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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-