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Anti-inflammatory and antioxidant activities, functional properties and mutagenicity studies of protein and protein hydrolysate obtained from *Prosopis alba* seed flour

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ABSTRACT

Prosopis species are considered multipurpose trees and shrubs by FAO and their fruit constitute a food source for humans and animals. According to the "Código Alimentario Argentino", "algarrobo flour" is produced by grinding the whole mature pod, but in the traditional process most of the seeds are discarded. In this paper, the flour from seed was obtained. Then, the proteins were extracted and enzymatic hydrolysis was carried out. According to their amino acid profile and chemical score (>100%), the *Prosopis alba* proteins, are not deficient in essential amino acids considering the amount of amino acid necessary by adults. The protein isolate showed a good solubility (pH 7.4–9), emulsificant capacity, oil binding capacity and water adsorption capacity. The antioxidant ability of proteins was significantly increased with hydrolysis (SC_{50} values: 50–5 μg/mL, respectively). Inhibitory activity of pro-inflammatory enzymes (lipoxygenase and phospholipase) was described. The mutagenicity/antimutagenicity of proteins and protein hydrolysates from seed flour were also analysed.

The results suggest that *P. alba* cotyledon flour could be a new alternative in the formulation of functional foods not only for its high protein content but also by the biological and functional properties of its proteins and protein hydrolysates.

1. Introduction

The genus *Prosopis* belongs to the Fabaceae family, subfamily Mimosoideae and involves about forty-four species distributed mainly in arid and semiarid tropical and subtropical regions of Southwest Asia, Africa and predominantly America. In America, *Prosopis* thrives in a large area that goes from the south-western part of the United States to the Argentinean Patagonia, being characteristic of the Monte desert in Argentina from Salta to Chubut provinces (Cabrera, 1994). *Prosopis* pods and leaves have economic significance for ruminant breeding (Abddel hafes & Abdullah, 2004; Obeidat, Abdullah, & Al-Lataifeh, 2008) and its wood is an important resource for construction, firewood and charcoal (Bogino & Villalba, 2008; Fagg & Stewart, 1994). *Prosopis alba* fruit is used to dissolve gallstones, and as an anti-bronchitic and laxative. Its flowers have diuretic properties; and the bark is used as an astringent and to heal eye infections (Pasiecznik, Harris, & Smith, 2004). *Prosopis* species fruits constitute a food source for humans and animals of Monte desert (Arenas, 2003; D'Antoni & Solbrig, 1977; Fagg & Stewart, 1994; Felger, 1977; Roic, Carrizo, & Palacio, 2002). Different food products are made from *P. alba* and *Prosopis nigra* pods: drinks (añapa, aloja and chicha), syrup, flour, sweets (arrope, patay, jam), etc. (Escobar, Estévez, Fuentes, & Venegas, 2009; Odibo, Ezeaku, & Ogbo, 2008; Roig, 1993). Felker, Takeoka, & Dao (2013) noted that the whole pods of different *Prosopis* species were ground for animal feeding, whereas the flour for human food is made only from the mesocarp fraction of washed and sorted pods. According to the "Código Alimentario Argentino", "algarrobo flour" is produced by grinding of whole mature pod of *P. alba* and *P. nigra*, but most of the seeds are discarded in the traditional process due to the hardness of the

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endocarp. In a previous paper we determined the nutritional and functional properties of flour obtained from the mesocarp fraction of *P. alba* and *P. nigra* that grow in Northwestern of Argentina (Cardozo et al., 2010). The use of *P. alba* cotyledons is little known in Argentina, probably due to the difficulties in obtaining them, a situation that could change by implementing a simple technology. The cotyledons and their flour would represent a real alternative as a protein source in the formulation of animal feeds and human food. Estévez, Escobar, and Ugarte (2000) and Escobar et al. (2009) studied the utilisation of *Prosopis chilensis* cotyledons to obtain some products for human alimentation with high protein content (cereal bars and cookies).

Antioxidants play a vital role in both food systems as well as in the human body to reduce oxidative processes. In food systems, antioxidants are useful in retarding both lipid peroxidation and the formation of secondary lipid peroxidation products and thus help to maintain flavour, texture, and, in some cases, the colour of the food products during storage (Samaranayaka & Li-Chan, 2011). Proteins and protein hydrolysates derived from sources like milk, soy, egg and fish have also shown antioxidant activity (Elias, Kellerby, & Decker, 2008; Hagen & Sandnes, 2004; Peña-Ramos & Xiong, 2003). Food-derived peptides also have potential for controlling and modulating some inflammatory diseases like hepatitis, inflammatory bowel disease like Crohn's disease and ulcerative colitis and other chronic intestinal inflammations (Chattertona, Nguyen, Bering, & Sangild, 2013; Sato, 2012). Phospholipase A_2 (PLA_2) activity serves to release arachidonic acid from membrane phospholipids, which can then be processed by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to generate leukotrienes, prostaglandins and hydroperoxides which are potent mediators of inflammation. Thus, the inhibition of these enzymes is one of the important cellular mechanisms of anti-inflammation.

Since there has recently been an increased interest in non-conventional natural food from plant origin with beneficial properties for health, seed proteins from *P. alba* could be used as functional food or food additive. In this study, we examine the procedure to obtain *P. alba* cotyledon flour, the functional properties and biological properties (potential as antioxidant, inhibitor of pro-inflammatory enzymes as well as antimutagenic) of proteins obtained from them and its hydrolysates. The genotoxicity of proteins and its hydrolysates was also analysed.

2. Materials and methods

2.1. Plant material

Ripe *P. alba* (Griseb.) pods were collected in Amaicha del Valle (Tucumán, Argentina) in April 2010. They were brushed to remove foreign material and dried at 50 °C until reaching a constant weight.

Dried pods were ground to powder (Helix mill, Metvisa) and were passed through 2 mm and 4 mm mesh sieves. Two fractions were obtained: mesocarp flour and seeds with endocarp. Since seed-stone (endocarp) thickness and hardness affect the process of cotyledon extraction, a given weight of seeds was treated with sulphuric acid (seed-to-solvent ratio of 1:5 w/v) for 5 min to soften the endocarp. Then, the seeds were separated from the endocarp and rinsed. They were kept in water for 24 h to produce their imbibition. The seed cotyledons, mucilage (endosperm) and episperm were separated.

2.2. Preparation of Prosopis cotyledon flour

The cotyledons were dried at 50 °C until reaching a constant weight. Then the dried cotyledons were ground to obtain cotyledon flour. The flour was stored in plastic screw-capped bottles at −20 °C until further use.

2.3. Determination of total protein content

The total nitrogen content of cotyledon flour and protein isolate were determined by Kjeldahl digests. A factor of 6.25 was used to determine the percentage of total protein.

2.4. Soluble protein determination

Soluble protein content was determined by Bradford (1976) (BIO-RAD®) method using bovine serum albumin (BSA) as a standard. Results were expressed as mg of BSA/g dry weight of cotyledon flour.

2.5. Isoelectric point determination

To determine the isoelectric point (pI), the sample was diluted to a 5 mg soluble protein/mL and fractioned in several tubes. Glacial acetic acid was added to bring each tube to reach different pH values. The content of the tubes was mixed and left to rest for 20 min. Next, the tubes were centrifuged and the supernatant was separated to quantify soluble protein.

2.6. Preparation of protein isolates from cotyledon flour

Prosopis cotyledons flour was extracted with 0.5% (w/v) aqueous sodium hydroxide (NaOH) (pH 10; flour-to-solvent ratio of 1:4; w/v) for 20 min with stirring in a cool bath. The preparation was centrifuged at 9692*g* for 35 min at 4 °C (Sorvall RC-50) and the pellet was re-extracted two fold with the same solution and centrifuged. All supernatants were pooled and adjusted to their pI. The pellet obtained by centrifugation at 9692*g* for 30 min at 4 °C was lyophilised and stored at −20 °C.

2.7. Enzymatic hydrolysis of protein isolates

A 2% (w/v) protein isolate (PI) suspended in 50 mM citrate-phosphate buffer pH 2.5 was subjected to enzymatic hydrolysis. The enzyme–substrate ratio was 1:20 w/w. A pepsin solution (Sigma–Aldrich) was added to the mixture and was incubated with continuous stirring for 2 h at 37 °C. Then the pH was adjusted to 7 with 1 M NaOH, a pancreatin solution (Sigma–Aldrich) in 50 mM citrate-phosphate buffer pH 7 was added and the mixture was incubated for 2 h at 37 °C. The reaction was stopped by heating at 80 °C for 20 min and the resultant *Prosopis* protein hydrolysate was frozen and stored at −20 °C. The hydrolysis treatment was performed in triplicate.

2.8. Hydrolysate fractionation by ultrafiltration

The hydrolysate solution was filtered through a membrane (MILLIPORE®) with a 3 kDa obtaining two new fractions, *Prosopis* protein hydrolysate <3 kDa and >3 kDa.

2.9. Electrophoresis

2.9.1. Tris-SDS–PAGE

SDS–PAGE (Laemmli, Amos, & Klug, 1976), was used to characterise the polypeptide profile of protein preparations. Typically, samples were electrophoresed on a 15% acrylamide separating gel with a 7% acrylamide stacking gel (1.0 cm × 10 cm × 1.5 mm). Protein samples were mixed with suitable volumes of SDS–PAGE sample buffer (0.05 M Tris–HCl, pH 6.8; 1% SDS; 0.01% bromophenol blue as the tracking dye; and 3% glycerol) containing 2% (v/v)

β-mercaptoethanol, heated for 3 min in a boiling water bath and cooled to room temperature. High and low molecular weight markers (Thermo Scientific, 10–170 kDa) were used in each gel run. After electrophoresis the gels were silver stained (Blem & Ramanarayanan, 1987).

2.9.2. Tricine–SDS–PAGE

Tricine–SDS–PAGE (Schägger, 2006) was used to characterise the polypeptide profile of protein hydrolysate and the ultrafiltrated fractions. The samples were electrophoresed on a 16% T 6% C monomer acrylamide separating gel (6 cm × 10 cm × 1.5 mm) with a 4% monomer acrylamide stacking gel (1.0 cm × 10 cm × 1.5 mm). For sample preparations 12 μL of each sample were mixed with 4 μL of sample buffer (0.15 M Tris–HCl, pH 7; 12% SDS; 0.05% Coomasie brilliant blue R-250 as the tracking dye; and 30% glycerol) containing 6% (v/v) β-mercaptoethanol, heated for 15 min in a 37 °C water bath and cooled to room temperature. Molecular weight markers for peptides (Sigma-weight range: 2.5–17 kDa) were used in each gel run. After electrophoresis the gels were silver stained.

2.10. Determination of hydrolysis degree (HD)

Hydrolysis degree quantification was carried out by the OPA assay (orto-phthaldialdehyde assay) (Nielsen, Petersen, & Dambmann, 2001) as follows: 100 μL of each sample dilution were mixed in a test tube with 750 μL of Pthaldialdehyde Reagent-Complete Solution (Sigma®) (1/1). They were shaken for 5 s and read 2 min later at 340 nm. Absorbance was determined as the average of three determinations.

2.11. Aminoacid analyses

Samples of isolate proteins were subjected to acid hydrolysis in the presence of 1 mL of 4 M methane sulphonic acid (MSA), 0.2% tryptamine (Creamer & Matheson, 1976) at 110 °C for 24 h. After that, the pH of the aminoacid (AA) extract was adjusted to 2.2 with NaOH and filtered using a syringe filter of 0.2 μm membrane. Amino acid concentrations were determined using a Biochrom 30 series amino acid analyzer (http://www.biochrom.co.uk). The basic principle of operation is the continuous flow chromatography procedure developed by Spackman (Moore, Spackman, & Stein, 1958).

The sample containing the mixture of amino acids was mixed with L-Norleucin as internal standard and loaded onto a column of cation-exchange resin. The column was eluted with buffers of different pH and ionic strength. The aminoacids were mixed with the Ninhydrin reagent. The amount of coloured compound produced is directly proportional to the quantity of amino acid present in the eluate. From the reaction coil, the eluate/ninhydrin mixture is fed to the photometer unit where the amount of each coloured compound is determined by measuring the amount of light absorbed. The light absorption is measured at two wavelengths, 570 nm and 440 nm, because imino acids produce coloured compounds which absorb light with a wavelength of 440 nm, whereas other amino acid coloured compounds absorb light at 570 nm.

Calibration curves were obtained using amino acid standard purchased from Sigma®. For quantification, peak areas were correlated with the concentrations according to calibration curves.

2.12. Determination of functional properties of protein from Prosopis cotyledon

2.12.1. Protein solubility

The protein isolate was mixed with water (ratio 1/20, w/v), and pH of the mixture was adjusted to 2.5–10.0 with 1.0 N NaOH and HCl and maintained with stirring at room temperature during 1 h, and then centrifuged at 3000*g* for 15 min. Soluble protein concentration in each supernatant was determined by Bradford method. The soluble protein content was calculated as percentage of total soluble protein.

2.12.2. Water absorption capacity (WAC)

Water absorption capacity was determined using the method of Beuchat (1977). One gram of protein isolate was weighed into a pre-weighed 15 mL centrifuge tubes. For each sample, 10 mL of distilled water were added and mixed using a vortex for 2 min. After the mixture was thoroughly wetted, samples were allowed to stand at room temperature for 30 min and then centrifuged at 3000*g* for 20 min. The supernatant was decanted and the centrifuge tube containing sediment was weighed. Water holding capacity (grams of water per gram of protein) was calculated.

2.12.3. Oil binding capacities (OBC)

Oil binding capacity was determined using the method of Chakraborty (1986). One gram of protein was weighed into pre-weighed 15 mL centrifuge tubes and thoroughly mixed with 10 mL of vegetable oil using a Vortex mixer. Samples were allowed to stand for 30 min. The protein–oil mixture was centrifuged at 3000*g* during 20 min. After centrifugation, the volume of supernatant was recorded. Fat absorption capacity (mL of oil per gram of protein) was calculated.

2.12.4. Emulsifying capacity

Emulsifying capacity (EC) was determined in triplicate according to the method described by Sathe and Salunkhe (1981) with modifications.

Protein isolate (0.1–0.25 g) were mixed with 50 mL of distilled water for 2 min using a blender at high speed before the addition of vegetable oil containing Red-O-dye. The oil was added slowly under continuous blending. Blending was stopped every 2 min to check for emulsion breakage. When a clear emulsion breakage was observed, the total volume of oil added was recorded and used to calculate the EC as volume (mL) of oil emulsified per gram of protein.

2.13. Bioactivity determinations of Prosopis cotyledon flour protein

2.13.1. Antioxidant activity

2.13.1.1. ABTS$^{\cdot+}$ scavenging activity. The antioxidant capacity assay was carried out by the improved ABTS$^{\cdot+}$ method as described by Re et al. (1999). The ABTS$^{\cdot+}$ was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature (23 °C) in the dark for 16 h. The ABTS$^{\cdot+}$ solution was obtained by dilution of the stock solution in 0.01 M sodium phosphate buffer pH 7.3 of the stock solution to an absorbance of 0.70 ± 0.02 at 734 nm. ABTS$^{\cdot+}$ solution (1 mL) was added to a final concentrations between 0 and 140 μg of soluble protein/mL and mixed thoroughly. Absorbance was recorded at 734 nm, at 1, 6 and 10 min after initial mixing. Antioxidant capacity was calculated as

$$\% = (A_{ABTS} - A_{sample}/A_{ABTS}) \times 100$$

SC_{50} values denote the sample concentration required to scavenge 50% ABTS free radicals.

2.13.1.2. DPPH scavenging activity. The DPPH radical scavenging activity was measured according to Zhang et al. (2009), with some modifications. DPPH solution 100 μL (0.125 mg/mL in 96° ethanol) was added to a concentrations between 0 and 900 μg of soluble protein/mL and shaken vigorously. It was kept at room temperature in the dark for 30 min and absorbance was measured at

550 nm. SC_{50} values were calculated as μg proteins required to scavenge 50% DPPH free radicals.

2.13.2. Determination of anti-inflammatory activity

2.13.2.1. Lipoxygenase inhibition. Anti-inflammatory activity was determined by measuring the inhibition of LOX (Taraporewala & Kauffman, 1990). The enzyme activity was determined using a spectrophotometric method based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide. Absorption at 234 nm was recorded every 30 s as a function of time for 5 min. The reaction mixture contained substrate (50 μM linoleic acid in 0.2 M borate buffer pH 9) and enzyme (500 U soy LOX-1, Sigma–Aldrich). The assay to obtain the 100% of LOX activity was performed with the vehicle as solvent control. Inhibitory assays were performed in presence of different concentrations of protein isolate and protein hydrolysate. The test compound concentration causing 50% inhibition of hydroperoxide-release (IC_{50}) was calculated from the concentration–inhibition response curve by regression analysis. The extinction coefficient of 25 mM^{-1} cm^{-1} was used for hydroperoxide quantification. Naproxen was used as reference anti-inflammatory compound.

2.13.2.2. Phospholipase A_2 inhibition. Secretory phospholipase A_2 ($sPLA_2$) activity was determined using 1,2 diheptanoylthio-glycerophosphocholine (1,2 dHGPC) and Triton X-100 as substrate (Reynolds, Huges, & Dennis, 1992). The buffer Tris–HCl (10 mM pH 8) with $CaCl_2$ (10 mM), KCl (100 mM) and Triton X-100 (0.3 mM) was used for reconstitution of substrate to achieve a final concentration of 1.25 mM. The mixture contained 50 μL buffer Tris–HCl (10 mM pH 8), 10 μL DTNB (10 mM), 10 μL enzyme $sPLA_2$ (1 μg/mL) from bee venom and 40–100 μg/mL of the sample (protein isolate or protein hydrolysate). The reaction was initiated by the addition of 150 μL of 1,2 dHGPC (1.66 mM) and maintained during 10 min. at 25 °C. The absorbance was read at 405 nm every one minute in a microplate reader (Biotek ELx 808).

2.14. Ames test

2.14.1. Mutagenicity assay

In the Ames test, $His^- \rightarrow His^+$ mutations are visualised by plating *Salmonella typhimurium* bacteria in a histidine poor growth medium. In this medium only His^+ mutants are able to form visible colonies. Different bacterial strains are available to identify different types of mutations. Strains TA98 and TA100 are currently most often used as they detect the great majority of mutagens. Strain TA98 gives an indication of frameshift mutations, while a positive response from strain TA100 indicates base pair substitution.

The mutagenicity assay with *S. typhimurium* was performed as described by Maron and Ames (1983). The experiments were performed with and without an exogenous metabolic system, the S9 fraction (Moltox – Molecular Toxicology Inc.).

Three different concentrations (between 22 and 208 μg protein/plate) of protein isolate and protein hydrolysate from *Prosopis* flour were evaluated in this assay. One hundred microliters of an overnight culture of bacteria (cultivated for 16 h at 37 °C, approximate cell density of 2–5 × 10^8 cells/mL), the different concentrations of protein and 500 μL of sodium phosphate buffer (0.2 M, pH 7.4 for assay without S9-mix) or 500 μL of S9-mix were added to 2 mL aliquots of top agar (containing traces of D-biotin and L-histidine). The resulting complete mixture was poured on minimal agar plates prepared as described by Maron and Ames (1983). The plates were incubated at 37 °C for 48 h (−S9) or 72 h (+S9) and the revertant bacterial colonies of each plate were counted. An extract was considered mutagenic if the number of revertants per plate was more than twice the number of colonies produced on the solvent control plates (spontaneous revertant frequency). Samples were tested in duplicate with two replicates. Sodium phosphate buffer was used as the negative control, 100 μL/plate and the positive controls employed were 4-nitro-o-phenylenediamine (4-NPD), 10 μg/plate and 2-aminofluorene (2-AF), 10 μg/plate.

To discriminate cytotoxicity, the number of surviving cells was determined by plating appropriate dilutions of treated bacterial suspension onto complete agar plates.

2.14.2. Antimutagenicity assay

The procedures for the antimutagenicity assays were similar to those described for the mutagenicity assays, except that in each tube of top agar containing the bacterial strain and the samples, the mutagenic agent was also added. The mutagen tested was a direct-acting genotoxic compound, 4-NPD (10 μg/plate).

2.15. Statistical analysis

Sampling and analyses were performed in triplicate, and the data are presented as mean ± standard deviation. GraphPad Prism 5.0 software was used to perform analysis of correlation between two variants by Pearson test with the level of significance set at $p < 0.05$ and of variance (ANOVA) with Tukey posttest at a confidence level of 95%.

3. Results and discussion

3.1. Proteins and protein hydrolysate obtained from cotyledon flour

In Argentina, *P. alba* flour is produced from whole dry pods. The hardness of the endocarp prevents seed grinding with the consequent yield loss. An efficient method to separate *P. alba* seeds from episperm or seed coat, endosperm, and cotyledon has not yet been well defined. A procedure in the obtention process from *Prosopis flexuosa* and *Prosopis chilensis* seeds was reported (Cosiansi, Milanesi, Da Riva, & Hayipanteli, 2002; Sáez Teuber, 2006). The main difficulties come from the very small size of *Prosopis* seeds and the strong adhesion of the seed coat to endosperm. In the present paper, a new pod processing technique to obtain *P. alba* cotyledons flour and flour proteins was designed.

The seed-endocarp fraction represents 47.7 ± 4.8% of *P. alba* pod dry weight, seeds constitute 12.8 ± 1.3% and cotyledon fractions make up 6.1 ± 0.6%. In our study, *Prosopis* cotyledon flour was obtained by grinding (Fig. 1). The crude protein fractions represent 62.1 ± 6.2% of the cotyledon flour. A similar value was obtained from *P. chilensis* cotyledons (Degussa Argentina, 2003; Sáez Teuber, 2006). Protein values from *P. alba* cotyledon flour were higher than those of soybeans (34.6%), broad beans (23.7%), lentils (25.4%), peas (22.9%), common beans (21.8%), chickpeas (18.5%) (Cosiansi et al., 2002) and *P. alba* and *P. nigra* (4.2%) flour of pods without seeds (epicarp and mesocarp fraction) (Cardozo et al., 2010). These results suggest that due to its high protein content, *P. alba* cotyledon flour (currently discarded) could be considered a new alternative in the formulation of foods or food supplements for both humans and animals, alone or combined with cereal proteins (cereal bars, cookies, coffee substitutes).

The proteins of cotyledon flour were extracted with 0.5% NaOH (W/V). The use of a strongly alkaline medium improved extraction procedure efficiency by increasing the amount of extracted protein in three extraction steps. The alkaline extraction showed 175.71 ± 48.65 mg soluble protein/g cotyledon flour.

The protein pattern of *P. alba* revealed a high number and intensity of bands. The molecular weight (MW) range of proteins was from 10 to 95 kDa (Fig. 2). The analysis of SDS–PAGE electrophoresis revealed the presence of three main proteins with MW of 17, 27 and 33 kDa. A protein isolate was obtained from alkali extraction

Fig. 1. (A) Seeds, (B) cotyledons and (C) cotyledon flour from *Prosopis alba*.

by acid precipitation at pH = 5 (pI of total protein extract). The total protein content for the protein isolate was 85.5% of cotyledon flour while the soluble protein content was 2.6% of cotyledon flour.

In order to simulate the effect of proteolytic enzymes normally produced during the digestion process, two intestinal proteases, pepsin and pancreatin, were contacted with the protein extract obtained from cotyledon flour for 4 h to obtain a protein hydrolysate. Then, the peptides of different molecular weight were separated by ultrafiltration in two fractions: one with MW > 3 kDa and another with MW < 3 kDa. The electrophoretic pattern of protein hydrolysate (hydrolysis degree value of 12.25%) showed bands with MW lower than 26 kDa (Fig. 2A and B).

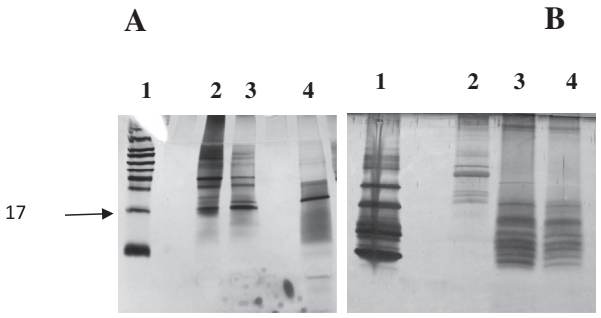

Fig. 2. (A) SDS–PAGE profiles of protein isolates from *P. alba* cotyledon flour. Lane 1: molecular weight markers (170; 130; 95;72; 55; 43; 34; 26; 17; 10 kDa), Lane 2: *P. alba* alkali extract, Lane 3: *P. alba* protein isolate and Lane 4: *P. alba* enzymatic protein hydrolysate. (B) Tricine–SDS–PAGE profiles of protein isolates from *P. alba* cotyledons flour, Lane 2: protein isolate, Lane 3: enzymatic protein hydrolysate, Lane 4: ultrafiltration fraction >3 kDa.

3.2. Aminoacid composition of protein hydrolysate

From a dietary standpoint, quality of a protein is influenced by two major factors: (1) the relative proportions of the essential amino acids in the food in relation to human amino acid/protein requirements; and (2) the extent to which the dietary protein is digested, absorbed and made available to synthesize proteins within the body. Nineteen amino acids were detected in protein isolate: asparagine (9.92%), threonine (1.59%), serine (5.94%), glutamine (20.11%), glycine (4.44%), alanine (4.35%), cysteine (1.49%), valine (3.87%), methionine (0.90%), isoleucine (2.82%), leucine (8.02%), tyrosine (3.29%), phenylalanine (4.15%), histidine (3.68%), tryptophan (0.92%), ornithine (0.48%), lysine (4.25%), arginine (14.14%), proline (5.61%). According to their amino acid profile and chemical score (>100%), the *P. alba* proteins are not deficient in essential amino acids considering the level of amino acid necessary by adults. Thus, a point of interest is that the amino acids of *P. alba* seed could serve as a supplemental source to improve the nutritional quality of grain protein (FAO/WHO/UNU, 1985). However, the chemical score of *P. alba* proteins (96%) based on the essential

Table 1
Essential amino acid pattern of *Prosopis alba* protein isolate compared to human requirement in amino acid.

Essential amino acid	Amino acid content (mg/g protein)			Relation [C/A]	Relation [C/B]
	Human requirement in aminoacid		Protein isolate (C)		
	Adult (A)	10–12 years (B)			
Hystidin	16	19	36.8 ± 0.1	2.30	1.94
Isoleucine	13	28	28.0 ± 0.1	2.09	1.00
Leucine	19	44	80.2 ± 0.2	4.22	1.82
Lysine	16	44	42.5 ± 0.1	2.66	0.96
Phenylalanine + Tyrosine	19	22	74.4 ± 0.1	3.91	3.38
Methionine + Cystine	17	22	23.9 ± 0.2	1.40	1.09
Threonine	9	28	27.0 ± 0.1	3.00	0.96
Tryptophane	5	9	9.2 ± 0.2	1.84	1.01
Valine	13	25	35.0 ± 0.1	2.69	1.40

amino acid pattern requirements for children (10–12 years) has two limiting aminoacids (lysine and threonine) (Table 1).

3.3. Functional properties of protein from Prosopis cotyledons

Functional properties of proteins are important in food processing and food product formulation, since they affect consumer acceptance. The functional properties of the protein depend on physicochemical characteristics (Rodrigues, Coelho, & Carvalho, 2012) such as the content of polar and nonpolar amino acids, which make protein a possible emulsifier, the surfactant possessing both hydrophilic and hydrophobic properties and being able to interact with both water and oil in food system. The proteins from *Prosopis* cotyledon flour were good emulsifier with an emulsifier capacity of 1000 mL oil/1 g protein. This high value might be due to a high exposure of more hydrophobic groups to water and oil interface, resulting in an increased EC and a stable emulsion.

The solubility is an important prerequisite of functional properties of food protein as a good index of potential applications in different systems. The pH had a significant effect on the solubility of *P. alba* protein. As shown in Fig. 3, the minimum solubility of *Prosopis* protein was observed at pH 4.5–5; the protein showed maximum solubility at pH 7.4–9 (45%).

The water adsorption capacity of *Prosopis* protein isolate was 3.92 g/g protein. Kinsella (1976) reported that the major factors affecting water adsorption capacity are protein denaturation and unfolding, and presence of carbohydrates and non-protein components.

Furthermore, high oil binding capacity is desirable for use in the cold meat industry, particularly for sausages, where the protein can bridge the fat and water in these products (Ogunwolu, Henshaw, Mock, & Santros, 2009). Kinsella (1976) stated that the oil binding mechanism can be explained as the physical entrapment of oil by capillary attraction. The protein concentrate of *Prosopis* showed good OBC (1.7 ± 0.5 g/g protein).

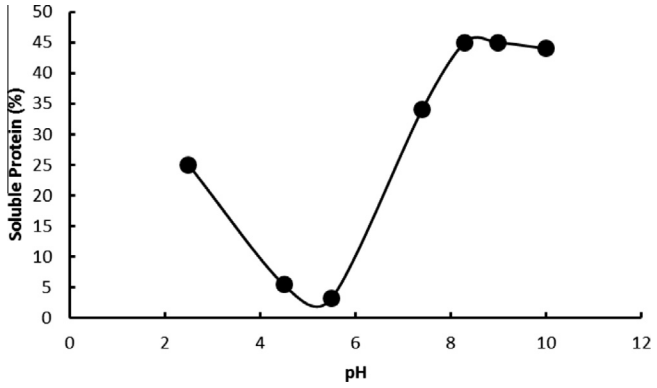

Fig. 3. pH-solubility profiles of protein isolate from cotyledon flour. Percentage of soluble protein was calculated at total protein basis in each sample.

3.4. Antioxidant activity

The free radical scavenger potential of different samples obtained from *P. alba* cotyledon flour was tested by the ABTS$^{\cdot+}$ and DPPH assays. Both assays are excellent tools for determining the antioxidant activity of hydrogen-donating compounds (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavenger of lipid peroxyl radicals) (Re et al., 1999; Zhang et al., 2009). The protein extracts obtained from *Prosopis* cotyledon flour were scavengers of ABTS cation-radical (Fig. 4A). The radical scavenging ability of proteins was significantly

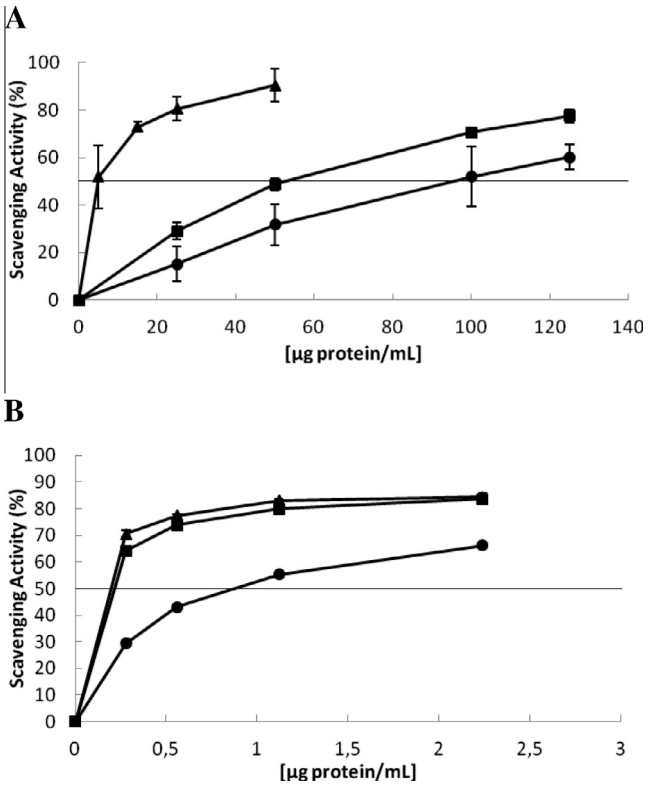

Fig. 4. ABTS cation radical scavenging activity of (A) crude protein extract (––●––), protein isolate (––■––) and enzymatic hydrolysate (––▲––) using different protein concentrations in 10 min of contact between sample-ABTS$^{\cdot+}$; (B) enzymatic hydrolysate with MW < 3 kDa at different contact time between sample and ABTS$^{\cdot+}$: 1 min (––●––), 6 min (––■––) and 10 min (––▲––).

Table 2
ABTS$^{\cdot+}$ and DPPH$^{\cdot}$ scavenging activity of protein isolates of soluble protein and its hydrolysate obtained from *Prosopis alba* cotyledons flours.

Samples	SC_{50} (μg protein/mL)	
	DPPH$^{\cdot}$ scavenging activity	ABTS$^{\cdot+}$ scavenging activity
Protein isolate	860	52
Protein hydrolysate	400	5
Protein hydrolysate with MW > 3 kDa	700	10
Protein hydrolysate with MW < 3 kDa	11.2	0.16

increased with the hydrolysis with SC_{50} values of 52 and 5 μg/mL, respectively (Table 2). Furthermore, the protein fraction with MW < 3 kDa was more active as antioxidant than the protein hydrolysate (SC_{50} values of 0.2 μg/mL and 5 μg/mL, respectively) (Table 2, Fig. 4A). Similar results were obtained with DPPH radical. The SC_{50} values were higher than those obtained with ABTS cation radical: 860, 400 and 11.2 μg/mL in protein isolate, protein hydrolysate and fraction with MW < 3 kDa, respectively.

The free radical scavenging ability of protein hydrolysates from *Prosopis* cotyledon flour was much higher than that of wheat germ (SC_{50} 1.3 mg/mL), chickpea (SC_{50} about 1.0 mg/ mL) and amaranthus protein hydrolysates (1.0 mg/mL) (Li, Jiang, Zhang, Mu, & Liu, 2008; Orsini Delgado, Tironi, & Añón, 2011; Zhu, Zhou, & Qian, 2006). Previous studies have pointed out that high radical scavenging activities for the protein hydrolysates or peptides are usually associated with hydrophobicity or high hydrophobic amino acid content (Rajapakse, Mendis, Byun, & Kim, 2005; Zhu et al., 2006). Furthermore, the antioxidant capacity of the protein fraction obtained from *P. alba* cotyledons was higher than that obtained from *P. alba* mesocarp (Cardozo et al., 2010).

3.5. Anti-inflammatory activity

The isolated protein obtained from cotyledon flour and its hydrolysates were selected for anti-inflammatory activity based on their relatively high antioxidant activity. While the isolated protein were inactive against LOX enzyme until 150 μg/mL, its hydrolysates decreased the LOX activity by 19 ± 2.6 and 52 ± 1.5% at 75 and 100 μg/mL, respectively (Fig. 5). The IC_{50} value for the protein hydrolysate was 100 μg/mL and for the control naproxen was 14 μg/mL. This would seem to indicate that the specific hydrolysis process used enhanced the bioactivity. However, the isolated protein and the protein hydrolysate did not modified significantly the $sPLA_2$ activity (Fig. 6).

The inhibition of lipoxygenases (LOXs) may influence the inflammation processes since these enzymes are involved in the formation of pro-inflammatory eicosanoids (leukotrienes and hydroperoxides) from fatty acids (Rossi et al., 2010) and participate in oxidative modifications of low-density lipoproteins (LDL) and in the development of atherosclerotic lesions (Sadik, 2005). Moreover, LOXs are sensitive to antioxidants and they commonly inhibit lipid hydroperoxide formation due to scavenging of lipid-oxy- or lipid-peroxy-radicals formed in the course of enzymatic peroxidation. This could limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX.

The LOX inhibition together with the antioxidant capacity of the hydrolysate may be useful in reducing or in preventing inflammatory complications. This is an important advance for the use of seed proteins from *P. alba* as a food supplement for treating inflammatory disease conditions.

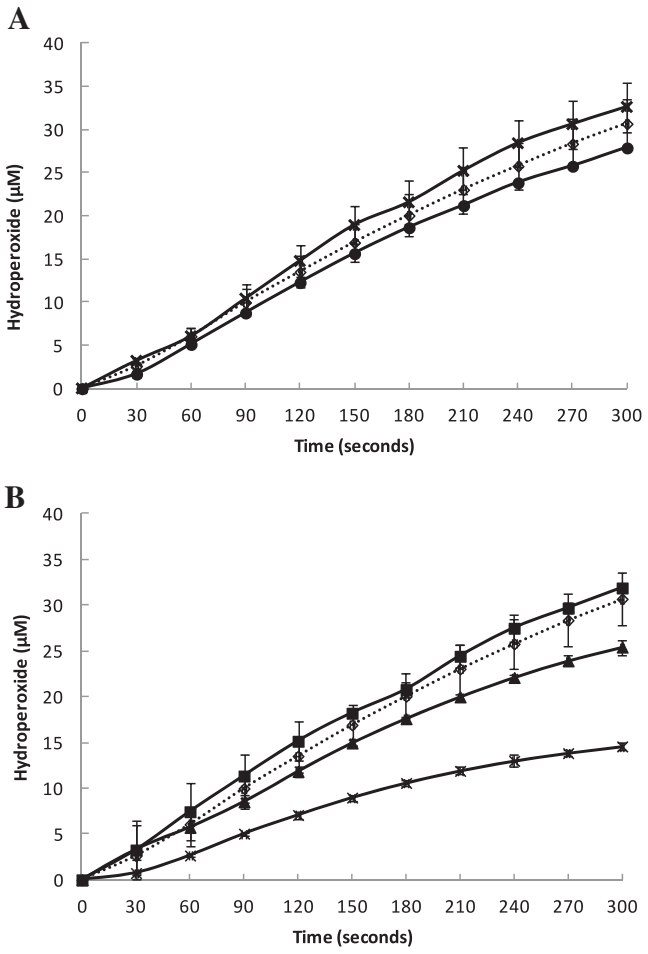

Fig. 5. Hydroperoxide production by lipoxygenase catalysed reaction in presence of different concentrations of protein isolate (A) and protein hydrolysate (B): 0 (◇), 50 (■), 75 (▲), 100 (×) and 150 (●) μg/mL.

3.6. Mutagenic/antimutagenicity activity

The mutagenicity of protein isolates and protein hydrolysate are not known. In this study, mutagenicity was evaluated by the Ames assay. In a series of experiments preceding the mutagenicity studies, it was ascertained that the different amounts of protein added to the indicator bacteria do not influence their viability (data not shown). Table 3 shows the number of revertants/plate after the

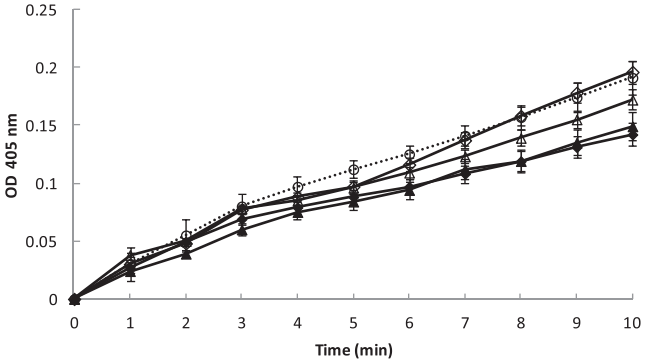

Fig. 6. Time course of phospholipase catalysed reaction in presence of 40 (filled symbols) and 100 (empty symbols) μg/mL protein isolate (▲) and protein hydrolysate (♦). Control (o).

Table 3
Revertant/plate in the strains TA98 and TA100 of *Salmonella typhimurium* after treatment with various doses of proteic extracts of *Prosopis* cotyledon with (+S9) and without (−S9) metabolic activation.

Samples	Treatment (μg protein/plate)	N° revertant/plate[a]			
		TA 98		TA 100	
		(−) S9	(+) S9	(−) S9	(+) S9
Protein isolate	52	24 ± 6	36 ± 4	180 ± 22	181 ± 18
	104	24 ± 8	37 ± 5	183 ± 7	184 ± 27
	208	27 ± 6	36 ± 8	173 ± 16	207 ± 28
Protein hydrolysate	22	19 ± 1	37 ± 6	215 ± 2	188 ± 20
	44	20 ± 3	38 ± 6	202 ± 0	184 ± 13
	88	27 ± 1	40 ± 10	199 ± 4	217 ± 24
Positive control[b]		1078 ± 89	890 ± 31	963 ± 62	810 ± 87
Negative control[c]		23 ± 5	35 ± 8	189 ± 11	210 ± 9

[a] Mean number of revertants [mean of four plates ± S.D.] (−S9) without and (+S9) with metabolic activation.
[b] Mean number of revertants induced by reference mutagens [2-AF, 2-aminofluorene (10 μg/plate), positive control for +S9; 4-NPD, 4-nitro-o-phenylenediamine (10 μg/plate) positive control for −S9].
[c] The number of spontaneous revertants was determined in assays without sample.

Table 4
Revertant/plate in the strains TA98 and TA100 of *Salmonella typhimurium* after co-treatment mutagen and proteins of *Prosopis* cotyledon flour.

Samples	Treatment (μg protein/plate)	N° revertant/plate[a]	
		TA 98	TA 100
Protein isolate	52	998 ± 32	1048 ± 105
	104	1073 ± 26	966 ± 35
	208	1016 ± 47	958 ± 27
Protein hydrolysate	22	1079 ± 62	933 ± 52
	44	1252 ± 21	876 ± 56
	88	1214 ± 54	989 ± 32
Positive control[b]		1078 ± 89	963 ± 62

[a] Mean number of revertants [mean of four plates ± S.D.].
[b] Mean number of revertants induced by the mutagen 4-NPD, 4-nitro-o-phenylenediamine (10 μg/plate).

treatments with the protein isolate and its hydrolysate in the two different strains of *S. typhimurium*, with or without metabolic activation. None of the preparations were mutagenic in strains TA98 or TA100 under the conditions used in this assay which indicates the inexistence of mutagens or pro-mutagens that cause base pair substitution (detected in TA100) and frameshift (detected in TA98) mutations. The absence of mutagenicity for the different protein preparations studied in the *Salmonella* tested strains indicates that DNA does not seem to be a relevant target for *Prosopis* protein. Antimutagenic effect was not observed at any sample concentration tested (Table 4).

4. Conclusions

The results suggest that *P. alba* cotyledon flour or protein isolate could be a new alternative in the formulation of foods for humans not only for its nutritive value and bioactivity but also by the functional properties such as emulsifying activity, WAC, OBC and solubility. This information is important for the development of value added products in the region, particularly as ingredients for the functional foods.

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