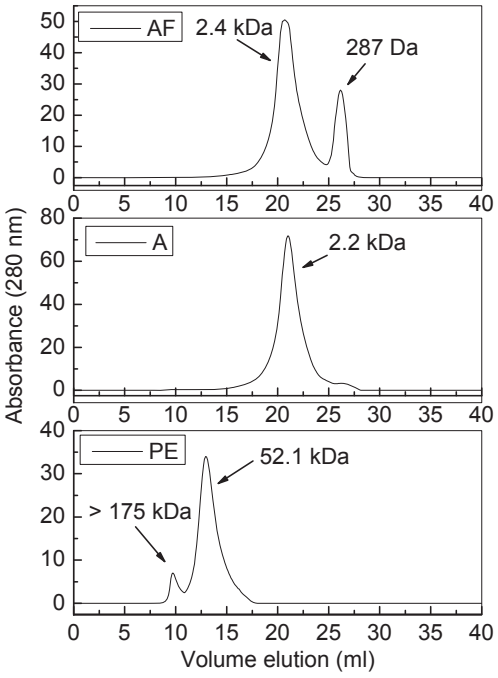

Fig. 2. FPLC gel filtration profiles of PE and its hydrolyzates (A and AF). Gel filtration chromatography was carried out with a Superdex 75 column. Injection volume was 100 μL (2.8 mg protein mL^{-1}). Elution was carried out using 50 mmol L^{-1} Potasium Phosphate Buffer pH 7.0 plus 150 mmol L^{-1} NaCl at 1 mL min^{-1}. The profile shown is representative of several that were carried out.

The study of inhibition mechanism using Lineweaver-Burk plots showed that A and AF peptides were uncompetitive inhibitors, generating almost parallel lines (Fig. 1D). These plots suggest that peptides from A and AF can bind only to substrate–enzyme complex and decrease the maximum enzyme activity, so it takes longer for the substrate or product to leave the active site (Jao, Huang, & Hsu, 2012). Similar results were found by Sato et al. (2002) for peptides Ala–Trp, Phe–Tyr and Ile–Trp obtained from *Undaria pinnatifida* (*wakame*) hydrolyzates with Protease S.

TEAC assay (ABTS+• radical cation decolorization assay) and DPPH inhibition were used to evaluate the capacity of PE and its hydrolyzates to quench radical species. As shown in Table 2, both A and AF exhibited higher scavenging effect than that of PF, indicating that peptides and phenolic compounds release during proteolysis increases the antioxidant capacity of substrate. In this way, Sheih et al. (2009) purified an antioxidative peptide (1.3 kDa), with DPPH radical scavenging activity from algae protein waste hydrolyzate. Also, several studies have shown that phenolic compounds have a potent radical scavenger activity. In this regard, Wang, Jónsdóttir, and Ólafsdóttir (2009) reported a high correlation between total polyphenolic content and DPPH radical scavenging activity from *Icelandic seaweeds* extracts, indicating an important role of algal polyphenols as chain-breaking antioxidants.

As shown in Table 2, the lower values of IC50% for ABTS+• and DPPH corresponded to AF. It is also observed, that IC50% values corresponding to A was 1.6 times higher than that obtained for sequential system for both radicals, indicating that A has lower antioxidant power than AF. As it is known, the antioxidant properties of peptides are highly influenced by the composition, sequence and molecular mass (Kim & Wijesekara, 2010). Most of the reported peptides exhibiting antioxidant activity were those with low molecular weights (Sheih et al., 2009). In this sense, Dávalos, Miguel, Bartolomé, and López-Fadiño (2004) suggested that hydrolyzates with high DH obtained with sequential proteases systems, such as AF, had higher proportion of low molecular weight peptides, which would access more easily to the oxidant system and lead to high values of TEAC and DPHH radical scavenger, having lower IC50% values. Taking into account that no significant differences in FPC of A and AF were found (Fig. 1), the higher antioxidant power activity of AF could be due to the presence of low molecular weight peptides in this hydrolyzate (287 Da). These results agree with those reported by Yust, Millán-Linares, Alcaide-Hidalgo, Millán, and Pedroche (2013), who hydrolyzed chickpea protein with different enzyme systems (Alcalase and Alcalase + Flavourzyme). In that work, the authors found that hydrolyzates obtained with the sequential system exhibited higher antioxidant capacity against DPPH than those obtained using Alcalase alone.

A and AF showed higher inhibition of β-carotene oxidation in the presence of copper than PE, indicating that these hydrolyzates had a high Copper-chelating activity (CC) (Table 2). However, the CC of A was lower than that obtained for AF, indicating that peptide size and amino acid compositions from hydrolyzates affect this property. Torres-Fuentes, Alaiz, and Vioque (2011) reported that a combination of high His contents, around 20–30%, and small peptide size provide the best chelating activities. In this sense, the His content of AF was significantly higher than that obtained from A. Also, FPLC profile of AF show components with lower MW around 287 Da (*di-peptides*). Thus, His residue and di-peptides should be involved in the formation of complexes with Cu^{2+} and thus decrease β-carotene oxidation. On the other hand, previous studies have shown that water extracts derived from seaweeds are potent ferrous ion chelators. However, metal chelation of some polyphenols play a minor role in the overall antioxidant activities (Wang et al. 2009).

The highest value of reducing power activity (RP) was obtained for A (Table 2), probably related with peptide composition of this hydrolyzate, which had the highest content of Met and Cys (Table 1). In this regard, reducing power activity has been attributed, among other amino acids, to Met and Cys because provide to peptides the redox couple S-S/-SH (Carrasco-Castilla et al., 2012).

The β-carotene bleaching inhibition (BCBI) of A was significantly higher than that obtained for AF (Fig. 3A). In this sense, A

Table 2
ACE inhibition and protein concentration required to produce 50% of inhibition (IC50) of ACE enzyme, radical cation $ABTS^{\bullet+}$, radical DPPH and protein concentration required to produce 50% of inhibition (IC50) of this radicals, copper chelating activity (CCA) and reducing power activity (RP) for protein extract (PE) and its hydrolyzates (A and AF) obtained from *P. columbina*.

Sample	ACE inhibition (%)	IC50-ACE inhibition (g L^{-1} of protein)	TEAC (mmol Trolox equivalent g^{-1} protein)	DPPH scavenging activity (mg BHT equivalent g^{-1} protein)	IC50-$ABTS^{+}$ (g L^{-1} of protein)	IC50-DPPH (g L^{-1} of protein)	CCA (mg EDTA equivalent g^{-1} protein)	RP (μg ascorbic acid equivalent g^{-1} protein)
PE	12.4 ± 0.5^{a}	5.4 ± 0.0^{c}	0.97 ± 0.0^{a}	21.6 ± 0.0^{a}	2.2 ± 0.1^{c}	2.1 ± 0.0^{c}	19.5 ± 0.9^{a}	11.5 ± 0.1^{a}
A	44.8 ± 2.4^{c}	1.2 ± 0.1^{a}	2.2 ± 0.1^{b}	68.5 ± 3.7^{b}	1.0 ± 0.0^{b}	1.6 ± 0.1^{b}	63.4 ± 7.4^{b}	53.8 ± 0.1^{c}
AF	35.2 ± 4.3^{b}	1.7 ± 0.0^{b}	4.1 ± 0.0^{c}	113.3 ± 5.8^{c}	0.6 ± 0.1^{a}	1.0 ± 0.1^{a}	81.2 ± 1.4^{c}	23.2 ± 1.9^{b}

Mean ± SD (n = 3). Different letters mean significant differences between samples ($p < 0.05$).

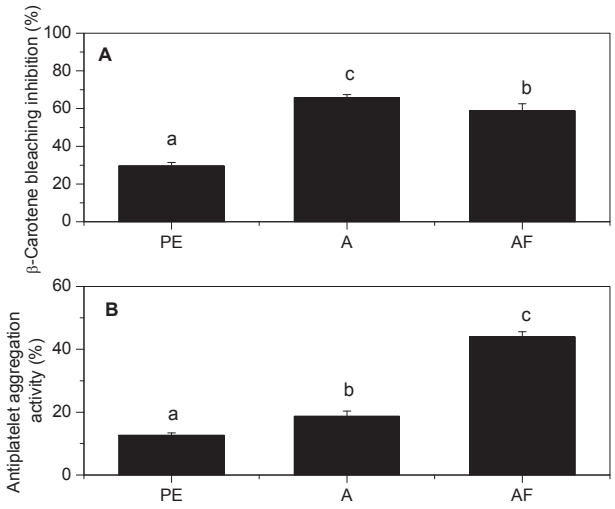

Fig. 3. β-carotene bleaching inhibition (A) and antiplatelet aggregation activity (B) of PE and its hydrolyzates (A and AF).

hydrolyzate has high content of hydrophobic amino acids (Tyr, Phe, Val, Ile and Leu), which could facilitate the access to lipid radicals from linoleic acid, giving higher β-carotene bleaching inhibition. This result was also found by Yust et al. (2012), who hydrolyzed chickpea protein with Alcalase and Alcalase + Flavourzyme. In that work, the authors observed a decrease of BCBI after adding Flavourzyme enzyme. This reduction was attributed to the hydrolysis of antioxidant peptides generated in the first stage with Alcalase. According to this, the lower BCBI obtained with AF system could be due to removal of active amino acids from A peptides. Regarding the peptide size, it could be assumed that the high proportion of low MW species (287 Da) and free amino acids of AF hydrolyzate may have an antagonist effect on BCBI. This fact was also observed by Pedroche et al. (2007) who assessed the BCBI in different peptide fractions obtained from *Brassica carinata* hydrolyzate. In that work, the antioxidant effect decreased with decreasing peptide size, achieving the lowest value of BCBI for peptide fractions with 500 Da.

As seen in Fig. 3B, the antiplatelet aggregation activity of AF was significantly higher than that obtained for A. It is important to note that platelet aggregation is mediated by the binding of fibrinogen to its receptor, platelet-membrane glycoprotein GPIIb/IIIa, which involves recognition of the Arg-Gly-Asp sequence of fibrinogen. It is conceivable, therefore, that peptides which contain Arg-Gly-Asp sequence might be capable of antagonizing the binding of fibrinogen to GPIIb/IIIa, resulting in the inhibition of platelet aggregation (Andronati, Karaseva, & Krysko, 2004). In this sense, Miyashita et al. (1999) synthesized eight mimetic peptides related to Arg-Gly-Asp sequence. They found that peptide Arg-Gly-Asp-Trp had the highest antiplatelet aggregation activity. Therefore, the higher antiplatelet aggregation activity found with AF system could be due to sequence of lower MW peptides (287 Da). Note that Trp content of AF was significantly higher than that found for A (Table 1).

3.3. Bio-accessibility of bioactive peptides from hydrolyzates

Table 3 shows the ACE inhibition results corresponding to A and AF dialysates (A_D and AF_D). As can be seen, the ACE inhibition rates of A_D and AF_D decreased respect to A and AF after treatment with gastrointestinal enzymes (12.5% and 5.4%, respectively). These results are in agreement with those found by Qu et al. (2010) for *Porphyra yezoensis* hydrolyzates. In that work, the authors found that ACE inhibition rates of peptides were decreased by 1.5% and 3.4% of the initial activity after pepsin or pepsin/trypsin system, respectively. In this way, Jao et al. (2012) reported that several ACE-I-inhibitory peptides with weak *in vitro* activity produce a strong antihypertensive effect *in vivo* while others, as is in this case, lose or reduce their activity. As can be seen, AF hydrolyzate was more resistant to gastrointestinal enzymes than A. This may be due to smaller size of peptides from AF (287 Da), which resists gastrointestinal proteolysis. In this sense, Sheih et al. (2009) reported that purified peptides from algae protein waste hydrolyzate remained ACE inhibitory activity after *in vitro* gastrointestinal digestion. The authors found that ACE inhibitory peptides having small molecular weight would be less susceptible to gastrointestinal proteolysis.

Results showed that Trolox equivalent antioxidant capacity (TEAC), DPPH scavenging activity and copper chelating activity (CCA) of A and AF not only was preserved after treatment with gastrointestinal enzymes but also was increased (Table 3). The increase in these antioxidants properties can be attributed to new peptides generated by gastrointestinal proteases from hydrolyzates, which would be bio-accessible like non-receptor-mediated process. Note that no significantly differences in FPC dialyzability for both dialysates were found (58.8 ± 4.2 and 57.0 ± 1.5%, for A_D and AF_D respectively).

RP and BCBI of A_D and AF_D were lower than that obtained for A and AF. This reduction is probably due to degradation of active peptides from hydrolyzates by gastrointestinal enzymes. These results agree with those reported by Orsini Delgado, Tironi and Añón (2011) who found that the antioxidant activity from amaranth hydrolyzate did not improve after gastrointestinal digestion.

Finally, A_D showed higher antiplatelet aggregation activity than that obtained for A. In addition, the digestion process did not decrease the antiplatelet aggregation activity of AF hydrolyzate. These results indicate that peptides from both hydrolyzates are potentially bio-accessible, maintaining the antiplatelet aggregation activity after gastrointestinal digestion.

4. Conclusions

Simulation of physiological digestion is a very useful tool in evaluating *in vitro* bioactive peptide stability against digestive enzymes. As far as we know, there is no literature related to bio-accessibility of bioactive peptides, resembling non-receptor-

Table 3
ACE inhibition, Trolox equivalent antioxidant capacity (TEAC), DPPH scavenging, reducing power activity (RP), copper chelating activity (CCA), β-Carotene bleaching inhibition (BCBI) and antithrombotic activity for hydrolyzates (A and AF) and its dialysates (A_D and AF_D) obtained from *P. columbina*.

Sample	ACE inhibition (%)	TEAC (μmol Trolox)	DPPH scavenging (mg BHT)	RP (μg ascorbic acid)	CCA (mg EDTA)	BCBI (%)	Antiplatelet aggregation activity (%)
A	44.8 ± 2.4^{c}	141.6 ± 11.0^{a}	1.23 ± 0.07^{a}	5.58 ± 0.09^{d}	1.00 ± 0.07^{a}	65.7 ± 1.7^{c}	18.7 ± 1.6^{a}
A_D	39.2 ± 1.6^{b}	609.7 ± 9.0^{b}	8.91 ± 0.80^{d}	3.79 ± 0.21^{b}	1.89 ± 0.09^{b}	37.4 ± 1.8^{a}	33.5 ± 2.3^{b}
AF	35.2 ± 4.3^{a}	132.1 ± 16.0^{a}	1.83 ± 0.50^{b}	4.87 ± 0.92^{c}	1.07 ± 0.08^{a}	58.9 ± 3.6^{c}	44.0 ± 1.6^{c}
AF_D	33.3 ± 0.9^{a}	624.8 ± 12.0^{b}	4.61 ± 0.70^{c}	1.99 ± 0.39^{a}	2.44 ± 0.02^{b}	33.0 ± 2.7^{b}	48.5 ± 1.6^{c}

Mean ± SD (n = 3). Different letters mean significant differences between samples ($p < 0.05$).

mediated process, from *P. columbina*. We have demonstrated that *P. columbina* hydrolyzates (A and AF) with antihypertensive, antioxidant and antiplatelet aggregation activity were potentially bioaccessible, maintaining overall the bioactivity after gastrointestinal digestion. However, *in vivo* studies in experimental animals must be done to confirm these results.

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