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DETERMINATION OF ANTIOXIDANT AND CHELATING ACTIVITY OF PROTEIN HYDROLYSATES FROM SPIRULINA (*Arthrospira maxima*) OBTAINED BY SIMULATED GASTROINTESTINAL DIGESTION

DETERMINACIÓN DE ACTIVIDAD ANTIOXIDANTE Y QUELANTE DE HIDROLIZADOS PROTEICOS DE ESPIRULINA (*Arthrospira maxima*) OBTENIDOS POR SIMULACIÓN DE DIGESTIÓN GASTROINTESTINAL

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Abstract

Spirulina is a cyanobacteria that has been used as food since ancient times, for example in Mexico it was consumed by the Aztecs. Its high protein content, distribution and amino acid composition suggests the presence of important peptides encrypted within the sequences of parent proteins, that after been released by digestive process they could show an antioxidant effect. Our present study examined the above hypothesis through the determination of the antioxidant and chelating activity of two Spirulina samples (SpRPh: Spirulina reduced of pholyphenols and PCBEx: extract of phycobiliproteins), subjected both to sequential hydrolysis with pepsin and pancreatin. At the end of the enzymatic action, extensive hydrolysates with a degree of hydrolysis (% DH) of 31.4 and 36.7%, for SpRPh and PCBEx respectively, were obtained. By determining the electrophoretic profiles, the degradation of characteristic bands of Spirulina proteins and the release of smaller peptides were observed. As a general trend, the antioxidant activity determined by different methods improved after simulating gastrointestinal digestion. On the other hand, protein hydrolysates from both groups showed Cu^{2+} and Fe^{2+} chelating activity.

Keywords: spirulina, protein hydrolysates, antioxidant activity, chelating activity.

Resumen

La espirulina es una cianobacteria que se ha utilizado en México como alimento desde la época de los aztecas. Su alto contenido de proteína, composición y secuencia de amino ácidos sugiere la presencia de péptidos encriptados dentro de las proteínas nativas, que después de ser liberados por la digestión gastrointestinal, pueden ejercer un efecto antioxidante. En el presente trabajo, se determinó la actividad antioxidante y quelante de dos muestras de espirulina (SpRPh: espirulina reducida en polifenoles y PCBEx: extracto de ficobiliproteínas), sometidas a hidrólisis secuencial con pepsina y pancreatina. Se obtuvieron hidrolizados extensivos que mostraron grados de hidrólisis de 31.4% y 36.7% para SpRPh y PCBEx respectivamente. A través de los perfiles electroforéticos, se observó la degradación de bandas características de las proteínas de espirulina y la liberación de péptidos de menor tamaño. En general, la actividad antioxidante determinada por diferentes métodos se incrementó por acción de la hidrólisis enzimática. Por otro lado, los hidrolizados proteicos de ambas muestras mostraron actividad quelante de Fe^{2+} y Cu^{2+}.

Palabras clave: espirulina, hidrolizados proteicos, actividad antioxidante, actividad quelante.

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1 Introduction

The oxidative stress is an imbalance between pro- and antioxidants, that exists when the concentration of the first ones increase inside an organism. A persistent oxidative environment increases the generation of reactive oxygen species (ROS). High levels of ROS, as well as reactive nitrogen species (RNS) such as nitric oxide (NO), which can damage the structure and function of DNA, resulting in genomic instability and cellular proliferation by alteration of cellular signal transduction pathways (Visconti and Grieco, 2009). The oxidative stress is implicated in the development and maintenance of several chronic and degenerative diseases including cancer, atherosclerosis, malaria, rheumatoid arthritis, Parkinson and Alzheimer (Jomova and Valko, 2011).

Under conditions of oxidative stress, the endogenous mechanism of defense, enzymatic and non-enzymatic, becomes insufficient for inhibit free radicals (Johansen and Harris, 2005). The consumption of antioxidants and/or diets enriched with these, seems to prevent or at least reduce the deterioration of the organism caused by excessive oxidative damage (Gutteridge and Halliwell, 2010).

In recent years there has been increased the interest in evaluate the antioxidant potential of protein hydrolysates and their possible application as functional foods and nutraceuticals (Samaranayaka and Li-chan, 2011). The antioxidative peptides can be released from different proteins of plant or animal origin during preparation of protein hydrolysates using exogenous or endogenous enzymes, food processing or during microbial fermentation, as well as during gastrointestinal digestion of food proteins (Korhonen and Pihlanto, 2003). The antioxidant activity of these peptides is due to their capabilities of radical scavenging, inhibition of lipid peroxidation and metal chelation (Sarmadi and Ismail, 2010).

Spirulina (*Arthrospira maxima*) is classified as a cyanobacterium or microscopic blue-green alga. For centuries, native people of Mexico and Africa have cultivated and consumed Spirulina as a food source (Chacon and González, 2010). At present, in addition to its use in human nutrition, microalgae can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals (Ponce *et al*., 2006). Spirulina contains around 60-70% protein, including all essential amino acids, although having reduced amounts of methionine, cysteine and lysine, compared to meat protein, egg or milk. However its content is superior to all vegetable proteins. We have attached this sentence to complete the information. It is also rich in vitamins, especially B12 and provitamin A (β-carotene), and minerals, especially iron, besides being one of the few dietary sources of γ-linolenic acid (Capelli and Cysewski, 2010).

The major fraction of the Spirulina protein has been found in the form of supramolecular arrangements known as phycobilisomes, which are constituted by pigmented (phycobiliproteins) and non-pigmented (binding peptides) polypeptides (Liu *et al*., 2005). The most important phycobiliproteins in Spirulina are C-phycocyanin (CPC) and allophycocyanin (APC), which together represent over 60% of the total protein.

The CPC has α and β subunits, with a molecular weight of 21.5 and 19.0 kDa, respectively, with a chromophore group attached to the α subunit (α 84) and two attached to the β subunit (β 84 and β 155) (Liao *et al*., 2011).

APC also has two α and β subunits, both subunits have a molecular weight of 17 kDa and a chromophore group bonded to residue 81. CPC has an absorption maximum at 620 nm, the chromophore groups impart a bright blue and red fluorescence (Sekar and Chandramohan, 2007). APC has maximum absorption at 652 nm, it presents purple and red fluorescence (Eriksen, 2008).

The assembly and stabilization of the phycobiliproteins within the phycobilisome is mediated by four different types of binding peptides: LC (core linker), LR (rod linker), LRC (rod-core linker) and LCM (core-membrane linker). The MW interval of binding peptides was very broad (LC <10 kDa, LR 27-35 kDa, LRC 25-27 kDa, and LCM 70-120 kDa) (Liu *et al*., 2005).

In the present work a simulation of the human gastrointestinal digestion process was carried out by using the sequential action of pepsin and pancreatin enzymes on the Spirulina samples with the aim of evaluating the potential antioxidant and chelating activities of the hydrolysates through of different techniques.

2 Material and methods

2.1 Materials

Spirulina (*Arthrospira maxima*) powder was acquired from the company Los Andes (Quito, Ecuador). The reagents: Gallic acid (G7384); ammonium

sulphate (V000261); dichlorodiphenyltrichloroethane (50-29-3); trichloroacetic acid (T6399); 2,2 -diphenyl-1-picrylhydrazyl (D9132); 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (A9941); potassium peroxodisulfate (77096PJ); trolox (238813); β-carotene (C9750); tween 20 (P1379); linoleic acid (L1376); ferrous chloride (44939); FerroZineTM (160601); pyrocatechol violet (P7884); copper sulphate pentahydrate (209198); and the enzymes pepsin E.C. 3.4.23.1 (P700) and pancreatin E.C. 232-468-9 (P1750) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). The reagents: Acetone (9006-05), HCl 1N (5620-02), NaOH 1N (5635-02) were obtained from JT Baker (Phillipsburg, NJ, USA). While reagents methanol (67-56-1) and chloroform (06205) were purchased from Meyer (México D.F., México) and Fermont (Monterrey N.L., México) respectively.

2.2 Determination of protein content

The protein content was determined by the micro Kjeldahl method (AOAC, 1997), by using a conversion factor of 6.25.

2.3 Preparation of Spirulina samples

2.3.1. Spirulina with decreased content of polyphenols (SpRPh)

With the purpose of decreasing the interference that may generate the phenolic compounds in the evaluation of the antioxidant ability of the protein hydrolysates, six extractions were performed by using acetone at 75% and 4°C. The ratio flour-solvent was maintained at 0.5:5 w/v. Each extraction was performed for 30 min with stirring. Afterwards, all the solvent was removed and consecutively substituted so that six extractions were completed. Samples were taken at the end of each extraction in order to determine the total content of polyphenols. After the six extractions with acetone, the solvent was eliminated by decantation. Then the residual acetone was removal with gaseous nitrogen. To ensure complete removal of the solvent, the material was exposed to the sun.

The total content of polyphenols was determined using of the Folin-Cicocalteu method as described by Singleton *et al*., (1999). A standard curve of gallic acid (from 0 to 0.5 mg/mL) was performed. The concentration of polyphenols was expressed as mg of gallic acid equivalent/g of dry Spirulina.

2.3.2.Obtention of the Spirulina phycobiliproteins extract (PCBEx)

The conditions for extraction were according to the method described by Silveira *et al*., (2007) a biomass-solvent ratio of 0.08 mg/mL, 25 °C for 24 h. The solvent used was distilled water.

With the purpose of obtaining the phycobiliproteins extract the method described by Bermejo *et al*., (2008) was used with modifications. The crude extract obtained was centrifuged (2500 g, 30 min, 4 °C); the supernatant was precipitated with ammonium sulphate at 50%, and centrifuged (2500 g, 30 min, 4 °C), the new supernatant was discarded and the blue precipitate was dissolved in distilled water, dialyzed and lyophilized for 24 h (40 °C and 0.834 mbar).

The degree of purity of the CPC and APC with respect to other components was calculated according to the Ec. (1) and Ec. (2) (Bennett and Bogorad, 1973):

$$PPC = \frac{A_{620}}{A_{280}} \quad (1)$$

$$PAPC = \frac{A_{652}}{A_{280}} \quad (2)$$

Where:
PPC = Purity ratio of CPC. PAPC = Purity ratio of APC. A_{620} = Sample absorbance at 620 nm. A_{280}= Sample absorbance at 280 nm. A_{652} = Sample absorbance at 652 nm.

2.4 Electrophoresis (microchips)

The electrophoretic analyses were carried out by using the equipment Bioanalyzer 2100 (Agilent Technologies, Germany). In order to operate the system, the Expert software 2100, version B.02.07.SI532 was used as well as the Protein 230 Reagent Kit (all from Agilent Technologies). Spirulina samples were denatured as specified in the guide of the reagent kit in the presence of dichlorodiphenyltrichloroethane (DTT) as reducing agent. The microchips were prepared according to the guide and directly analysed with the Bioanalyzer 2100.

2.5 Enzymatic hydrolysis of the Spirulina samples

Spirulina samples were sequentially hydrolysed with pepsin (90 min) and pancreatin (120 min) according

to Megias *et al*., (2008). The pH of the protein suspension in water (5% w/v) was adjusted to 2.5 with hydrogen chloride (HCl) 1N and then the pepsin was added at an enzyme-substrate ratio of 1:20 w/w, considering this moment as the initial time; afterwards, the pancreatin was added (and the pH was adjusted to 7.5 by using sodium hydroxide NaOH 1N) for 120 min at 37 °C. Aliquots of the reaction mixture were taken at different times (0, 5, 10, 20, 30, 45, 60, 90, 105, 120, 150, 180 and 210 min). With the aim of inactivating the enzyme, the mixture was boiled for 10 min. Finally, the hydrolysates were adjusted to the isoelectric point of the phycobiliproteins and then centrifuged, so that the supernatant containing the peptide was maintained at -20 °C for subsequent analysis

2.6 Determination of the degree of hydrolysis

The degree of hydrolysis (% DH) was determined through the trichloroacetic acid (TCA) method. An aliquot of 2 mL of hydrolysate and one of 2 mL of TCA 20% were mixed. The mixture was stirred and then centrifuged; the total nitrogen content was determined in the supernatant through the micro Kjeldahl method. The calculation of % DH was performed by using the Ec. (3):

$$\%DH = \frac{\text{Nitrogen soluble in TCA 20\%}}{\text{Total nitrogen}} \times 100 \quad (3)$$

2.7 Antioxidant activity assay

2.7.1.Determination of 2,2 -diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

This parameter was determined according to the method described by Shimada *et al*., (1992) after some modifications. An aliquot of 200 μL of hydrolysate was mixed with 2 mL of DPPH (125 μM in methanol 80%). After stirring, the mixture was kept at room temperature for 60 min in the dark. Subsequently, the tubes were shaken and the absorbance was read at 520 nm by using an UV-VIS spectrophotometer (Jenway 6505, UK). The DPPH free radical scavenging activity (% SA) was calculated as the percentage of inhibition of this radical, by employing the Ec. (4):

$$\%SA = \frac{A_{control} - A_{sample}}{A_{control}} \quad (4)$$

Where: $A_{control}$ = absorbance of the control solution without the hydrolysate, and A_{sample}= absorbance of the hydrolysates.

2.7.2. Determination of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging activity

The TEAC assay is based on the reduction of the cation radical ($ABTS^+$) by antioxidant compounds and was performed according to Re *et al*., (1999); after minimal modifications. First, the $ABTS^+$ radical was generated by mixing a 7 mM solution of ABTS and a 2.45 mM solution of potassium peroxodisulfate. After 12 to 16 h of reaction, the solution was diluted with absolute methanol up to obtaining 0.70 ± 0.02 absorbance at 734 nm. The antioxidant activity was determined by mixing 10 μL of hydrolysate and 990 μL of the $ABTS^+$ dilution. Absorbance was measured at 0 and 6 min using a UV-VIS spectrophotometer. The degree of decoloration was considered as the magnitude of radical scavenging ability of the hydrolysate and was calculated by using a standard curve prepared with 50, 100, 250, 500 and 1000 mM of Trolox.

2.7.3. Inhibition of β-carotene bleaching

This test simulates the lipid oxidation of the cell membrane, so it is considered a good model of lipid peroxidation. In this water-in-oil emulsion system, the linoleic acid acts as a free radical generator (peroxyl); these kind of radicals oxidate the β-carotene resulting in a whitening effect, which can be inhibited by a radical scavenger.

This assay was performed by using the method developed by Velioglu *et al*., (1998) after certain modifications. Portions of β-carotene (2 mg) were placed in a test tube and dissolved with 1 mL of chloroform; then, 200 μL of Tween 20 and 20 μL of linoleic acid were added, and the mixture was vigorously stirred by using a vortex. After removing the chloroform using nitrogen, 20 mL of oxygen-rich water were added. From this solution, it was prepared a solution diluted with oxygen-rich water whose absorbance reported 1.4-1.5 at 450 nm. The determination was carried out in a microplate, by placing 200 μL of the diluted solution of β-carotene and then adding 50 μL of hydrolysate (100 μg protein). The microplate was incubated at 40 °C for 60 min; afterwards, absorbance was read at 450 nm in a microplate reader (Multiskan Spectrum, ThermoLab Systems, MA, USA). The degradation rate (DR) of β-

carotene was calculated with the Ec. (5):

$$DR = \frac{\ln(A_0/A_{60})}{60} \quad (5)$$

Where: A_0 represents the hydrolysate absorbance at 0 min and A_{60} is the hydrolysate absorbance at 60 min. Once the DR was obtained, the antioxidant activity (AA) was determined as the percentage of inhibition with respect to the control sample, by using the formula:

Where DRcontrol is the degradation rate observed in the control sample (i.e. without hydrolysate) and DRsample is the degradation rate observed with the hydrolysate.

2.8 Chelating activity assay

2.8.1. Determination of the ferrous ion (Fe^{2+})

The Fe^{2+} chelating activity was determined by measuring the formation of the metal complex Fe^{2+}-ferrozine (Carter, 1971). The sample (100 μg) was mixed with 250 μL of acetate buffer at pH 4.9, and 30 μL of ferrous chloride ($FeCl_2$) (0.01% w/v). Ferrozine was added after incubation for 30 min at room temperature. The generation of a metal complex given by the binding of iron ions with ferrozine was measured at 562 nm, by means of a microplate reader (Multiskan Spectrum, ThermoLab Systems, MA, USA). The Fe^{2+} chelating activity was calculated by applying the Ec. (6):

$$\%Fe^{2+} \text{ chelating activity} = \frac{A_{control} - A_{sample}}{A_{control}} \quad (6)$$

Where: $A_{control}$= absorbance observed for the EDTA control solution, and A_{sample}= absorbance.

2.8.2. Determination of the cupric ion (Cu^{2+})

The Cu^{2+} chelating activity was determined according to the method proposed by Saiga *et al*., (2003); 290 μL of acetate buffer at pH 6 (50 mM), 6 μL of pyrocatechol violet (4 mM and separately prepared by using the same acetate buffer), and copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) (1 μg) were added to the hydrolysate (100 μg). The Cu^{2+} chelating activity was determined by the change in absorbance at 632 nm by using the microplate reader. The Cu^{2+} chelating activity was calculated as done for the Fe^{2+} one.

2.9 Statistical analysis

All the assays were conducted with three replicates. Data were expressed as mean ± standard deviation.

3 Results and discussion

3.1 Characterization of Spirulina samples

Many studies report the presence of phenolic compounds in Spirulina like (salicylic, trans-cinnamic, chlorogenic, synaptic and caffeic acids), which have demonstrated antioxidant activity (El-Baky, 2009). Thus, in this study Spirulina was treated with the aim of decreasing the content of these compounds by the elaboration of six extractions with acetone at 75%, in order to avoid the interference of phenolic compounds in the antioxidant activity of the protein hydrolysates. The initial content of phenolic compounds was 3.57 mgGAE/g, with the treatment decreased to 1.49 mgGAE/g, the six extractions allowed to eliminate 56.6% of phenolic compounds. The protein content was modified from 57.1 % to 63.0% after extractions with acetone.

On the other hand, the phycobiliproteins extract (PCBEx) was obtained from the untreated Spirulina material. The final product was a powder of blue colour, characteristic of phycobiliproteins, with a protein content of 72.3 ± 0.2 g/100 g (which represents almost 26% more of protein concentration than the initial dry material) and a purity ratio of 0.52 and 0.15 for CPC and APC, respectively.

The electrophoretic profile of Spirulina samples, i.e. PCBEx and SpRPh was performed (see Fig. 1). As seen in the lanes 1-3, the samples showed a band with a molecular weight (MW) between 17-19 kilodalton (kDa), which probably corresponds to the subunits α and β of CPC and APC, the major proteins in Spirulina.

Also, it was observed that the intensity of the band corresponding to the phycobiliproteins in the protein extract (Fig. 1, band B) was higher than the rest, since its concentration was 1620.3 ng/μL, which is four times higher than the one found in Spirulina samples with low content of polyphenols (band A, 435 ng/μL) and without treatment (band C, 348 ng/μL).

Besides the characteristic band of phycobiliproteins, other bands were observed. They might be proteins of no interest for this study or binding peptides, which are responsible for the assembly and maintenance of the phycobilisome structure (Liu *et al*., 2005). The MW interval of binding peptides was very broad so that they could be distributed over almost the entire gel.

The presence of a band in all samples, which is immediately below the 240 kDa, may correspond

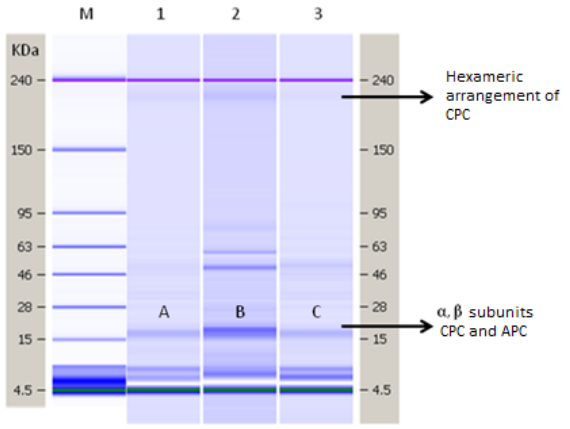

Figure 1. Electrophoretic profiles of the proteins of spirulina samples carried out in a Bioanalyzer 2100. M: molecular weight markers (values in vertical rows are in kDa), 1: SpRPh, 2: PCBEx, 3: Spirulina without treatment.

to the hexameric arrangement of CPC (232 kDa), possibly due to the aggregation and dissociation effects undergone by this protein during its extraction. The state of the protein in solution depends on its source, concentration and isolation conditions (Jiang *et al*., 2001); the hexameric arrangement is the one which tends to prevent denaturation since this arrangement is the *in vivo* functional unit (Chaiklahan *et al*., 2012).

3.2 Protein hydrolysates from Spirulina

Enzymatic hydrolysis was employed in this work with the aim of releasing the peptides with antioxidant activity from SpRPh and PCBEx (see Fig. 2). The use of simulated gastrointestinal digestion by the sequential action of pepsin (90 min) and pancreatin (120 min), allowed to observe that the hydrolysis performance was similar in the two samples of Spirulina, being 2.1 and 2.8 times higher respectively after using pancreatin. The pepsin allowed to reach 11.2 and 17.1% degree of hydrolysis in SpRPh and PCBEx, respectively; these values increased to 31.4 and 36.7% after the second stage of the sequential enzymatic hydrolysis using pancreatin. The increase in the degree of hydrolysis is due to the specificity of pepsin is different form that of the proteolytic mixture contained in pancreatin. Therefore, it is more likely to have more hydrolytic effect after the sample was contacted with a mixture of proteases than with a single one.

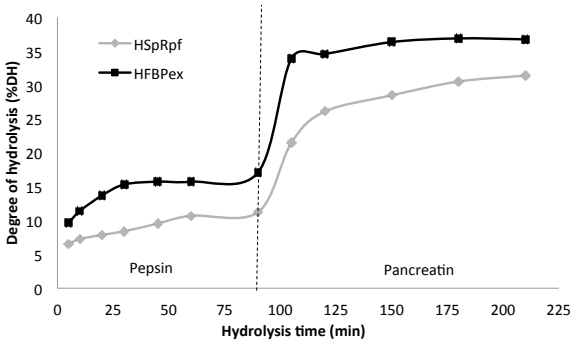

Figure 2. Sequential enzymatic hydrolysis with pepsin and pancreatin of spirulina samples: SpRPh and PCBEx. 5% w/v protein concentration and 1:20 w/w ratio enzyme-sustrato. Each value is expressed as mean ± S.D. (n = 3).

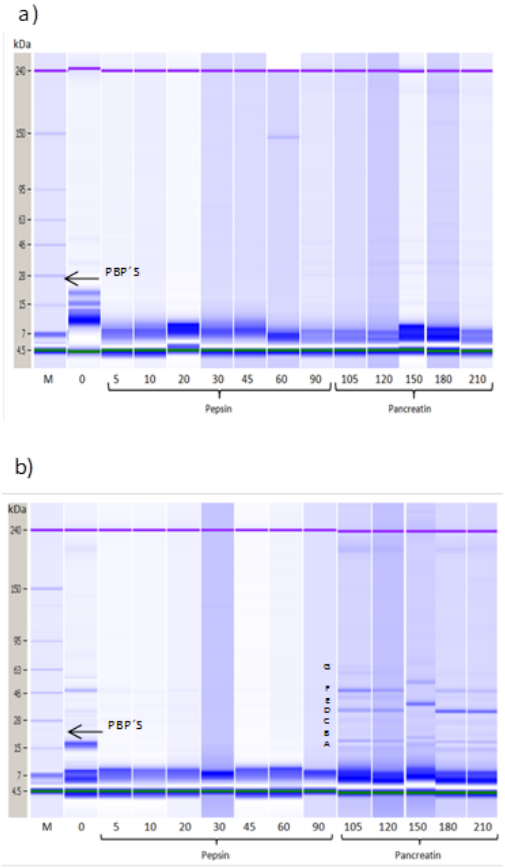

Figure 3. Electrophoretic pattern of SpRPh (a) and PCBEx (b) protein hydrolysates obtained by using pepsin (0, 5, 10, 20, 30, 45, 60 y 90 min) and pancreatin (105, 120, 150, 180 y 210 min) M: molecular weight markers (kDa). PBP'S (Phycobiliproteins 17-19 KDa), A: 14.9 KDa; B: 18.5 kDa; C: 27.7 kDa; D: 35.1 kDa; E: 44.0 kDa; F: 48.9 kDa and G: 61.0 kDa.

In Fig. 3a and b, the electrophoretic profile of SpRPh and PCBEx hydrolysates is shown. In both gels it was observed the presence of the band characteristic of the phycobiliproteins (17-19 kDa), and some which may correspond to binding peptides or to arrangements of the same proteins, as mentioned above.

Although this fact was clearer for PCBEx, these bands were also found in the hydrolysates of SpRPh. In both cases, it was observed that after 5 min this characteristic band disappeared and, subsequently, smaller bands corresponding to the free peptides were produced. As observed, the concentration of <10 kDa peptides considerably increased after the action of pancreatin.

In the case of PCBEx (Fig. 3b), the protein ranging 34.9-35.2 kDa was observed throughout the entire hydrolysis process. According to the above described, this band could correspond to a LR binding peptide, which is characterized by having molecular weights from 27 to 35 kDa and it participates in the assembly of the peripheral rods of the phycobilisome substructure. It is commonly assumed that these binding peptides are located in the formed inner cavity when two trimers produce a 'face to face' type assembly (Liu *et al.*, 2005); this could have derived into a protective effect against hydrolysis of the binding peptide.

For the PCBEx hydrolysates (Fig. 3b), new bands were observed (A-F) from the start of hydrolysis with pancreatin; probably, those bands belong to protein aggregates generated by the sequential hydrolysis with the two enzymes. Phycobiliproteins, and especially binding peptides, might produce irreversible aggregates via hydrophobic interactions, given the high amount of hydrophobic residues which become exposed through the hydrolytic process.

3.3 Antioxidant activity of protein hydrolysates from Spirulina

3.3.1. DPPH free radical scavenging capacity

Fig. 4 depicts the results obtained from determining the antioxidant capacity by the DPPH method. In both types of samples, the hydrolysates obtained after the use of pepsin in the first minutes (5-60) had low antioxidant activity, namely 9.4% (SpRPh) and 24.9% (PCBEx) Trolox equivalents, i.e. the DPPH free radical scavenging capacity was lower (less than half)

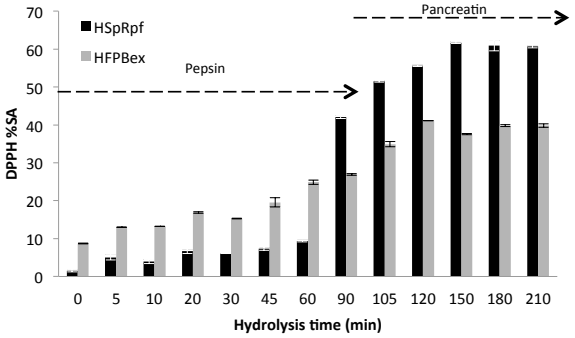

Figure 4. DPPH radical scavenging activities of *in vitro* sequential digestion of SpRPh and PCBEx protein. Each value is expressed as mean ± S.D. (n = 3).

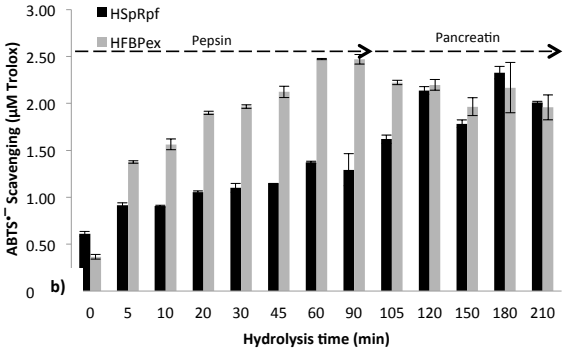

Figure 5. $ABTS^{\cdot-}$ scavenging capacity of *in vitro* sequential digestion of SpRPh and PCBEx protein. Each value is expressed as mean ± S.D. (n = 3).

in SpRPh than in PCBEx hydrolysates. However, at 90 min, there was an increase of more than four times in SpRPh, whereas the increase in PCBEx was almost nil. When pancreatin was added, the antioxidant activity in both samples gradually increased up to reaching 41.4 and 61.7% at 120 and 150 min, for PCBEx and SpRPh, respectively.

In the case of SpRPh hydrolysates, the rise in antioxidant capacity occurs because the major determinant reaction in this method is the steric accessibility to the DPPH free radical by smaller molecules such as the peptides generated through hydrolysis, the better access to the radical the higher antioxidant activity (Klompong *et al.*, 2007).

3.3.2. Scavenging of $ABTS^{+}$ radical

In Figure 5, the results obtained by the ABTS method are presented. Both samples reported antioxidant activity: 610.7 (SpRPh) and 364.9

(PCBEx), considered as mM Trolox equivalents, at time 0 of hydrolysis. This activity may be attributed to the chromophores of phycobiliproteins and, in the case of (SpRPh), to the phenolic compounds that remained after the extraction (43.4%).

With this technique, it was possible to observe an increase in the antioxidant activity of hydrolysed samples; this fact was more discernible in SpRPh, while the PCBEx showed a slight reduction in its antioxidant activity after adding pancreatin. This was possibly due to the formation of protein aggregates (Fig. 3), which could have affected the spatial orientation of amino acids required to inhibit the $ABTS^{+}$ radical. The highest antioxidant capacity for PCBEx occurred after 90 min of hydrolysis (2.47 mM Trolox), while for SpRPh it was at 180 min (2.33 mM Trolox).

3.3.3. Inhibition of β-carotene bleaching

In Fig. 6, the results obtained in this determination for SpRPh and PCBEx hydrolysates, are shown. All hydrolysates inhibited the degradation of β-carotene in more than 38%. In the case of SpRPh, better inhibition values were obtained for hydrolysates prepared with pepsin followed by pancreatin at 105-210 min. With PCBEx, the highest activity was observed in the hydrolysates obtained at 45 min with pepsin as well as with sequential hydrolysis at the last three sampling times (150, 180 and 210 min).

The variation of the values obtained at different times of hydrolysis can be due to the difference in size and sequence of the peptides that are generated, as well as to the influence that these features exert on the antioxidant activity of peptides/proteins, over the lipid peroxidation mediated by free radicals (Qian *et al*., 2008).

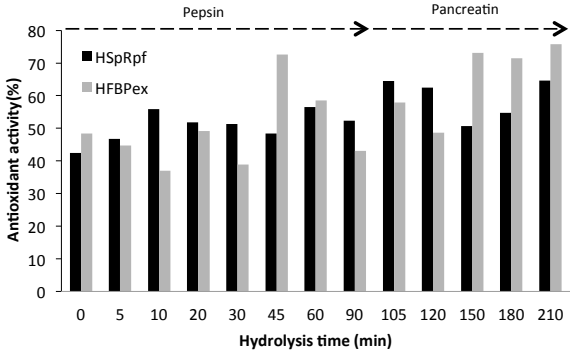

Figure 6. Antioxidant activity of SpRPh and PCBEx protein hydrolysates using the β-carotene bleaching assay. Each value is expressed as mean ± S.D. (n = 3).

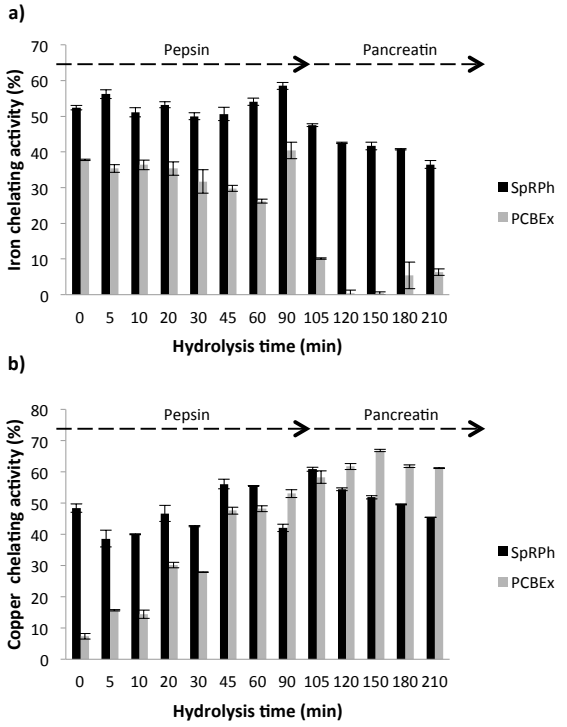

Figure 7. Fe^{2+} (a) and Cu^{2+} (b) chelating activity of SpRPh and PCBEx protein hydrolysates. Each value is expressed as mean ± S.D. (n = 3).

3.4 Chelating activity of protein hydrolysates from Spirulina

3.4.1. Fe^{2+} chelating activity

Fig. 7a contains the results obtained from the determination of chelating activity over Fe^{2+} An inverse relationship was observed between the time of hydrolysis and the metal chelating activity, during pancreatin hydrolysis for both samples. This phenomenon also observed in PCBEx with pepsin, but not in SpRPh with the same enzyme. Some authors report which that chelating activity increases with decreasing molecular weight by effect of enzymatic hydrolysis (Klompong *et al*., 2007 and Dong *et al*., 2008).

Moreover, the Spirulina has been considered as a good material for metal chelation. May be due to functional groups present on the surface of its cells, mainly carboxyl groups of proteins and amino acid side chains such as histidine, cysteine, aspartic and glutamic acid (Ashmead *et al*., 1985). The decrease in the chelating activity over Fe^{2+} observed in the present work can be attributed to a disruption of these functional grousps caused by

the enzymatic hydrolysis. Nonetheless, especially for SpRPh hydrolysates, high percentages of Fe^{2+} chelation were observed.

3.4.2. Cu^{2+} chelating activity

In Fig 7b, the chelating activity over Cu^{2+} reported by Spirulina hydrolysates is depicted. In general, the hydrolysates obtained from PCBEx showed a direct relationship between the hydrolysis time and the chelating activity, being the hydrolysates obtained by the sequential action of the two enzymes (105-210 min) the most active ones. The SpRPh samples did not show a defined activity. There was a greater chelating effect (66.8%) in the PCBEx hydrolysate at 150 min, whereas for the SpRPh hydrolysate the Cu^{2+} chelating percentage was 60.8% at 105 min of enzyme action.

Conclusions

In view of the results obtained from the current study, the sequential enzymatic hydrolysis resulted in an increase on antioxidant activity as determined through DPPH, ABTS and inhibition of β-carotene bleaching techniques. In addition to these changes, the variation in the antioxidant activity of the samples can be attributed to the complex mixture of peptides and, in some cases, free amino acids, which were generated by the hydrolysis process. The size of the peptides, their solubility, amino acid sequence and composition may play an important role in the development of their antioxidant activity.

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