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Enzyme proteolysis enhanced extraction of ACE inhibitory and antioxidant compounds (peptides and polyphenols) from *Porphyra columbina* residual cake

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Abstract The traditional method to obtain phycocolloids from seaweeds implies successive extraction steps with cold and hot water. The residual cake derived from phycocolloids obtaining process of red seaweed *Porphyra columbina* is a waste containing 27 % protein and 10.7-mg gallic acid equivalents $(100\ g)^{-1}$. Seaweeds contain functional proteins, and the enzymatic hydrolysis of these proteins has been shown to release bioactive peptides. The aims of this study were to extract bioactive peptides and polyphenols after enzymatic hydrolysis of the residual cake and to evaluate their ACE inhibitory and antioxidant capacities (TEAC, DPPH, and copper-chelating activity). Residual cake hydrolysate has low molecular weight peptides containing Asp, Glu, Ala, and Leu. Residual cake hydrolysate had higher protein solubility than residual cake. ACE inhibition (≈45 %) and radical scavenging activity (TEAC and DPPH inhibition) were attributed mainly to low molecular weight peptides (500 Da) and polyphenols compounds released during proteolysis. The 50 % inhibition protein concentration value (IC50) corresponded to residual cake hydrolysate was 1.01±0.02 and 0.91±0.01 g L^{-1}, for ABTS and DPPH, respectively. Also, residual cake hydrolysate had high copper-chelating activity (≈97.5 %). Hydrolysis could be used as a means to obtain ACE inhibitory and antioxidant compounds (peptides and polyphenols) from algae protein waste and add value to the phycocolloids extraction process.

Keywords Red seaweeds · Rhodophyta · Residual cake · Hydrolysates · ACE inhibitory activity · Antioxidant capacity

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Introduction

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 (e.g., Mohamed et al. 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 antioxidant properties or the production of functional peptides through enzyme-mediated hydrolysis in batch reactors (Ngo et al. 2011). The application of hydrolytic enzymes has shown a great potential to improve the extraction yield, enhance the release of secondary plant metabolites, and preserve the bioactive properties of the extracts. Enzymatic extraction has also been reported to increase the extractability of bioactive compounds from several brown algae (Wang et al. 2010).

At present, various ACE inhibitory peptides have been isolated from different marine protein sources such as algae protein (Sheih et al. 2009), shark meat (Wu et al. 2008), and oyster protein (Wang et al. 2008). These peptides have proven to be safer than synthetic ones (Qu et al. 2010). Also, phlorotannins from brown alga *Ecklonia cava* have shown to possess ACE inhibitory activity (Athukorala and Jeon 2005). Antioxidant peptides from marine food sources also are gaining attention as new antioxidant alternatives (Sheih et al. 2009), and algal polyphenols have shown to possess radical scavenging activities (Wang et al. 2009).

Porphyra columbina is a red edible macroalga found on hard substratum in Patagonia Argentina coasts (Pérez et al. 2007), mainly used as food. The residual cake derived from phycocolloid-obtaining process of red seaweed *P. columbina* is a waste, and there are no reported studies about ACE inhibitory and antioxidant activity of peptides or polyphenols derived from it. The underutilized residual cake contains polyphenols and more than 27 % protein, but has low economical value as animal feed due to its low protein digestibility (≈59 %). However, the protein and polyphenol content makes the residual cake a potential source of ACE inhibitory and antioxidant bioactive compounds. The use of proteolytic enzymes is aimed basically at the collection of peptides. However, proteolysis can release other bioactive compounds such as polyphenols bound to proteins. For example, Wang et al. (2010) noted that the enzymatic proteolysis of *Palmaria palmata* enhanced the extraction of polyphenols and other bioactive components.

The aim of the present study was to use the enzymatic hydrolysis to obtain bioactive peptides from the residual cake of *P. columbina*. The potential of enzyme treatments to obtain bioactive compounds from underutilized residual cake was evaluated by ACE inhibition, ABTS inhibition, DPPH radical scavenging, and copper-chelating activity.

Materials and methods

Flavourzyme was obtained from Sigma Chemical Co. (USA), and fungal protease concentrate was provided by Genencor (Arroyito, Argentina). The other reagents were obtained from Sigma Chemical Co.

Sampling and preparation of hydrolysate from residual cake

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 in plastic bags. To remove adherent seawater, sediment, organic debris, macrofauna, and epibiota, the algae were scraped and rinsed with distilled water. *P. columbina* samples were dried to constant weight (100± 4 °C) and ground to obtain a powder with a particle size lower than 1 mm, using a laboratory hammer mill. Then, samples were passed through a 0.85-mm mesh sieve and stored at 4 °C in plastic bags until analysis.

P. columbina was dispersed at 50 g kg^{-1} in distilled water and then centrifuged at 3,000×*g* for 30 min. The pellet was subjected to various extraction steps with cold and hot water. This methodology is the traditional approach intended to obtain phycocolloids. Subsequently, the residual cake was hydrolyzed. The criteria for the selection of suitable enzymes was made taking into account a possible change to industrial scale, so highly available commercial ones, with proper capacity to hydrolyze this substrate, were selected. We used an acid protease (fungal protease concentrate) because the alkaline protease tested showed low activity toward this substrate. In addition, an exoprotease (Flavourzyme) was added in a sequential way, in order to favor amino acid release. It is appropriate to note that Flavourzyme has endopeptidase and exopeptidase activity, which allows a high degree of hydrolysis (DH) to be obtained (In et al. 2002).

The enzymatic hydrolysis parameters were selected according to manufacturer's guidelines and previous assays. The reaction time was selected to obtain a high DH in a short time, since although it is possible to obtain higher DH with longer times, the trend of DH vs. time is a plateau. Working conditions for the enzymes were temperature, 55 °C; pH4.3; E/S ratio, 50 g kg^{-1} and temperature, 55 °C; pH7.0; E/S ratio, 20 g kg^{-1} for fungal protease concentrate and Flavourzyme, respectively. Residual cake hydrolysate was prepared using fungal protease concentrate enzyme for 3 h and then Flavourzyme enzyme for 4 h sequentially, with a total reaction time of 7 h.

Once the hydrolysis was complete, the enzymes were inactivated by thermal treatment following the manufacturer guidelines. The inactivated hydrolysate was centrifuged at 2,000×*g* for 30 min, and the supernatant was frozen and lyophilized. Free amino groups were measured using OPA, according to Nielsen et al. (2001), and the degree of hydrolysis (DH) was calculated as follows:

$$DH(\%) = \left[\frac{(h - h_0)}{h_{tot}}\right] \times 100$$

where h_{tot} is the total number of peptide bonds in the protein substrate calculated from amino acids profile (8.47 mEq g^{-1} protein), h is the number of peptide bonds cleaved during hydrolysis, and h_0 is the content of free amino groups in the substrate.

Residual cake and residual cake hydrolysate characterization

Protein content was determined using AOAC (1995) procedures. The elemental composition (C, H, N, S, and O) was determined using LECO microanalyzer (Leco Corporation, USA).

Protein solubility was measured at different pH (from 2.0 to 11.0) according to Drago and González (2001) and expressed as the percentage of proteins in the soluble fraction with respect to the protein content of the sample. Protein content of residual cake hydrolysate was determinate by Lowry method (Lowry et al. 1951), using bovine serum albumin as standard.

For amino acid analysis, samples (2 mg) were hydrolyzed with 4 mL of 6 N 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 et al. (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, USA) 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 % *w*/*v*) pH6.0 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).

Gel filtration chromatography was carried out in an AKTA purifier system equipped with a Superdex peptide column (GE Life Sciences, USA). Injection volume was 200 μL (10 mg protein PRC and its hydrolysate m L^{-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 (6,512 Da), bacitracin (1,450 Da), cytidine (246 Da), and glycine (75 Da).

Three different extraction systems were used to determine the free phenolic content of residual cake and residual cake hydrolysate: methanol (100), acetone:water (80:20), and acetone:water:acetic acid (80:15:5). Residual cake and residual cake hydrolysate were dispersed at 20 % (*w*/*w*) and 5 % (*w*/*w*) in each system, respectively. The extracts were stirred for 30 min and then centrifuged at 8,000×*g*. Free phenolic content from supernatant was quantified according to Schanderl (1970) with modifications, using Folin–Ciocalteu reagent. A standard curve with serial gallic acid solutions was used for calibration. Results were expressed as mg gallic acid equivalents g^{-1} of extract.

Bioactive properties of residual cake hydrolysate

Angiotensin-converting enzyme (ACE) inhibition ACE was prepared according to Hayakari et al. (1978) with modifications. Rabbit lungs were used as starting material. Tissue samples (1 g) were diced and homogenized in 5-mL ice-cold 0.1-M potassium phosphate buffer, pH8.3, containing 0.25 M sucrose and 0.1 mM PMSF. The homogenate was centrifuged at 12,000×*g* for 5 min, and the resulting supernatant was used as the source of ACE.

ACE activity was determined according to Hayakari et al. (1978) with modifications. This method relies on the colorimetric reaction of hippuric acid with 2,4,6-trichloro[1,3,5] triazine (TT), developed in a 1.995-mL incubation mixture containing a 175-μL 0.1-M potassium phosphate buffer, pH8.3, 15 μL sodium chloride (5 M), 20 μL of HHL (3.3 g L^{-1}), and 20 μL of enzyme extract. Incubation was carried out for 45 min at 37 °C. The reaction was completed by the addition of 665 μL of TT (30 g L^{-1}) in dioxane, followed by 1.1 mL of 0.1-M phosphate buffer, pH8.3. The reaction mixtures were centrifuged at 12,000×*g* for 10 min, and the absorbance measured at 382 nm. The ACE inhibitory activity of residual cake hydrolysate was evaluated in 20 μL of 2.37 g L^{-1} of protein. ACE inhibitory activity expressed as ACE inhibition (%) was calculated according to Cian et al. (2011).

Antioxidant capacity For ABTS radical scavenging assay and copper-chelating activity, the residual cake hydrolysate was dissolved in distilled water, while for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals scavenging activity assay, the residual cake hydrolysate was dissolved in acetone:water (80:20).

To estimate the antioxidant capacity, $ABTS^{\bullet+}$ radical cation decolorization assay was used. The inhibition rate of $ABTS^{\bullet+}$ radical cation was calculated according to Cian et al. (2011). To estimate the Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalent antioxidant capacity (TEAC), a concentration–response curve for $ABTS^{\bullet+}$ absorbance at 734 nm as a function of 0–3.5 mmol L^{-1} Trolox concentration was performed. The absorbance reading was taken at 6 min after initial mixing. TEAC from residual cake hydrolysate was determined at 1.8 g L^{-1} of protein. To determine the concentration causing an inhibition of 50 % (IC50%), serial dilutions of residual cake hydrolysate from 0 to 2.37 g L^{-1} protein were

made, and the data were fitted with the following equation:

$$y = a - (bc^x)$$

where y is the inhibition rate; a, b, and c are the regression parameters; and x is the protein concentration (g L^{-1}). The IC50% value was obtained as:

$$IC_{50} = \ln[(a - 50\%)/b]/\ln c$$

DPPH radical scavenging activity was measured according to the method of Sánchez-Moreno et al. (1998). An aliquot of 10 μL of residual cake hydrolysate at 0.9 g L^{-1} of protein was mixed with 190 μL of methanol solution containing the 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 at 517 nm between the blank and the sample was used for calculating the scavenging activity as inhibition (%). As a positive control, 50.5 μmol L^{-1} ascorbic acid was used. To determine the concentration causing a DPPH inhibition of 50 % (IC50%), serial dilutions of residual cake hydrolysate from 0 to 0.9 g L^{-1} protein were made and the data fitted with the following equation:

$$y = a - bx$$

where y is the inhibition rate, a and b are the regression parameters, and x is the protein concentration (g L^{-1}). The IC50% value was obtained as:

$$IC_{50} = (a - 50\%)/b$$

The copper-chelating activity was determined by the assay of β-carotene oxidation according to Megías et al. (2008). A solution of β-carotene was prepared by dissolving 10 mg in 1 mL of chloroform. After addition of 1 mL Tween 20, chloroform was evaporated under nitrogen. Aliquots from this solution were dissolved in 0.1-N pH7.0 phosphate buffer. A 50-μmol L^{-1} solution of $CuSO_4$ was prepared, and 10 μL were added to each well. The assay mixture was 200 μL β-carotene, 10 μL $CuSO_4$, and 200 μL residual cake hydrolysate. The concentration of residual cake hydrolysate was 1 g L^{-1} of protein. The degradation of β-carotene was monitored by recording the decrease in absorbance at 470 nm. A positive control, 200 μL β-carotene+10 μL $CuSO_4$, and a negative control, 200 μL β-carotene+10 μL buffer phosphate, were run.

Statistical analysis

All analyses were performed in triplicate from each sample (n=3), and results are expressed as mean±SD. The experimental data from each analysis were analyzed by one-way analysis of variance, using the software Statgraphics Plus 3.0. The statistical differences between samples were determined using the least significant difference (LSD) test. The significance was established at P<0.05.

Results and discussion

Residual cake and residual cake hydrolysate characterization

Table 1 shows the protein content and elemental composition of residual cake and residual cake hydrolysate. 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 and Ching 2000). The protein content of residual cake was similar to that obtained for *P. columbina* (24.09±1.0 g100 g^{-1} dw). This higher value in residual cake may be due to high fiber content of *P. columbina* (48.02±1.13 g100 g^{-1} dw), which could interact strongly with the protein (Urbano and Goñi 2002) and prevent its extraction.

The degree of hydrolysis (DH) from residual cake hydrolysate obtained with the enzymatic system used (fungal protease concentrate+Flavourzyme) was 15.04±0.29 %. This high DH 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 endoprotease alone (In et al. 2002). The production of protein hydrolysates by sequential action of endoprotease and exopeptidase coupled with the development of post-hydrolysis procedures is considered the most effective way to obtain protein hydrolysates with defined characteristics (Kong et al. 2008).

As shown in Table 1, S, C, H, and protein content from residual cake and residual cake hydrolysate were different because residual cake hydrolysate is the soluble fraction obtained by hydrolysis.

Table 2 shows the amino acids profile of residual cake and residual cake hydrolysate. Aspartic acid, glutamic acid, alanine, and leucine were the most abundant amino acids in residual cake and residual cake hydrolysate. For most

Table 1 Protein content and elemental composition of residual cake and residual cake hydrolysate

Components	Residual cake (g (100 g)$^{-1}$ dw)	Residual cake hydrolysate (g (100 g)$^{-1}$ dw)
S	1.8±0.1^a	2.1±0.1^b
C	37.2±0.2^a	40.8±0.3^b
H	6.8±0.1^a	9. 5±0.1^b
N	4.1±0.1^a	4. 4±0.2^a
Protein (Kjeldahl) (factor=6.25)	25.5±0.3^a	27.4±1.0^b

Means±SD (n=3)

dw dry weight

Different letters in the same row means significant differences between samples (p<0.05)

Table 2 Amino acids (AA) profile of residual cake and residual cake hydrolysate, and the ratio of AA residual cake hydrolysate to AA residual cake

Amino acids	Total amino acids of residual cake (g $(100\ g)^{-1}$ protein)	Total amino acids of residual cake hydrolysate (g $(100\ g)^{-1}$ protein)	Ratio AA residual cake hydrolysate/ AA residual cake
Asp	11.3±0.2	12.3±0.1	1.1
Glu	9.0±0.2	10.3±0.0	1.1
Ser	6.7±0.0	6.4±0.1	1.0
His	1.2±0.0	1.0±0.0	0.8
Gly	9.7±0.1	9.6±0.2	1.0
Thr	6.2±0.0	6.4±0.0	1.0
Arg	6.8±0.1	6.4±0.1	0.9
Ala	10.3±0.1	12.1±0.1	1.2
Pro	3.4±0.2	3.5±0.2	1.0
Tyr	2.9±0.0	2.3±0.0	0.8
Val	6.7±0.0	6.8±0.0	1.0
Met	1.9±0.1	1.5±0.1	0.8
Cys	1.7±0.0	0.9±0.0	0.5
Ile	3.2±0.0	2.9±0.1	0.9
Trp	0.3±0.0	0.64±0.0	2.1
Leu	8.4±0.0	8.0±0.0	1.0
Phe	4.3±0.0	3.6±0.1	0.8
Lys	6.0±0.0	5.4±0.0	0.9

Means±SD (n=3)

seaweed, acidic amino acids constitute a large part of the amino acid fraction (Fleurence 1999). In this waste product (residual cake) and in its hydrolysate (residual cake hydrolysate), the sum of acidic amino acids was 20.3 and 22.6 $(100\ g)^{-1}$, respectively. Similar results were obtained for other red seaweeds such as *Porphyra acanthophora* ($19.5(100\ g)^{-1}$ of total amino acids; Lourenço et al. 2004) and *Gracilaria birdiae* (22/100 g of total amino acids; Gressler et al. 2010). The predominance of acidic amino acids over basic amino acids is typical of red seaweed (Mabeau et al. 1992). Alanine and the essential amino acid leucine were present in relatively high levels in residual cake and residual cake hydrolysate. These results were similar to those found by Galland-Irmouli et al. (1999) for *P. palmata* (Ala, 12.2/100 g total amino acids and Leu, $8.2(100\ g)^{-1}$ total amino acids).

The ratio of residual cake hydrolysate to residual cake for the content of each amino acid is approximately 1.0. Therefore, for each amino acid, its content in residual cake hydrolysate is similar to that obtained for residual cake. However, the content of Trp in residual cake hydrolysate was higher than residual cake (2.1), while the Cys content in residual cake hydrolysate was lower (0.5).

Figure 1 shows the FPLC gel filtration profile of residual cake and residual cake hydrolysate. Residual cake profile

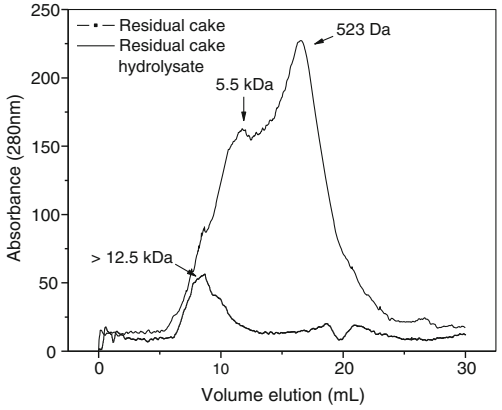

Fig. 1 FPLC gel filtration profile of *Porphyra columbina* waste (residual cake) and its hydrolysate (residual cake hydrolysate). The profile shown is representative of several that were carried out

shows one main peak corresponding to components with molecular weight (MW) higher than 12.5 kDa (proteins) since their components presented a volume elution lower than that corresponding to volume exclusion. These components have a molecular weight around 20 kDa observed in SDS-PAGE electrophoresis (data not shown). The profile of residual cake hydrolysate exhibited two main peaks. The first one corresponds to peptide fractions with molecular weight (MW) around 5.5 kDa and the second corresponds to peptides of low MW (500 Da). Considering 120 Da the average MW of amino acids, this fraction could be formed by tetrapeptides.

Table 3 shows the protein solubility of residual cake and residual cake hydrolysate at different pH (from 2.0 to 11.0). For residual cake, water solubility was very low in all evaluated pH. This low solubility is expected since residual cake is the product of extraction process which combines different extractive stages with cold and hot water. This process favors the solubilization and extraction of soluble proteins in water. In this way, in residual cake, there are only proteins linked mainly to fiber or other components, which affect its water solubilization.

Solubility of residual cake hydrolysate is significantly higher than that of the substrate. This could be explained taking into account that hydrolysis promotes exposition of more polar groups and also generates new polypeptides molecules of smaller size than that of substrate, and consequently, solubility is increased (Kabirullah and Wills 1981). Also, when the algal cell wall or its components are disrupted, the constituents including proteins are released (Wang et al. 2010).

Figure 2 shows free phenolic contents of residual cake and residual cake hydrolysate (a and b, respectively). For both samples, the lowest values of phenolic content were obtained with methanolic extraction system, and the highest value of phenolic content was obtained with the acetone/

Table 3 Protein solubility of residual cake and residual cake hydrolysate at different pH (from 2.0 to 11.0)

pH	Residual cake solubility	Residual cake hydrolysate solubility
2.0	0.0±0.0[a]	33.8±0.3[b]
4.0	0.1±0.0[a]	33.6±0.1[b]
6.0	0.2±0.0[a]	35.5±0.0[b]
8.0	0.4±0.0[a]	34.9±0.1[b]
11.0	1.3±0.1[a]	34.9±0.1[b]

Means±SD (n=3)

Different letters in the same row means significant differences between samples ($p<0.05$)

water extraction system. Therefore, this system is most suitable to extract phenolic compounds from residual cake and its hydrolysate. These results were similar to that found by Uma et al. (2010) for *Lawsonia inermis*. In this case, free phenolic content for acetone/water (80:20) extraction system was higher than that obtained for acetone alone. Similar results have been observed in others vegetables; Turkmen et al. (2007) obtained higher yields of polyphenol extraction for black tea with acetone/water, ethanol/water, and methanol/water extraction system than pure solvents. Finally, Chew et al. (2008) found for three seaweeds (*Panaeolus antillarum*, *Caulerpa racemosa*, and *Kappaphycus alvarezii*) higher values of polyphenol content with methanol/water extraction system (50:50) than methanol alone (100 %). The addition of small quantity of water to organic solvent usually creates a more polar medium, which facilitate the extraction of polyphenols (Uma et al. 2010). The mixture

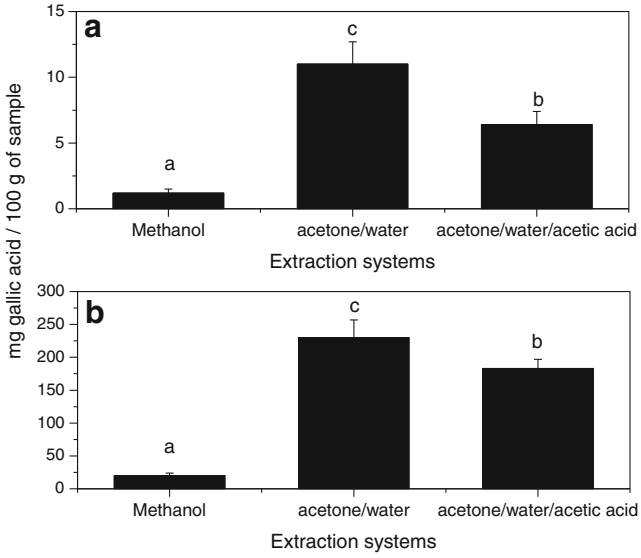

Fig. 2 Total polyphenolic content from residual cake (**a**) and residual cake hydrolysate (**b**) with different extraction systems (100 % methanol; 80 % acetone/20 % water; and 80 % acetone/15 % water/5 % acetic acid). Data are expressed as mean±SD; *letters* with $P<0.05$ means significant differences

acetone/water is able to extract phenolic compounds with the highest and lowest polarity as well as those of moderate polarity (Zhang et al. 2007), resulting in a higher content of polyphenols extracted from residual cake and residual cake hydrolysate.

As shown in Fig. 2, the free polyphenols extracted with acetone/water system were higher for residual cake hydrolysate than for residual cake. As a result of protease treatment, peptides and polyphenols interactions decrease, and hence, trapped polyphenols with proteins are more easily extracted. This result was also observed by Wang et al. (2010) for the red alga *P. palmata* hydrolysates.

Bioactive properties

The bioactive properties of residual cake could not be assessed due to the low solubility of the sample (Table 3). Hence, only the bioactive properties of residual cake hydrolysate were evaluated.

Residual cake hydrolysate showed a relatively good ACE inhibitory activity (45.65±0.95 % of ACE inhibition). This value was higher than the observed by Khantaphant et al. (2011), for a protein hydrolysate from *Lutjanus vitta* produced by sequential hydrolysis (Alcalase for 2 h plus Flavourzyme for 2 h). Murakami et al. (2004) found that peptide WE80BG derived from whey proteins showed the strongest anti-hypertensive activity, with a medium level of ACE inhibitory activity (53.6 %). This peptide was purified by reverse-phase HPLC, and it was found to be a tetrapeptide (Ala-Leu-Pro-Met) with 570 Da. Hence, the fraction of 500 Da (tetrapeptides) from residual cake hydrolysate may be primarily responsible for ACE inhibition. Also, Suetsuna and Chen (2001) isolated a tetrapeptide Ile-Val-Val-Glu with ACE inhibitory activity from a hydrolysate of *Chlorella vulgaris*. As ACE-I inhibitory peptides are usually between 2 and 30 amino acids in size (Wilson et al. 2011), our peptide fraction of 5.5 kDa (≈45 amino acids) would not be responsible for ACE inhibition.

The ACE-preferred substrates contain 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). The hydrophilic amino acid residues in the peptide sequence may 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 et al. 2009). 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. Residual cake hydrolysate has a high content of Asp, Glu, Ala, and a relatively high content of Leu (Table 2). Also, Trp content from residual cake hydrolysate was higher than residual cake. The abundance of the above-mentioned amino acids in the peptides from hydrolysate could be responsible for their ACE inhibition.

The free polyphenolic content of residual cake hydrolysate in water was 4.4 mg gallic acid g^{-1} hydrolysate. Polyphenolic compounds also inhibit ACE activity through sequestration of the enzyme metal factor, Zn^{2+} ion (Wijesekara and Kim 2010). Hence, polyphenolic compounds released during proteolysis of residual cake might form a complex with small peptides and inhibit ACE activity. Athukorala and Jeon (2005) have reported that Flavourzyme enzymatic digest of brown seaweed *E. cava*, which contains high content of phlorotannins, is a potent ACE inhibitor. They have hydrolyzed seven marine brown algal species and analyzed ACE inhibitory activities. Most of algal species showed potent ACE inhibitory activities. However, *E. cava* was the most potent ACE inhibitor among them due to its rich content in polyphenols (Ngo et al. 2011). Hence, ACE inhibitory activity observed in residual cake hydrolysate may be due to the sum of the activity of produced peptides and released polyphenols.

Antioxidant capacity

ABTS•$^{+}$ inhibition of residual cake hydrolysate was 61.4± 0.9 % at 1.8 g L^{-1} of protein, and the Trolox equivalent antioxidant capacity (TEAC) value was 2.78±0.04 mmol Trolox L^{-1}. This antioxidant capacity may be due to the release of low molecular weight peptides (500 Da) and polyphenols during proteolysis process. Several studies have shown that low molecular weight hydrolysates generally possess higher radical scavenging capacity than high molecular weight hydrolysates (Wang et al. 2010). Dávalos et al. (2004) and Moosmann and Behl (2002) suggested that hydrolysates with high DH obtained with sequential proteases systems had higher proportion of low molecular weight peptides which would access more easily to the oxidant system and lead to high values of TEAC. Also, the hydrolysis process improves the extraction efficiency of polyphenolic compounds which have been reported to be potent radical scavengers (Ngo et al. 2011).

The TEAC value of residual cake hydrolysate was similar to those reported for other proteins sources. You et al. (2009) found a TEAC value of 2.5 mmol Trolox L^{-1} for fish protein hydrolysate (*Misgurnus anguillicaudatus*). However, You et al. (2010) found a higher TEAC value for gelatin hydrolysate (9.8 mmol Trolox L^{-1}) than that reported by us. This different TEAC values could be explained by the fact that antioxidant capacity of hydrolysates is affected by the amino acid sequence of the produced peptides, which depends on protease specificity (Cian et al. 2011).

Figure 3 shows absorbance at 517 nm vs. time, from DPPH, DPPH+residual cake hydrolysate, and DPPH+ ascorbic acid. The results for DPPH+residual cake hydrolysate at 0.9 g L^{-1} of protein show that as time reaction increases, absorbance at 517 nm decreases and then tends to a plateau when time is around 60 min. It should be noted

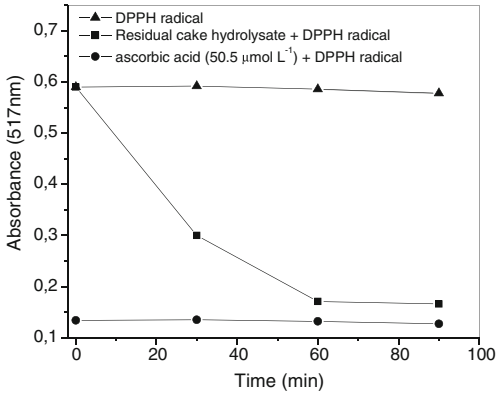

Fig. 3 DPPH radicals scavenging activity assay of residual cake hydrolysate. Residual cake hydrolysate was evaluated at 0.9 g L^{-1} of protein and ascorbic acid at 50.5 μmol L^{-1}. The reaction time was 90 min, and the absorbance was reading at 517 nm

that DPPH is a stable free radical with a maximum absorbance at 517 nm in methanol. When DPPH encounters a proton-donating substance, the radical is scavenged and the absorbance is reduced (You et al. 2009). Therefore, the results indicated that the residual cake hydrolysate acted as a good electron donor and could react with free radicals to terminate the radical chain reaction. However, ascorbic acid (50.5 μmol L^{-1}) reduces instantly DPPH radical, and absorbance at 517 nm decreased immediately (Fig. 3). This means that residual cake hydrolysate and ascorbic acid have a very different behavior as antioxidant agents.

DPPH inhibition for residual cake hydrolysate was 49.4± 2.4 % at 0.9 g L^{-1} of protein. As mentioned above, the radical scavenging capacity of hydrolysate could be due to low molecular weight peptides (around 340 Da), free amino acids, and polyphenols released during the hydrolysis process. In this way, Sheih et al. (2009) observed an antioxidative peptide with DPPH radicals scavenging activity from algae protein waste hydrolysate. The MW of this peptide was 1,309 Da and its peptide sequence Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe. Also, Wang et al. (2009) reported a high correlation between total polyphenolic contents and DPPH radical scavenging activity from Icelandic seaweeds extracts, indicating an important role of algal polyphenols as chain-breaking antioxidants.

Figure 4a shows the influence of protein concentration of residual cake hydrolysate on ABTS•$^{+}$ inhibition. The results show that as protein concentration increases, inhibition rate of residual cake hydrolysate increases and then tends to a plateau when protein concentration is around 1.75 g L^{-1} of protein. This behavior was also observed in other protein sources such as alga protein waste (Sheih et al. 2009), wheat gluten (Wang et al. 2007), alfalfa leaves (Xie et al. 2008), and bovine hemoglobin (Cian et al. 2011). The IC_{50} value for residual cake hydrolysate was 1.01±0.02 g L^{-1} of protein. This value was lower than that reported by Ren et al.

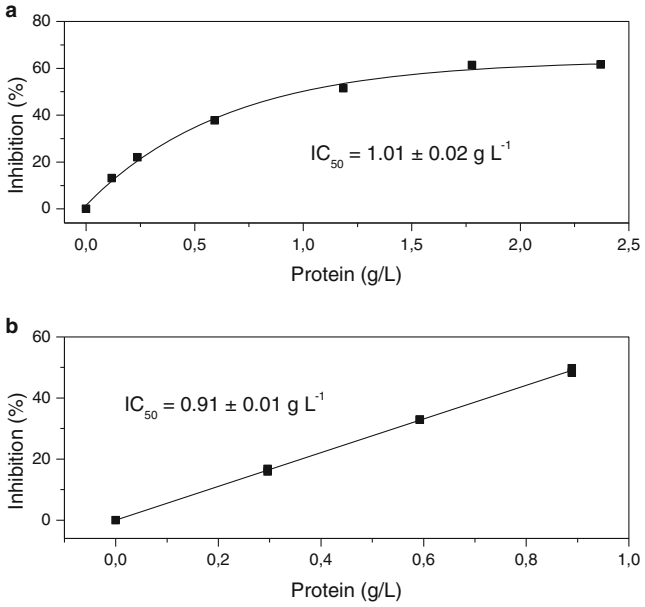

Fig. 4 Effect of residual cake hydrolysate protein concentration on ABTS inhibition (**a**) and DPPH inhibition (**b**). Residual cake hydrolysate protein concentration was evaluated from 0 to 2.37 g L^{-1} and 0 to 0.9 g L^{-1}, for ABTS and DPPH, respectively

(2008) for grass muscle hydrolysates (2.11±0.10 g L^{-1} of protein). However, Sheih et al. (2009) reported an IC_{50} value of 0.04 g L^{-1} of protein for microalgae *C. vulgaris* hydrolysate.

Figure 4b shows the influence of protein concentration of residual cake hydrolysate on DPPH inhibition. Residual cake hydrolysate showed a concentration-dependent increase of antioxidant activity for concentrations up to 0.9 g L^{-1} of protein. This linear behavior has been observed by other researchers for different hydrolysates (Song et al. 2008; Balti et al. 2011). The IC_{50} value was 0.91±0.01 g L^{-1} of protein and was lower than that reported by Song et al. (2008) for *Arca subcrenata* hydrolysate (6.23 g L^{-1} of protein).

Figure 5a shows the degradation of β-carotene (measured as decrease in absorbance at 470 nm) vs. time, for negative control (without Cu^{2+}), Cu^{2+}+residual cake hydrolysate, and positive control (with Cu^{2+}). The results for negative control show as time reaction increases, absorbance at 470 nm decreases slightly. This may be due to oxidative degradation of β-carotene as a result of autoxidation initiated by light in the presence of oxygen or by peroxides (Megías et al. 2008). However, for positive control, the absorbance at 470 nm decreases rapidly with the reaction time, indicating the strong oxidation of β-carotene in presence of copper. Residual cake hydrolysate showed a high inhibition of β-carotene oxidation in the presence of copper. The residual cake hydrolysate behavior was very similar to negative control, and there were no significant differences with negative control at 240 min (Fig. 5b).

Torres-Fuentes et al. (2011) reported that the combination of high His content (around 20–30 %) and small peptide size provides the best peptide chelating activities. However, the content of His in residual cake hydrolysate is relatively low. However, as previously mentioned, residual cake hydrolysate has high content of acid amino acids, which may be primarily responsible for the high chelating activities. At pH 7.0, carboxyl residues of acidic amino acids (Asp, p*K*a= 3.86; Glu, p*K*a=4.25) are charged to form anions (Saiga et al. 2003). Thus, these residues should be involved in the formation of complexes with Cu^{2+} making it unsuitable to catalyze lipid oxidation.

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). Therefore, the chelating activity could be mainly due to the peptides present in residual cake hydrolysate.

Antioxidative properties of peptides are related to their composition, structure, hydrophobicity, and molecular weight. Tyr, Trp, Met, Lys, Cys, and His are examples of amino acids having antioxidant activity. Also, it has been reported that Ala, Leu, and Pro (with non-polar aliphatic groups) have high reactivity to hydrophobic PUFAs radicals, and hydrogen donors such as Asp and Glu are able to quench unpaired electrons or radicals by supporting protons (Quian et al. 2008). Most of the reported peptides exhibiting antioxidative activity were those with low molecular

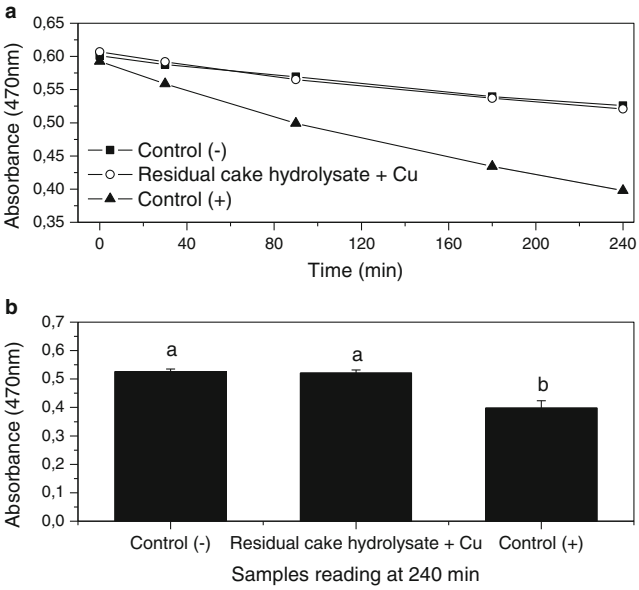

Fig. 5 Copper-chelating activity by assay of β-carotene oxidation of residual cake hydrolysate. **a** The reaction time was 240 min, and the absorbance was reading at 470 nm. Copper-chelating activity by assay of β-carotene oxidation of residual cake hydrolysate at 240 min and 470 nm (**b**). Residual cake hydrolysate was evaluated at 1.0 g L^{-1} of protein, and Cu^{2+} was aggregated at 50 μmol L^{-1}. Data are expressed as mean±SD; *letters* with $P<0.05$ indicate significant differences

weights (Sheih et al. 2009; Quian et al. 2008). In this sense, residual cake hydrolysate has peptides with low molecular weight of approximately four amino acids and also contains Asp, Glu, Ala, and a relatively high content of Leu, which would be responsible of antioxidant capacity.

Finally, in order to better understand the contribution of peptides and polyphenols to the overall ACE and antioxidant activity, further fractionation, such as ultrafiltration process, should be done.

In conclusion, we have demonstrated that the proteolysis is a good alternative to obtain bioactive peptides and to increase the extraction of other compounds like polyphenols from residual cake of *P. columbina*, which exhibited ACE inhibitory and antioxidant properties. As proteolytic enzymes used are commercially available in large amounts, it could be possible to scale up this process and add value to a phycocolloid process waste.

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Conflicts of interest The authors declare no conflicts of interest.

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