Food Research International 49 (2012) 364–372

Contents lists available at SciVerse ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

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

Raúl E. Cian [a,b,*], Olga Martínez-Augustin [c], Silvina R. Drago [a,b]

[a] Instituto de Tecnología de Alimentos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, 1° de Mayo 3250, (3000) Santa Fe, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[c] Department of Biochemistry and Molecular Biology II, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Universidad de Granada, Campus de Cartuja s/n, 18071 Granada, Spain

ARTICLE INFO

Article history:
Received 9 April 2012
Accepted 1 July 2012
Available online 17 July 2012

Keywords:
Immunomodulation
ACE inhibition
Antioxidant capacity
Hydrolysates
Red seaweeds

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.

© 2012 Elsevier Ltd. All rights reserved.

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

* Corresponding author at: Instituto de Tecnología de Alimentos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, 1° de Mayo 3250, (3000) Santa Fe, Argentina. Tel.: +54 342 4571164 (int 2585); fax: +54 342 4571164.
E-mail address: rec_704@yahoo.com.ar (R.E. Cian).

0963-9969/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodres.2012.07.003

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

compounds in the PF extracts. The extraction was performed by triplicate.

2.4.4.2. Stock standard solutions. Stock standards of individual polyphenol compounds: gallic acid (1000 mg L^{-1}), catechin (400 mg L^{-1}), epicatechin (400 mg L^{-1}), p-coumaric acid (1000 mg L^{-1}), ferulic acid (2000 mg L^{-1}), rutin (640 mg L^{-1}), cinnamic acid (1000 mg L^{-1}), ellagic acid (200 mg L^{-1}), quercetin (620 mg L^{-1}), kaempferol (135 mg L^{-1}), cyanidin (2000 mg L^{-1}) and perlagonidin (1300 mg L^{-1}) were prepared by exactly weighing and dissolving the standards in HPLC grade methanol. These solutions protected against light and stored at −20 °C were proved to be stable for a period of 2 month.

2.4.4.3. Phenolic compound profile of PF. Compounds were separated on a 150 mm × 4.6 mm, 5 μm particle size, Supelcosil LC-ABZ column, and an Alltech C18 5 μm guard column. The mobile phase was a gradient prepared from formic acid in water (2%, pH 3, solvent A) and formic acid in methanol (2%, pH 3, solvent B). The gradient program was 0.01–8.00 min 15% B isocratic; 8.01–25.00 min 15–50% B; 25.01–40.0 min 50% B isocratic; 40.01–50.0 min 50–90% B; 50.01–55.00 min 90–15% B. 10 min of equilibration was required before the next injection. The flow rate was 0.7 mL min^{-1} and the analyses were done at room temperature (25 °C). The injected volume was 20 μL. The detector was set at 280, 320, 360 and 520 nm for simultaneous monitoring of the different groups of phenolic compounds. Peak identification was performed by comparison the retention times and diode array spectral characteristics with external standards. Data were processed using Shimadzu LC solution software.

2.5. Bioactive properties of PF and PFH

2.5.1. Angiotensin-converting enzyme activity (ACE) inhibition

Angiotensin-converting enzyme activity inhibition was determined according to Hayakari, Kondo, and Izumi (1978) and antihypertensive activity was expressed as ACE inhibition (%). The antihypertensive activity from PF and PFH were evaluated in 20 μL of 4.0 g L^{-1} of proteins.

2.5.2. Immunomodulation properties

2.5.2.1. Spleen mononuclear cell isolation. Female Wistar rats were obtained from Janvier S.A.S (Le Genest Saint Isle, France), sacrificed by cervical dislocation and the spleen was extracted aseptically. Cell suspensions were obtained by disrupting the tissues between dissecting forceps in medium. After centrifuging, cells were cleared of erythrocytes by suspension on hypotonic lysis buffer (0.15 mol L^{-1} NH_4Cl, 10 M $KHCO_3$, 0.1 mol L^{-1} $Na_2EDTA{\bullet}2H_2O$, pH 7.3) for 30 min on ice. Mononuclear cells were washed and suspended in RPMI medium supplemented with 10% FBS, 2 mmol L^{-1} L-glutamine, 100 U mL^{-1} penicillin, 0.1 g L^{-1}streptomycin, 2.5 g L^{-1} amphotericin B and 0.05 mmol L^{-1} β-mercaptoethanol. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was approved by the Animal Welfare Committee of the University of Granada (Granada, Spain).

2.5.2.2. Cytokine determination. For cytokine determinations the cells suspensions (10^6 cells mL^{-1} in RPMI medium) were cultured in the presence or absence of PF and PFH (1.0 and 0.1 g L^{-1} of protein) and stimulated with concanavalin A (ConA, 5 μg mL^{-1}) or LPS (1 μg mL^{-1}). Cell culture medium was collected after 24 or 48 h, cleared by centrifugation (3000 × *g*, 5 min, 4 °C) and frozen at −80 °C until assayed for cytokine content by commercial ELISA kits (Biosource Europe, Nivelles, Belgium and Becton Dickinson, Franklin Lakes, NJ, USA). In all the experiments, samples were run in triplicate and results are expressed as cytokine concentration (pg mL^{-1}).

2.5.2.3. Lactate dehydrogenase assay. Cellular toxicity was measured as the release of lactate dehydrogenase. Cells were cultured in the conditions described above and lactate dehydrogenase activity in supernatants was measured spectrophotometrically using sodium pyruvate (25 mmol L^{-1}) as substrate in 50 mmol L^{-1} sodium phosphate buffer (pH = 7.5) (Halprin & Ohkawara, 1966).

2.5.3. Antioxidant capacity

2.5.3.1. Trolox equivalent antioxidant capacity (TEAC). To estimate the antioxidant capacity, $ABTS^{\bullet+}$ radical cation decolorization assay according to Re et al. (1999) was used. To estimate the TEAC a concentration-response curve for the absorbance at 734 nm for $ABTS^{\bullet+}$ 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. The absorbance reading was taken at 6 min after initial mixing. TEAC from PF and PFH was determined at 4 g L^{-1} of protein. To determine the concentration causing an inhibition of 50% (IC50%) serial dilutions of PF and PFH from 0 to 9 g L^{-1} protein were made.

2.5.3.2. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay. DPPH radical scavenging activity assay was measured according to the method of Brand-Williams, Cuvelier, and Berset (1995). An aliquot of 10 μl of PF and PFH at 4 g L^{-1} protein was mixed with 190 μl of methanol solution containing 0.08 mmol L^{-1} DPPH radical. The mixture was allowed to stand for 30 min in the dark, and the absorbance was monitored at 517 nm. The difference between the blank and the sample was used for calculating the scavenging activity as percentage inhibition. Serial dilutions of PF and PFH from 0 to 4 g L^{-1} protein were made to determine the concentration causing an inhibition of 50% (IC50%).

2.5.3.3. Oxygen radical absorbance capacity (ORAC) assay. ORAC assay was measured according to the method of Cao, Alessio, and Cutler (1993). The reaction was carried out in 75 mmol L^{-1} phosphate buffer (pH 7.4). Sample solution (20 μL) and 96 nanomol L^{-1}Fluorescein (150 μL) was pre-incubated for 5 min at 37 °C, and the ORAC analysis was initiated by adding 30 μL of 320 mmol L^{-1} 2,2′-Azobis(−amidinopropane) dihydrochloride (AAPH). Trolox was used as a calibration solution, and Fluorescein decay curves between the blank and the sample was performed. The oxidant activity from PF and PFH was evaluated at 9.0 g L^{-1} of proteins. All values were expressed as Trolox equivalents.

2.5.3.4. 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) with modifications. A solution of β-carotene 10 g L^{-1} in chloroform was prepared. After addition of 1 mL Tween 20, chloroform was evaporated under nitrogen and then dissolved in 0.1 mol L^{-1} pH 7.0 phosphate buffers. A solution 50 μmol L^{-1} $CuSO_4$ was prepared and 10 μl were added to each well. The assay mixture was: 200 μl β-carotene, 10 μl $CuSO_4$, 200 μl PF or PFH. The concentration of PF and PFH was 1 g L^{-1} of protein. The degradation of β-carotene was monitored by recording the decrease in absorbance at 470 nm.

2.6. Statistical analysis

All results are 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 PF

Table 1 shows amino acids profile of PF. The sum of aspartic and glutamic acids content was 29.8 g/100 g of total amino acids and were the most abundant amino acids. For most seaweed, aspartic and glutamic acids constitute together a large part of the amino acid fraction (Fleurence, 1999). Similar results were obtained for other red seaweeds such as *Porphyra acanthophora* (27 g/100 g of total amino acids) (Galland-Irmouli et al., 1999). The predominance of acidic amino acids over basic amino acids is typical of red seaweed and the high levels of them are responsible for the special flavor and taste of seaweeds (Galland-Irmouli et al., 1999). On the other hand, PF has a high content of alanine and a relatively high content of threonine, leucine, serine, glycine and proline. However, cysteine, methionine, histidine and tryptophan were in low amounts.

Table 2 shows phenolic compound profile of PF. PF showed a low content of epicatechin, p-coumaric acid, quercetin and kaempferol. Also were not detected gallic acid, catechin, ferulic acid, rutin, cinnamic acid and ellagic acid. However, after acid hydrolysis the content of gallic acid, catechin and epicatechin increased. This increase could be to the acid hydrolysis process which disfavors protein-polyphenol interactions and allows the depolymerization of phenolic compounds. It is important to take into account that polyphenols may interact with proteins via hydrogen bonding, π-bonding, hydrophobic interactions, ionic and covalent linkage (Wang et al., 2010). When the algae cell wall is disrupted, the intracellular constituents including proteins are released from the cells, which are prone to complex with polyphenols, leading to aggregation and ultimate precipitation (Siriwardhana et al., 2008). Finally, the decrease in the content of coumaric acid, quercetin and kaempferol may be due to degradation of these phenolic compounds during acid hydrolysis (Nuutila, Kammiovirta, & Oksman-Caldentey, 2002).

3.2. Characterization of PFH

In the present study, PF was separately hydrolyzed by trypsin (T) and alcalase (A) and sequentially hydrolyzed by trypsin (T) + alcalase (A) and alcalase (A) + trypsin (T). The extent of protein degradation by proteolytic enzymes was estimated by assessing the degree hydrolysis (DH) which was observed to be 43.5 ± 1.9%, 57.7 ± 1.8%, 48.3 ± 0.5% and 64.3 ± 1.9% for T, A, TA and AT respectively. DH corresponding to A was higher than that obtained for T, for the same hydrolysis time. This indicates that A enzyme was more active than T on PF. A similar result was reported by Qu et al. (2010) for *Porphyra yezoensis* hydrolysates.

The highest DH value was obtained whit AT system. When the enzymatic order was reversed, first t and then an enzyme (i.e. TA), the DH obtained was slightly higher than that reached with T enzyme. The poor action of A enzyme on an hydrolyzed substrate could be due to the fact that a is an endoprotease and after T action lower specific sites could be available.

The fourth derivative analysis from visible spectrum for PF and PFH was obtained and compared with those corresponding to hydrosoluble pigments characteristic of red algae, such as phycoerithryn (R-PE) and phycocyanin (C-PC). As shown in Fig. 1C, PF mainly exhibits 12 peaks. Peaks detected in PF are present in the R-PE spectrum (Fig. 1A) and C-PC profile (Fig. 1B). Thus PF has R-PE and C-PC which belong to the phycobilin family of fluorescent heteroproteins present in Rhodophytas (Niu, Wang, Zhou, Lin, & Chen, 2007). However, there are other peaks that not correspond to either R-PE or C-PC and match with additional pigments in PF (such as soluble chlorophyll without phytol). Similar results were reported by Sampath-Wiley and Neefus (2007) with an aqueous extract of *Porphyra purpurea* in a 0.1 M phosphate buffer (pH 6.8). However in the case of PF hydrolysates (Fig. 1D), peaks detected in FP are not present, indicating that enzymatic hydrolysis process degraded phycobiliproteins. It is appropriate to note that during hydrolysis process chromophores could be released from phycobiliproteins and be easily oxidized modifying the PF visible spectrum.

Fig. 2 shows the FPLC gel filtration profile of PF and PFH. PF profile shows two main peaks. The first peak corresponds to components with molecular weight (MW) higher than 12.5 kDa (proteins) and the second one to components of MW 484 Da. The presence of low MW components in PF may be due to possible interactions between these components with proteins. Therefore, some components of very low MW are retained in PF.

PFH profile shows effective degradation by enzymatic hydrolysis process of 12.5 kDa compounds present in PF and increasing of the proportion of intermediate MW species and peptides. This is evidenced as a widening of the base of the second peak (Fig. 2), which was around 340 Da. Considering 120 Da the average MW of amino acids, this fraction could be form by di-peptides. The emergence of a third peak in PF hydrolysates profile corresponding to free amino acids: 76 Da, 84 Da, 92 Da and 79 Da for T, A, TA and AT respectively.

3.3. Immunomodulation properties

As a routine procedure, the possible effect of PF and PFH on splenocyte cell viability was assessed. As shown in Table 3, an increase in Lactate dehydrogenase activity was observed in the culture medium when PF and PFH were added at 1 g L^{-1}. This is typically the consequence of enzyme release secondary to cell lysis or membrane leakage. However, there was no toxicity with the concentration of 0.1 g L^{-1}

Table 1
Amino acids profile of protein fraction (PF) obtained from *P. columbina*.

Amino acids	Total amino acids (g 100 g^{-1} protein dw)
Asp	13.54 ± 0.08
Glu	16.30 ± 0.48
Ser	5.59 ± 0.04
His	0.81 ± 0.03
Gly	8.84 ± 0.03
Thr	7.10 ± 0.04
Arg	3.99 ± 0.02
Ala	15.75 ± 0.03
Pro	5.16 ± 0.01
Tyr	1.59 ± 0.03
Phe	2.43 ± 0.10
Val	3.93 ± 0.03
Met	1.49 ± 0.06
Cys	1.79 ± 0.03
Ile	1.99 ± 0.01
Trp	0.27 ± 0.03
Leu	5.39 ± 0.07
Lys	4.04 ± 0.05

Mean ± SD (n = 3). dw (dry weight).

Table 2
Phenolic compound profile of protein fraction (PF) obtained from *P. columbina* before and after acid hydrolysis (6 mol L^{-1} HCl, 50 min at 90 °C).

Phenolic compound	Concentration before acid hydrolysis (mg kg^{-1} dw)	Concentration after acid hydrolysis (mg kg^{-1} dw)
Gallic acid	ND	2.47 ± 0.02
Cathechin	ND	0.21 ± 0.01
Epicatechin	0.14 ± 0.01	5. 20 ± 0.02
Ferulic acid	ND	ND
Coumaric acid	0.23 ± 0.01	ND
Rutin	ND	ND
Cinnamic acid	ND	ND
Ellagic acid	ND	ND
Quercetin	0.17 ± 0.01	0.06 ± 0.00
Kaempferol	0.02 ± 0.00	ND

Mean ± SD (n = 3), dw (dry weight). ND: no detected.

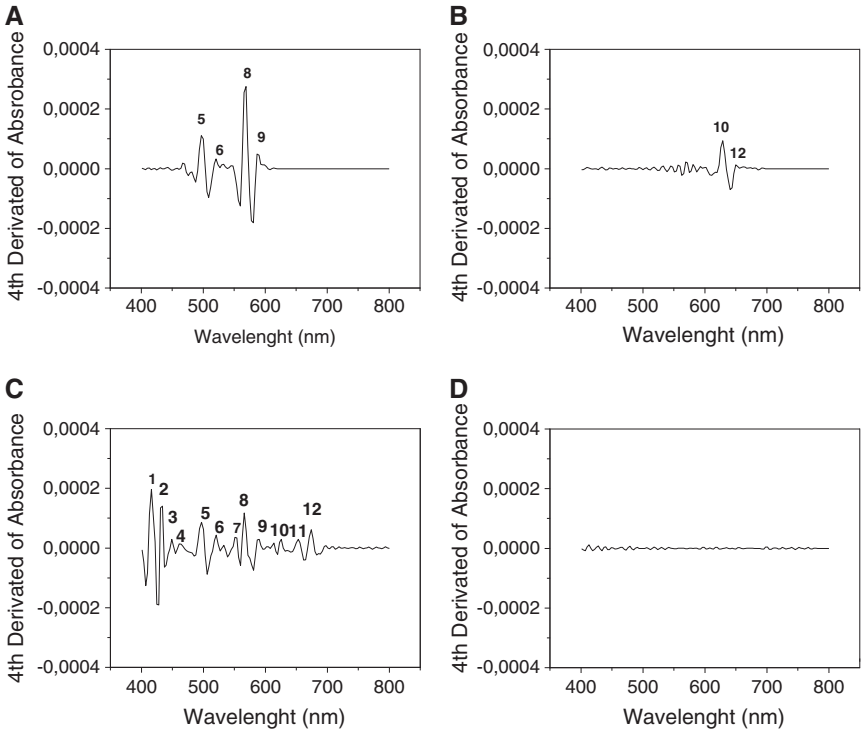

Fig. 1. Fourth derivate spectra from R-phycoerythrin (A), C-phycocyanin (B), protein fraction (PF) (C) and protein fraction hydrolysates (D).

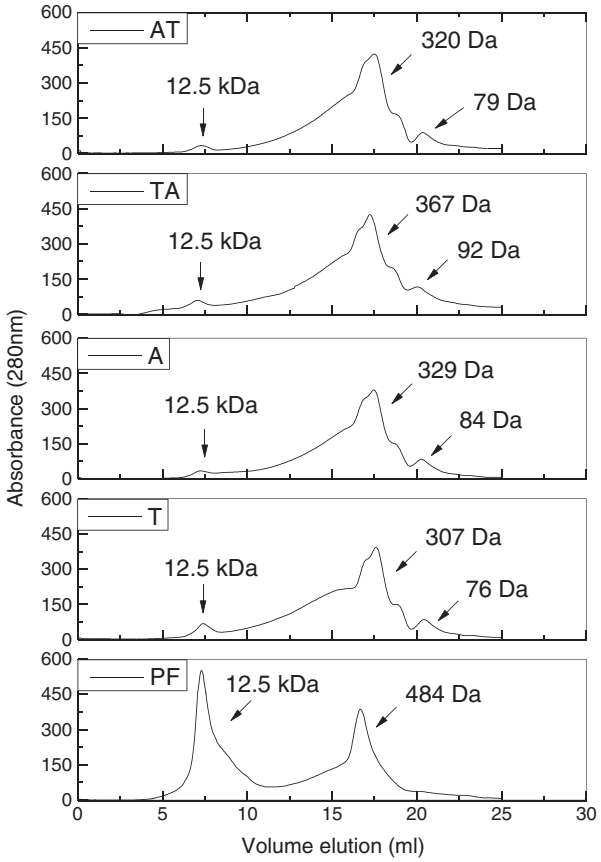

Fig. 2. FPLC gel filtration profile of protein fraction (PF) and PF hydrolysates (T, A, TA and AT). Gel filtration chromatography was carried out with a Superdex peptide column. Injection volume was 200 μL (10 mg protein mL^{-1}). Elution was carried out using 0.75 mol L^{-1} ammonium bicarbonate at 1 ml min^{-1}. The profile shown is representative of several that were carried out.

both in basal condition and in the presence of cell stimulus (LPS, ConA). Therefore further experiments were performed at this protein concentration (0.1 g L^{-1}).

Fig. 3 shows the effect of PF and PFH on the production of IL-10 by rat splenocytes. All the assayed products, exhibited a stimulatory effect on IL-10 secretion in basal conditions (Fig. 3A). The effect on IL-10 secretion of A, TA and AT hydrolysates was higher than that of substrate (PF). When spleen macrophages or T lymphocytes in the splenocyte preparation were stimulated with LPS or ConA respectively, a large surge in IL-10 release was observed (Fig. 3B and C). This was further increased by the addition of PF and PFH, being the effects of the hydrolysates higher than those of PF. Since LPS and ConA act as macrophage and T cell stimuli, our results suggest that IL10 production in both cell types is increased by PF and PFH, and that the release of bioactive peptides from the PF by enzymatic hydrolysis may enhance this effect.

Table 3
Level of lactate dehydrogenase (LDH) (mU μl^{-1}) in the culture medium from splenocyte with or without protein fraction (PF) obtained from *P. columbina* and PF hydrolysates (1 or 0.1 g L^{-1} of protein).

Sample	Protein (g L^{-1})	Basal	ConA	LPS
PF	0	20.2 ± 2.7^{a}	20.1 ± 2.0^{a}	22.5 ± 0.4^{a}
	0.1	21.1 ± 1.3^{a}	19.7 ± 1.1^{a}	21.8 ± 1.0^{a}
	1	113.8 ± 2.0^{b}	138.0 ± 4.1^{b}	139.6 ± 0.5^{b}
T	0	29.4 ± 1.1^{a}	27.1 ± 0.4^{a}	29.4 ± 1.1^{a}
	0.1	28.4 ± 0.8^{a}	27.3 ± 3.6^{a}	29.4 ± 1.8^{a}
	1	116.3 ± 1.6^{b}	142.2 ± 0.5^{b}	107.2 ± 7.4^{b}
A	0	29.4 ± 1.1^{a}	27.4 ± 0.4^{a}	29.4 ± 1.1^{a}
	0.1	29.1 ± 0.5^{a}	27.9 ± 21.7^{a}	27.4 ± 1.4^{a}
	1	159.1 ± 1.2^{b}	134.6 ± 0.1^{b}	229.5 ± 3.4^{b}
TA	0	29.4 ± 1.1^{a}	27.1 ± 0.4^{a}	29.4 ± 1.1^{a}
	0.1	29.8 ± 1.5^{a}	25.7 ± 2.1^{a}	28.5 ± 1.0^{a}
	1	113.1 ± 4.8^{b}	444.5 ± 27.2^{b}	263.5 ± 8.6^{b}
AT	0	29.4 ± 1.1^{a}	27.1 ± 0.4^{a}	29.4 ± 1.1^{a}
	0.1	29.2 ± 2.3^{a}	28.4 ± 2.1^{a}	28.9 ± 0.6^{a}
	1	146.7 ± 0.5^{b}	304.3 ± 2.7^{b}	229.2 ± 1.4^{b}

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

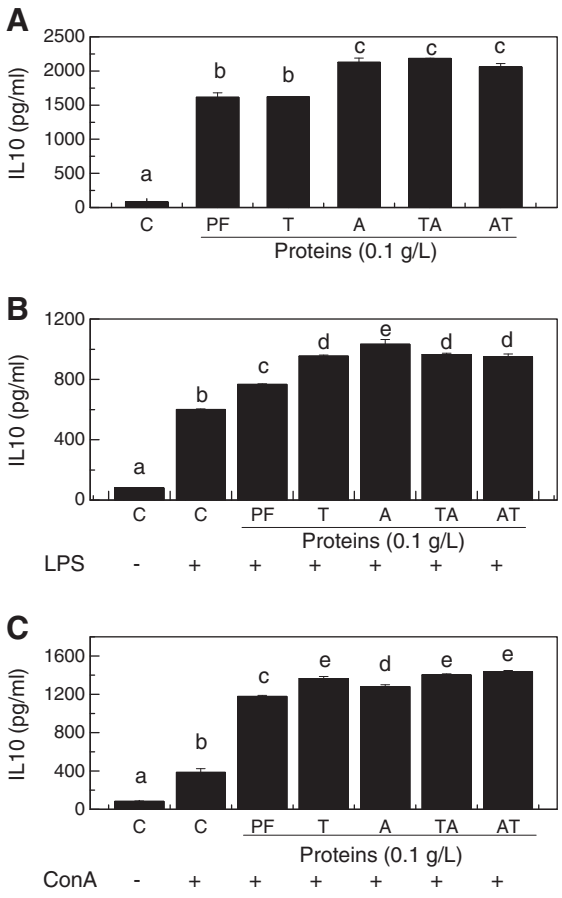

Fig. 3. Effect of protein fraction (PF) and PF hydrolysates (T, A, TA and AT) on the production of IL-10 by splenocyte. Splenocytes were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PF or its hydrolysates (0.1 g L^{-1} of protein) in absence (A), or presence of bacterial lipopolysaccharide (LPS, 1 μg/mL) (B), Concanavalin A (ConA, 5 μg/mL) (C). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as mean ± SD.; different letters means significant differences ($p<0.05$).

We further examined the possible effect of PF and PFH on the production of the proinflammatory cytokines TNFα and IFNγ by splenocytes stimulated with LPS or ConA respectively (Fig. 4). Although T and A hydrolysates increased TNFα release under LPS stimulation, PF and the hydrolysates TA and AT had a marked inhibitory effect, with a ~50% of cytokine release being blocked by AT hydrolysate.

As shown in Fig. 4B, the addition of PF and PFH to splenocytes decreased IFNγ secretion in the cell culture medium under ConA stimulation. This effect was much more pronounced after PFH than after PF addition, with a maximal inhibition of approximately 80% for TA and AT hydrolysates.

IL-10 is expressed by spleen macrophages and regulatory T lymphocytes (Tregs). It has been described that IL-10 has potent inhibitory effects on a number of immune cells including macrophages and lymphocytes. Thus, IL-10 down-regulates the production of TNFα by LPS stimulated spleen macrophages and of IFNγ by T lymphocytes (Feng, Tang, Chang, & Wilson, 1993). Our results are in agreement with this facts and indicate that PF and PFH (TA and AT) exert immunosuppressive effects on splenic macrophages inducing the production of IL-10 and suppressing TNFα. ConA is known for its ability to stimulate T- cell subsets giving rise to different T cell populations (Dwyer & Johnson, 1981). Among these populations Tregs are immunosuppressive T cells that produce IL-10 and T helper 1 (Th1) cells are proinflammatory T cells that produce IFNγ (Kaiko, Horvat, Beagley, & Hansbro, 2008). Our results indicate that PF and PFH may induce spleen T cell differentiation to Tregs inhibiting, at the same, time the differentiation to Th1. The striking effects of PF and PFH described here are consistent with a significant immunosuppressive action *in vitro,* which could be related with an anti-inflammatory activity *in vivo.* Further studies are needed to evaluate this possibility and to further characterize the responsible peptides in PFH and their bioavailability.

3.4. Angiotensin-converting enzyme activity (ACE) inhibition

Table 4 shows the ACE inhibition results corresponding to PF and PFH (T, A, TA and AT). Hydrolysates showed a relatively good antihypertensive activity (higher than 35% of ACE inhibition), while PF enhanced ACE activity (around −24% of ACE inhibition). The values

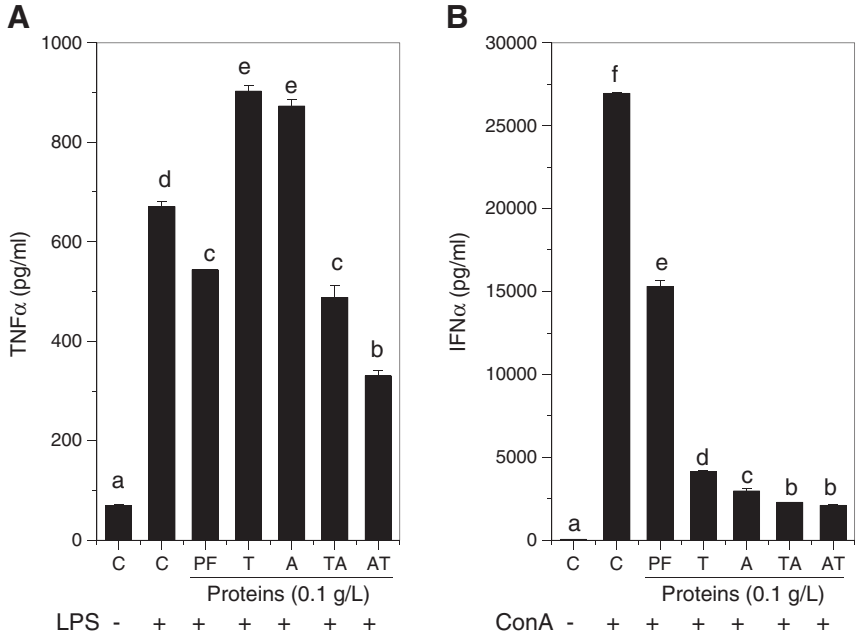

Fig. 4. Effect of protein fraction (PF) and PF hydrolysates (T, A, TA and AT) on the production of TNFα and IFNγ by splenocytes. Splenocytes were plated in 24-well plates (0.5×10^6 cells/well) and cultured with PF or its hydrolysates (0.1 g L^{-1} of protein) in presence of bacterial lipopolysaccharide (LPS, 1 μg/mL) (A), Concanavalin A (ConA, 5 μg/mL) (B). After incubation, culture medium was collected and frozen at −80 °C until ELISA analysis. Data are expressed as mean ± SD.; different letters means significant differences ($p<0.05$).

Table 4
ACE inhibition and protein concentration required to produce 50% of inhibition (IC50) of radical cation ABTS•+ and radical DPPH for protein fraction (PF) and PF hydrolysates (T, A, TA and AT) obtained from *P. columbina*.

Sample	ACE inhibition (%)	IC50-DPPH (g L^{-1} of protein)	IC50-ABTS•+ (g L^{-1} of protein)
PF	−23.9 ± 1.0[a]	4.2 ± 0.3[b]	5.6 ± 0.4[b]
T	36.8 ± 2.4[b]	2.8 ± 0.2[a]	2.4 ± 0.2[a]
A	35.6 ± 0.3[b]	2.7 ± 0.2[a]	2.2 ± 0.1[a]
TA	38.1 ± 1.8[b]	2.7 ± 0.2[a]	2.1 ± 0.2[a]
AT	35.2 ± 3.0[b]	3.0 ± 0.6[a]	2.1 ± 0.1[a]

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

obtained for PFH were very similar to those published by Qu et al. (2010) for *Porphyra yezoensis* hydrolysates, using Alcalase as enzyme.

Similar results of ACE inhibition were obtained for all hydrolysates (around 35–38%). This could be due to the similar size of the peptide fractions obtained in the different hydrolysates. As mentioned above, the fraction around with MW around 340 Da could be form by di-peptides which would be primarily responsible of ACE inhibition (Wilson, Hayes, & Carney, 2011). In this sense, Sato et al. (2002) found for brown alga (*Undaria pinnatifida*) seven kinds of ACE inhibitory di-peptides obtained with Protease S.

PF has a high content of aspartic, glutamic, alanine and a relatively high content of amino acids threonine, leucine and proline (Table 1). The abundance of the above-mentioned amino acids in the di-peptides sequence for PFH could be responsible for ACE inhibition. Peptides with high ACE inhibition activity have a high content of branched and aromatic amino acids such as Pro, Glu, Val, Phe, and Tyr in its peptide sequence. ACE prefer substrates containing branched amino acid residues at the N-terminal position, and hydrophobic amino acid residues (aromatic or branched-side chains) at the C-terminal position (Sato et al., 2002; Wijesekara, Qian, Ryu, Ngo, & Kim, 2011). The hydrophilic amino acid residues in the peptide sequence could also affect inhibitory activity by disrupting the access of the peptide to the active site of ACE. The hydrophilic–hydrophobic partitioning in the sequence was also a critical factor in the inhibitory activity (Sheih, Fang, & Wu, 2009).

3.5. Antioxidant capacity

DPPH is a relatively stable radical that is widely used to test the ability of compounds to scavenge free radicals and therefore act as antioxidants (Tsopmo, Cooper, & Jodayree, 2010). We used this assay to evaluate the capacity of PF and PFH to quench radical species. As shown in Fig. 5A, all PFH exhibited higher scavenging effect than that of PF. The higher radical scavenging capacity from hydrolysates could be due to the low MW peptides (around 340 Da) and free amino acids released during the hydrolysis process. Several studies have shown that low MW hydrolysates generally possess higher DPPH radical scavenging capacity than high MW hydrolysates (Wang et al., 2010). In addition, the radical scavenging activity was concentration-dependent (data not shown) and the IC50 value of PFH was lower than that of PF (Table 4). This indicates that hydrolysates have a higher antioxidant capacity than substrate.

The TEAC assay (ABTS + • radical cation decolorization assay), was widely applied to evaluate the total antioxidative activity in both lipophilic and hydrophilic samples (Sheih et al., 2009). For PF and PFH, the inhibition of ABTS + • radical cation tended to a plateau when concentration reached 8 g L^{-1} of protein (data not shown), and it was significantly higher for PFH than for PF (Fig. 5B). As shown in Table 4, the lower values of IC50 corresponded to PFH. It is also observed, that IC50 values corresponding to PF for DPPH and ABTS assay were 2 times higher than those obtained for PFH, which indicates the lower capacity of this protein fraction to inhibit these radicals.

The radical scavenging activity was also determined using ORAC, another commonly used assay to evaluate the antioxidant activity of

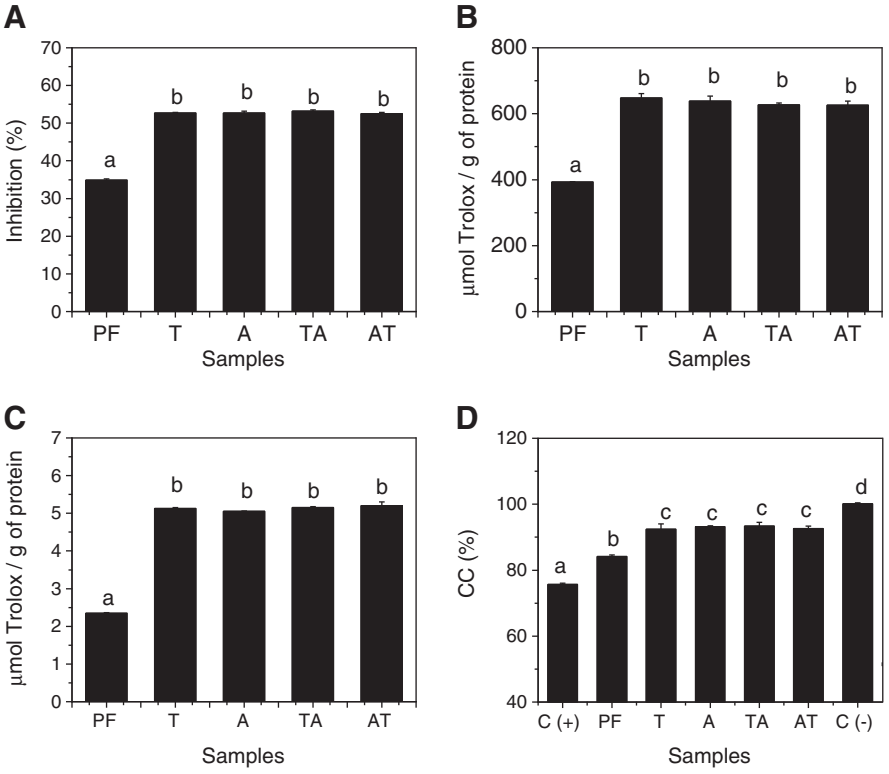

Fig. 5. Antioxidant capacity of protein fraction (PF) and PF hydrolysates (T, A, TA and AT). DPPH radicals scavenging activity assay (A), ABTS scavenging assay (B), oxygen radical absorbance capacity (ORAC) assay (C) and copper-chelating (CC) activity (D). PF and PF hydrolysates were evaluated at 4 g L^{-1} of protein, 4 g L^{-1} of protein, 9 g L^{-1} of protein and 1 g L^{-1} of protein, for DPPH, TEAC, ORAC and CC respectively.

food components (Tsopmo et al., 2010). It was found that ORAC values from PFH were significantly higher than that of PF (Fig. 5C). The peroxyl radical scavenging activity obtained for PFH was higher than that reported by Wang et al. (2010) for hydrolysates from red seaweeds *Palmaria palmate*.

The antioxidative properties of peptides are highly influenced by molecular mass and molecular structure. Most of the reported peptides exhibiting antioxidative activity were those with low molecular weights (Qian et al., 2008; Sheih et al., 2009).

PFH have peptides with low molecular weight, and also have a high content of aspartic, glutamic, alanine and a relatively high content of amino acids threonine, leucine and proline. It had been reported that alanine, leucine, proline with non-polar aliphatic groups have high reactivity to hydrophobic PUFAs radicals, and hydrogen donors such as aspartic and glutamic acid are able to quench unpaired electrons or radicals by supporting protons (Qian et al., 2008).

As observed in Fig. 5D, PFH showed higher inhibition of β-carotene oxidation in the presence of copper. These hydrolysates possessed a high Copper-chelating activity (CC≈95%), which may be due to peptide size and amino acid compositions. In this sense, 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. However, the content of His in PF is relatively low, but the content of other chelating amino acids such as Asp and Glu is high. At pH 7.0, carboxyl residues of acidic amino acids (Asp, pKa = 3.86; Glu, pKa = 4.25) are charged forming anions (Saiga, Tanabe, & Nishimura, 2003). Thus, these residues should be involved in the formation of complexes with Cu^{2+} and thus suppress β-carotene oxidation.

It should be noted that the antioxidant capacity of PF could be due to the presence of phycobiliproteins. In this sense, Bermejo, Pinero, and Villar (2008) demonstrated the antioxidant properties of the phycobiliprotein phycocyanin isolated from a protein extract of the green alga *Spirulina platensis* and suggest these bioactive properties are attributed to protein's ability to chelate metal and to scavenge free radicals.

4. Conclusions

Many bioactive peptides have been discovered from enzymatic hydrolysates of different food proteins, but so far, there has been no research focused on phycobiliproteins byproducts of *P. columbina*. The proteolysis is a good alternative to obtain bioactives peptides from phycobiliproteins byproducts of *P. columbina*. Here we have demonstrated that PFH rich in low molecular weight peptides exhibited immunosuppressive, antihypertensive, and antioxidant properties. Thus, hydrolysis could be used as a mean to obtain bioactive peptides from algae phycobiliproteins byproducts and add value to extraction process of phycocolloids.

Acknowledgments

This study was partially supported by the Ministry of Science and Innovation (SAF2011-22812, AGL2008-04332), by funds from Junta de Andalucía (CTS-6736, CTS235 and CTS164), and by the Fundación Ramón Areces. REC was partially supported by ERASMUS MUNDUS EXTERNAL COOPERATION WINDOW. The authors ALSO thank the project CAI + D 2009-PI-54-258 of the Universidad Nacional del Litoral for the partial support of this research in Argentina and EADIC — LOTE 16. CIBERehd is funded by the Instituto de Salud Carlos III.

REC conducted research. OMA and SRD designed research. REC, OMA and SRD wrote the paper and had primary responsibility for final content. All authors read and approved the final manuscript. The authors are thankful to PhD Javier Vioque, PhD Manuel Alaiz, Biochem. Franco Van de Velde and PhD Mercedes González for the technical assistance.

References

Alaiz, M., Navarro, J., Giron, J., & Vioque, E. (1992). Amino acid analysis by high-performance liquid chromatography after derivatization with diethylethoxymethylenemalonate. *Journal of Chromatography. A*, *591*, 181–186.

Bermejo, P., Pinero, E., & Villar, A. M. (2008). Iron chelating ability and antioxidant properties of phycocyanin isolated from a protein extract of *Spirulina platensis*. *Food Chemistry*, *110*, 436–445.

Brand-Williams, W., Cuvelier, M., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft und Technologie*, *28*, 25–30.

Cao, G., Alessio, H., & Cutler, R. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology & Medicine*, *14*, 303–311.

Cian, R., López-Posadas, R., Drago, S., Sánchez de Medina, F., & Martínez-Augustin, O. (2012). A *Porphyra columbina* hydrolysate upregulates IL-10 production in rat macrophages and lymphocytes through an NF-κB, and p38 and JNK dependent mechanism. *Food Chemistry*, *134*, 1982–1990.

Dwyer, J., & Johnson, C. (1981). The use of concanavalin A to study the immunoregulation of human T cells. *Clinical and Experimental Immunology*, *46*, 237–249.

Feng, L., Tang, W., Chang, J., & Wilson, C. (1993). Molecular cloning of rat cytokine synthesis inhibitory factor (IL-10) cDNA and expression in spleen and macrophages. *Biochemical and Biophysical Research Communications*, *192*, 452–458.

Fitzgerald, C., Gallagher, E., Tasdemir, D., & Hayes, M. (2011). Heart health peptides from macroalgae and their potential use in functional foods. *Journal of Agricultural and Food Chemistry*, *59*, 6829–6836.

Fleurence, J. (1999). Seaweed proteins: biochemical, nutritional aspects and potential uses. *Trends in Food Science and Technology*, *10*, 25–28.

Galland-Irmouli, A., Fleurence, J., Lamghari, R., Luçon, M., Rouxel, C., Barbaroux, O., Bronowicki, J., Villaume, C., & Guéant, J. (1999). Nutritional value of proteins from edible seaweed *Palmaria palmate* (Dulse). *The Journal of Nutrition and Biochemistry*, *10*, 353–359.

Gómez-Ordóñez, E., Jiménez-Escrig, A., & Rupérez, P. (2010). Dietary fibre and physicochemical properties of several edible seaweeds from the northwestern Spanish coast. *Food Research International*, *43*, 2289–2294.

Halprin, K., & Ohkawara, A. (1966). Carbohydrate metabolism in psoriasis: an enzymatic study. *The Journal of Investigative Dermatology*, *47*, 222–229.

Hayakari, M., Kondo, Y., & Izumi, H. (1978). A rapid and simple spectrophotometric assay of angiotensin-converting enzyme. *Analytical Biochemistry*, *84*, 361–369.

Kaiko, G., Horvat, J., Beagley, K., & Hansbro, P. (2008). Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology*, *123*, 326–338.

Kim, S., & Wijesekaraa, I. (2010). Development and biological activities of marine-derived bioactive peptides: a review. *Journal of Functional Foods*, *2*, 1–9.

Megías, C., Pedroche, J., Yust, M., Girón-Calle, J., Alaiz, M., Millán, F., & Vioque, J. (2008). Production of copper-chelating peptides after hydrolysis of sunflower proteins with pepsin and pancreatin. *LWT- Food Science and Technology*, *41*, 1973–1977.

Mohamed, S., Hashim, S., & Rahman, H. (2012). Seaweeds: a sustainable functional food for complementary and alternative therapy. *Trends in Food Science and Technology*, *23*, 83–96.

Ngo, D., Wijesekara, I., Vo, T., Ta, Q., & Kim, S. (2011). Marine food-derived functional ingredients as potential antioxidants in the food industry: an overview. *Food Research International*, *44*, 523–529.

Nielsen, P., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science*, *66*, 642–646.

Niu, J., Wang, G., Zhou, B., Lin, X., & Chen, C. (2007). Purification of R-phycoerythrin from *Porphyra haitanensis* (Bangiales, Rhodophyta) using expanded-bed absorption. *Journal of Applied Phycology*, *43*, 1339–1347.

Nuutila, A., Kammiovirta, K., & Oksman-Caldentey, K. (2002). Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. *Food Chemistry*, *76*, 519–525.

Qian, Z., Jung, W., & Kim, S. (2008). Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana Shaw*. *Bioresource Technology*, *99*, 1690–1698.

Qu, W., Maa, H., Pan, Z., Luo, L., Wanga, Z., & He, R. (2010). Preparation and antihypertensive activity of peptides from *Porphyra yezoensis*. *Food Chemistry*, *123*, 14–20.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying and improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, *26*, 1231–1237.

Saiga, A., Tanabe, S., & Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *Journal of Agricultural and Food Chemistry*, *51*, 3661–3667.

Sampath-Wiley, P., & Neefus, C. (2007). An improved method for estimating R-phycoerythrin and R-phycocyanin contents from crude aqueous extracts of *Porphyra* (Bangiales, Rhodophyta). *Journal of Applied Phycology*, *19*, 123–129.

Sato, M., Hoskawa, T., Yamaguchi, T., Nakano, T., Muramoto, K., & Kahara, T. (2002). Angiotensin I-converting enzyme inhibitory peptides derived from wakame (*Undaria pinnatifida*) and their antihypertensive effect in spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry*, *50*, 6245–6252.

Sheih, I., Fang, T., & Wu, T. (2009). Isolation and characterization of a novel angiotensin I converting enzyme (ACE) inhibitory peptide from the algae protein waste. *Food Chemistry*, *115*, 279–284.

Siriwardhana, N., Kim, K., Lee, K., Kim, S., Ha, J., Song, C., Lee, J., & Jeon, Y. (2008). Optimization of hydrophilic antioxidant extraction from *Hizikia fusiformis* by integrating treatments of enzymes, heat and pH control. *International Journal of Food Science and Technology*, *43*, 587–596.

Souza, B., Cerqueira, M., Bourbon, A., Pinheiro, A., Martins, J., Texeira, J., Coimbra, M., & Vicente, A. (2012). Chemical characterization and antioxidant activity of sulfated

polysaccharide from the red seaweed *Gracilaria biridiae*. *Food Hydrocolloids*, *27*, 287–292.

Tarola, A., Van de Velde, F., Salvagni, L., & Preti, R. (2012). Determination of phenolic compounds in strawberries (*Fragaria ananassa* Duch) by high performance liquid chromatography with diode array detection. *Food Analytical Methods*. http://dx.doi.org/10.1007/s12161-012-9431-5.

Torres-Fuentes, C., Alaiz, M., & Vioque, J. (2011). Affinity purification and characterization of chelating peptides from chickpea protein hydrolysates. *Food Chemistry*, *129*, 485–490.

Tsopmo, A., Cooper, A., & Jodayree, S. (2010). Enzymatic hydrolysis of oat flour protein isolates to enhance antioxidative properties. *Advance Journal of Food Science and Technology*, *2*, 206–212.

Wang, T., Jónsdóttir, R., Kristinsson, H., Hreggvidsson, G., Jónsson, J., Thorkelsson, G., & Ólafsdóttir, G. (2010). Enzyme-enhanced extraction of antioxidant ingredients from red algae *Palmaria palmata*. *LWT- Food Science and Technology*, *43*, 1387–1393.

Wijesekara, I., Qian, Z., Ryu, B., Ngo, D., & Kim, S. (2011). Purification and identification of antihypertensive peptides from seaweeds pipefish (*Syngnathus schlegeli*) muscle protein hydrolysate. *Food Research International*, *44*, 703–707.

Wilson, J., Hayes, M., & Carney, B. (2011). Angiotensin-I-converting enzyme and prolyl endopeptidase inhibitory peptides from natural sources with a focus on marine processing by-products. *Food Chemistry*, *129*, 235–244.

Yust, M., Pedroche, J., Girón-Calle, J., Vioque, J., Millán, F., & Alaiz, M. (2004). Determination of tryptophan by high-performance liquid chromatography of alkaline hydrolysates with spectrophotometric detection. *Food Chemistry*, *85*, 317–320.