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Bioactive properties of peptides obtained by enzymatic hydrolysis from protein byproducts of *Porphyra columbina*

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ABSTRACT

The traditional method to obtain phycocolloids from seaweeds implies successive extraction steps with cold and hot water. The first cold water extract has no phycocolloids but is rich in proteins and is considered a waste. Four hydrolysates were obtained using trypsin, alcalase and a combination of both sequentially added from a first cold water protein extract (PF) derived from *Porphyra columbina*. PF hydrolysates (PFH) were enriched in peptides with low molecular weight containing Asp, Ala and Glu. Both PF and PFH showed immunosuppressive effects on rat splenocytes as they enhanced IL-10 production while the production of TNFα and IFNγ was inhibited. These immunosuppressive effects were higher for PFH. PFH had antihypertensive activity (>35% of ACE inhibition) and antioxidant capacity (DPPH, TEAC, ORAC and copper-chelating activity). The hydrolysis could be used as a mean to obtain bioactive peptides from algae protein byproducts and to add value to the phycocolloids extraction process.

1. Introduction

Edible seaweeds are rich in bioactive compounds, antioxidants, soluble dietary fibers, proteins, minerals, vitamins, phytochemicals, and polyunsaturated fatty acids (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010). Seaweeds have been traditionally used as gelling and thickening agents in food or pharmaceutical industries, but the knowledge of their diverse bioactive compounds, has opened up potential opportunities for these industries (Souza et al., 2012). Red, brown and green seaweeds have been shown to have a plethora of therapeutic properties for health and disease management, such as anticancer, antidiabetic, antihypertensive, antioxidant, anticoagulant, anti-inflammatory, antifungal, antibacterial and tissue healing properties *in vivo*. Active compounds include sulphated polysaccharides, phlorotannins, carotenoids (e.g. fucoxanthin), minerals, peptides and sulfolipids, with proven benefits against degenerative metabolic diseases (Mohamed, Hashim, & Rahman, 2012).

The marine bioprocess industry has evolved and novel technologies have been developed to convert and utilize marine food byproducts. These technologies allow the isolation of substances with antioxidative properties or the production of functional biopeptides through enzyme-mediated hydrolysis in batch reactors (Ngo, Wijesekara, Vo, Ta, & Kim, 2011).

Bioactive peptides usually contain 3–20 amino acid residues, and their activities are based on their amino acid composition and sequence (Qian, Jung, & Kim, 2008). These short chains of amino acids are inactive within the sequence of the parent protein, but can be released during gastrointestinal digestion, food processing, or fermentation. Marine-derived bioactive peptides have been obtained widely by enzymatic hydrolysis and have shown to exert many physiological functions, including antioxidant, antihypertensive, anticoagulant, and antimicrobial activities (Kim & Wijesekaraa, 2010).

Porphyra columbina is a red seaweed which has a high protein content (approximately 30% dry weight) and total dietary fiber (39–54% dry weight). Among red algae proteins, phycobiliproteins have drawn attention because of their bioactive properties (Fitzgerald, Gallagher, Tasdemir, & Hayes, 2011). However, no research is available on the production of bioactive peptides from *P. columbina* phycobiliproteins. Phycocolloids are gelatinous chemicals produced by seaweeds and the traditional method to obtain them comprises successive extraction steps with cold and hot water. The first cold water extract has no phycocolloids and is considered a waste. Nevertheless, it has proteins including phycobiliproteins which can be used as source of bioactive peptides. Cian, López-Posadas, Drago, Sánchez de Medina, and Martínez-Augustin (2012) used enzyme hydrolysis to obtain bioactive peptides from remaining proteins in the final cake, which were insoluble in cold and hot water whit good inmunomodulatory activity. However, these proteins were no phycobiliproteins. Therefore, the aims of

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this study were to obtain bioactive peptides by enzymatic hydrolysis from *P. columbina* water soluble proteins (phycobiliproteins) obtained as a byproduct of phycocolloid process and to evaluate their bioactive properties: immunomodulatory properties, ACE inhibition and antioxidant capacity.

2. Materials and methods

2.1. Reagents

Trypsin (T) and Alcalase (A) were obtained from Novozymes Spain S.A. (Madrid, Spain). The other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Sigma (Barcelona, Spain).

2.2. Preparation of protein fraction (PF) from byproducts of P. columbina

One kilogram of different specimens of *P. columbina* was hand-picked in Punta Maqueda (Comodoro Rivadavia, Argentina). The collection was carried out in October 2010. Samples were taken to the laboratory at 4 °C inside plastic bags. To remove adherent seawater, sediment, organic debris, macro fauna and epibiota, they were scraped and rinsed with distilled water. *P. columbina* samples were dried to constant weight (100 ± 4 °C), ground to obtain a powder with a particle size lower than 1 mm, using a laboratory hammer mill (Retsch, Haan — Germany). Then samples were passed through a 0.85 mm mesh sieve and stored at 4 °C in plastic bags until analysis.

The red marine algae *P. columbina* was dispersed at 50 g kg^{-1} in distilled water for 2 h and then centrifuged at 3000 × *g* for 30 min at 20 °C. The supernatant was ultra-filtered using 10 kDa cut-off Molecular/Por® Cellulose-Ester membrane and Molecular/Por® Stirred Cell S-43-70 system. The volume reduction factor (VCR) was 2. The fraction with molecular weight > 10 kDa (protein fraction, PF) was used as substrate for enzymatic hydrolysis.

2.3. Preparation of hydrolysates

Hydrolysates were obtained using 25 mL batch thermostatic reactor. The reaction pH was adjusted to 8.0 with 0.5 mol L^{-1} buffer Tris. The substrate concentration was 1% (w/w) in every case. Working conditions for T and A enzymes were: temperature 50 °C, pH 8.0, enzyme/substrate (E/S) ratio 5% (w/w). Once the hydrolysis was finished, the enzyme was inactivated by thermal treatment following the manufacturer guidelines and the hydrolysates were lyophilized. PF hydrolysates (PFH) were prepared using the following systems:

- Simple hydrolysis
  Hydrolysate T: Hydrolysis with T enzyme during 4 h.
  Hydrolysate A: Hydrolysis with A enzyme during 4 h.

- Sequential hydrolysis
  Hydrolysate TA: Hydrolysis with T enzyme during 2 h + hydrolysis with A enzyme during 2 h; total reaction time, 4 h.
  Hydrolysate AT: Hydrolysis with A enzyme during 2 h + hydrolysis with T enzyme during 2 h; total reaction time, 4 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 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 PF and PFH

2.4.1. Spectroscopic measurements

PF, PFH, R-phycoerythrin (R-PE, Sigma 52412-F) and C-phycocyanin (C-PC, Sigma 52468-F), were dispersed at 1 g L^{-1} in 0.1 mol L^{-1} phosphate buffer (pH 6.8). Absorption spectra were recorded with a UV–visible spectrophotometer (Milton Roy Genesys 5) against a blank containing phosphate buffer. Fourth derivative analysis of absorbance scans from samples was performed to resolve the overlapping peaks of individual pigment spectra according to Sampath-Wiley and Neefus (2007).

2.4.2. Amino acid analysis

Samples (2 mg) were hydrolyzed with 4 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 600E multi-system with a 484 UV–vis detector (Waters, Milford, MA) 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. The solvents used were (A) sodium acetate (25 mmol L^{-1}) containing sodium azide (0.02% w/v) 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).

2.4.3. Fast protein liquid chromatography (FPLC)

Gel filtration chromatography was carried out in an AKTA purifier system equipped with a Superdex peptide column (GE Life Sciences, Piscataway, NJ, USA). Injection volume was 200 μL (10 mg protein PF and its hydrolysates mL^{-1}) and elution was carried out using 0.75 mol L^{-1} ammonium bicarbonate at 1 ml min^{-1}. Elution was monitored at 214 nm and molecular mass was estimated using molecular weight (MW) standards from Pharmacia: blue dextran (2,000,000 Da), cytochrome C (12,500 Da), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da) and glycine (75 Da).

2.4.4. Phenolic compound

2.4.4.1. Sample extraction and hydrolysis. The phenolic compounds determination was carried out according to Tarola, Van de Velde, Salvagni, and Preti (2012). Phenolic compounds were extracted from 0.2 g of PF in 5 mL of HPLC grade methanol at room temperature. The extract was sonicated for 15 min, centrifuged at 3000 × *g* for 15 min at room temperature, and supernatant was collected. The insoluble material was re-extracted three times with 5 mL HPLC grade methanol. All fractions collected were concentrated by a nitrogen flow at room temperature to 5 mL and finally filtered through a Millipore 0.45 μm pore size filter before to be injected in the HPLC system. These fractions were used for the analysis of free aglycones or non conjugated or non condensed phenolic compounds naturally present in PF. After taking these samples, an acid hydrolysis was performed over all extracted obtained with the objective of releasing the aglycone portion from glycosylated phenolic compounds and hydrolyzing the conjugated and condensed ones. This hydrolysis was carried out heating 2 mL extracted sample and 1 mL HCl 6 mol L^{-1} for 50 min in an oven at 90 °C. After hydrolysis, the extracts were allowed to cool and were ready to be injected in the HPLC system for the analysis of total aglycones from glycosylated phenolics or conjugated or condensed phenolic