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Amaranth protein films from thermally treated proteins

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ABSTRACT

The usefulness of amaranth protein isolates, native and thermally treated, in edible films preparation was studied. Protein films were prepared by casting using glycerol as plasticizer. Films from amaranth native protein isolates showed low water vapor permeability (WVP) but poor mechanical properties. In order to improve this functionality, proteins were treated at 70 and 90 °C which corresponds to the denaturation temperature of the protein fractions present in the isolates. The unfolded conformation of these thermally treated proteins, when partially or totally denatured, favors the interactions between polypeptide chains during the film formation. These interactions lead to a greater cross-linking degree, which was reflected in the lower amount of water-soluble free peptides that were linked to the matrix. In these thermally treated protein films, a greatest contribution of disulfide and hydrogen bonds to the films stabilization was observed. These changes in the films structural properties would confer them a greater tensile strength and lower water solubility but higher thickness and WVP.

1. Introduction

The use of agricultural biopolymers for the development of edible and/or biodegradable films could be an opportunity to increase their applications, to create new markets, and to contribute to decrease the environmental pollution by substituting nondegradable synthetic plastic in pharmaceutical and food applications. These biopolymers have been used to prepare edible films and coatings that hold promise for innovative uses in food protection and preservation. Their utility lies in their capacity to act as an adjunct for improving overall food quality, extending shelf-life, and possibly improving cost–benefit of packaging materials (Guilbert, 1986; Kester and Fennema, 1986; Petersen et al., 1999).

Numerous proteins such as corn zein, wheat gluten, soy, peanut, cottonseed, sunflower, rice bran, serum albumin, egg white, collagen, gelatin, myofibrils, casein and whey proteins, and others of limited availability, have been studied as potential film forming agents (Krochta, 1997; Cuq et al., 1998; Shih, 1998; Gennadios, 2002). The film-forming ability of a protein can be influenced by the amino acid composition, distribution and polarity, ionic cross-links between amino and carboxyl groups, hydrogen bonds as well as intra- and intermolecular disulfide bonds (Gennadios and Weller, 1991). The interconnection of protein molecules during the drying process leads to the formation of the film matrix. Therefore, the extension or unfolding of the protein molecules could favor the interaction between them and where the junction zones could be formed to a higher extent (Hoque et al., 2010).

Amaranth (*Amaranthus hypochondriacus*) is a crop whose seeds have a protein content that is higher than that of other cereals (approximately 14%), a well-balanced composition in essential amino-acids and an important content of sulfur-containing amino-acids (Bressani, 1989), which add to their well known agronomic advantages (Lehmann, 1996). The main protein fractions present in the amaranth grain are albumins, 11S-globulin, P-globulin, and glutelins (Scilingo et al., 2002). To date, no works on the use of these proteins for edible films preparation have been published.

Tapia-Blácido et al. (2005) have produced amaranth flour films with interesting mechanical and water vapor barrier characteristics that were later attributed to the interactions formed between their polymers (starch and proteins) and lipids, to the distribution of these interactions within the film matrix and to the natural concentrations of each component in the film (Tapia-Blácido et al., 2007).

Avanza et al. (2005) have studied the gel-forming properties of amaranth proteins at different thermal conditions and protein concentrations. They reported that minimum conditions for gelation were 7% w/v of protein isolates and a temperature of 70 °C. They observed that a rapid denaturation of globulins followed by sulfhydryl/disulfide interchange reactions between protein molecules conduced to a gelation phenomenon enhanced by protein aggregation. Gels prepared under these critical conditions presented a strong gel-like behavior. Although these gels presented low adhesiveness, they were elastic in nature ($\tan\delta < 0.1$) and they had high hardness, fracturability and cohesiveness.

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It has been reported that films formed by heat treated proteins from soybean, whey, bean or squid gelatin showed improved mechanical properties and in some cases, they also present lower solubility and water vapor permeability (WVP) (Stuchell and Krochta, 1994; Pérez-Gago and Krochta, 2001; Choi and Han, 2002; Hoque et al., 2010). It is expected that a protein displaying a higher degree of unfolding in its initial structure, promote interactions between chains by increasing the cross-linking. The latter fact has implications for the resulting film properties, especially the mechanical and barrier ones.

Considering the studies mentioned above, the aim of this work was to evaluate the usefulness of amaranth protein isolates in the preparation of edible films and to study the effect of using thermally modified amaranth proteins on the functional properties of the resulting films.

2. Materials and methods

2.1. Plant material

Seeds of *A. hypochondriacus*, (cultivar 9122) used in this work were obtained from Estación Experimental del Instituto Nacional de Tecnología Agropecuaria (INTA), Anguil, La Pampa, Argentina.

2.2. Flour preparation

Seeds were ground and screened by 0.092 mm mesh. The resulting flour was defatted with hexane at 25 °C for 5 h (100 g/L suspension) under continuous stirring. After drying at room temperature, the flour was stored in hermetic containers in a chamber at 4 °C up to a month, when it was used for protein isolates preparation.

2.3. Preparation of amaranth protein isolate

Amaranth protein isolate (**API**) used in this study was prepared according to Martínez and Añón (1996). Briefly, defatted flour was suspended in water (100 g/L) and pH adjusted to 11.0 with 2 N NaOH. The suspension was stirred for 60 min at room temperature and then centrifuged 20 min at 9000*g* at 15 °C. The supernatant was adjusted to pH 5.0 with 2 N HCl and then centrifuged at 9000*g* for 20 min at 4 °C. The pellet was suspended in water, neutralized with 0.1 N NaOH and freeze-dried. API was stored in hermetic containers in a chamber at 4 °C up to 2 months until used. Its protein content was determined by Kjeldahl (method 954.01 AOAC, 1990), $N = 5.85$ (Segura-Nieto et al., 1994).

2.4. Thermal treatment

API was dispersed in water (1% w/v) and dispersions-placed in Erlenmeyer flasks covered with aluminum foil- were heated in a water bath at 70 ± 2 °C and 90 ± 2 °C for 20 min under continuous stirring. After heating, the flasks were immediately cooled by immersion in an ice bath and were freeze-dried, obtaining **API70** and **API90**, respectively.

2.5. Protein isolates characterization

2.5.1. SDS–PAGE electrophoresis

This analysis was performed using the procedure described by Martínez et al. (1997). Runs were carried out using a separating gel (12% w/v in polyacrylamide) and a stacking gel (4% w/v in polyacrylamide) in minislab arrangement (Bio-Rad Mini-Protean II Model). The following continuous buffers system were used: 0.375 mol/L Tris–HCl, 1 g/L SDS, pH 8.8, for the separating gel; 0.025 mol/L Tris–HCl, 0.192 mol/L glycine and 1 g/L SDS, pH 8.3, for the running buffer; and 0.125 mol/L Tris–HCl, 200 ml/L glycerol, 10 g/L SDS, 0.5 g/L bromophenol blue (p.a., Sigma Chemical Co.), pH 6.8, as sample buffer. For runs under reducing conditions the sample buffer also contained 50 ml/L 2-mercaptoethanol (2-ME) (p.a., Sigma Chemical Co.) and samples were heated for 60 s in a boiling water bath. Protein molecular weights were estimated using low MW markers (p.a., Pharmacia, Amersham, England) that included phosphorylase b (94.0 kDa), albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Gels were fixed and stained with R-250 Coomassie blue (0.2% w/v) in water/methanol/acetic acid (5:5:2) overnight, and destained with 25% v/v methanol and 10% v/v acetic acid. Gels images were acquired with a HP Scanjet G2710 scanner.

2.5.2. Differential Scanning Calorimetry (DSC)

A TA Instrument DSC Q100 V9.8 Build 296 (New Castle, DE, USA) was used for these studies. Temperature and heat flow calibration of the equipment was carried out according to ASTM standards, using lauric and stearic acid and indium as standards, respectively. Hermetically sealed aluminum pans containing 10–15 mg of samples (20% w/v of amaranth protein isolates) were prepared and scanned at 10 °C/min over the range of 30–120 °C. Denaturation enthalpies (ΔH_d) and temperatures in the minimum signal of the peak (T_d in °C) were taken from the corresponding thermograms (Universal Analysis V4.2E, TA Instruments, New Castle, DE, USA). Enthalpy values (ΔH_d) were expressed as J/g protein, taking into account the dry weight (determined by perforating the pans and heating overnight at 105 °C) and the protein content of sample (Molina et al., 2004).

2.6. Film Formation

Films were prepared by dispersing API, API70 and API90 (5% w/v) and glycerol (1.25% w/v, Anedra, Argentina) in distilled water. Dispersions were magnetically stirred for 1 h at room temperature, their pH was adjusted to pH 10.5 with 2 mol/L NaOH, and they were stirred again for additional 20 min. Ten mL of each film forming dispersion were poured onto polystyrene Petri dishes (64 cm^2) and dried at 60 °C for 3 h in an oven with air flow and circulation (Yamato, DKN600, USA). The dry films were conditioned at 20 °C and 58% relative humidity in desiccators with saturated solutions of NaBr for 48 h before being peeled from the casting surface for characterization.

2.7. Film characterization

2.7.1. Moisture Content (MC)

MC was determined after drying in an oven at 105 °C for 24 h. Small specimens of films collected after conditioning, were cut and placed on Petri dishes that were weighed before and after oven drying. MC values were determined in triplicate for each film, and calculated as the percentage of weight loss based on the original weight (ASTM D644-94, 1994).

2.7.2. Film thickness

Film thickness was measured by a digital coating thickness gauge (Check Line DCN-900, USA). Measurements were done at five positions along the rectangular strips for the tensile test, and at the center and at eight positions round the perimeter for the WVP determinations. The mechanical properties and WVP were calculated using the average thickness for each film replicate.

2.7.3. Film color

Film colors were determined using a Minolta Chroma meter (CR 300, Minolta Chroma Co., Osaka, Japan). A CIE Lab color scale was used to measure the degree of lightness (L), redness ($+a$) or greenness ($-a$), and yellowness ($+b$) or blueness ($-b$) of the films. The instrument was standardized using a set of three Minolta calibration plates. Films were measured on the surface of the white standard plate with color coordinates of $L = 97.3$, $a = 0.14$ and $b = 1.71$. Total color difference (ΔE) was calculated from:

$$\Delta E = \sqrt{(L_{film} - L_{standard})^2 + (a_{film} - a_{standard})^2 + (b_{film} - b_{standard})^2} \quad (1)$$

Values were expressed as the means of nine measurements on different areas of each film.

2.7.4. Opacity

Each film specimen was cut into a rectangular piece and placed directly in a spectrophotometer test cell, and measurements were performed using air as the reference. A spectrum of each film was obtained in an UV–Vis spectrophotometer (Beckman DU650, Germany). The opacity of the film (UA/mm) was calculated by dividing the absorbance at 500 nm by the film thickness (mm) (Cao et al., 2007). All determinations were performed in triplicate.

2.7.5. Mechanical properties

The tensile strength, Young's modulus and elongation at break of the films were determined following the procedures outlined in the ASTM methods D882-91 (ASTM, 1991), taking an average of six measurements for each film and using at least two films per formulation. The films were cut into 6 mm wide and 80 mm long strips, and mounted between the grips of the texture analyzer TA.XT2i (Stable Micro Systems, Surrey, England). The initial grip separation was set at 50 mm and the crosshead speed at 0.4 mm/s. The tensile strength (force/initial cross-sectional area) and elongation at break were determined directly from the stress–strain curves using Texture Expert V.1.15 software (Stable Micro Systems, Surrey, England), and the Young's modulus was calculated as the slope of the initial linear portion of this curve.

2.7.6. Solubility in water

Solubility was measured by immersion of film disks (2.0 cm in diameter) in water containing sodium azide, at 25 ± 2 °C for a period of 24 h (Gontard et al., 1992). The amount of dry matter in the initial and final samples was determined by drying the samples at 105 °C for 24 h. All determinations were performed in triplicate.

2.7.7. Water vapor permeability (WVP)

Water vapor permeability tests were conducted using ASTM method E 96-80 (1989) with some modifications (Gennadios et al., 1994). Each film sample was sealed over a circular opening of 0.00177 m^2 in a permeation cell that was stored at 25 °C in a desiccator. To maintain a 75% relative humidity (RH) gradient across the film, anhydrous silica (0% RH) was placed inside the cell and a saturated NaCl solution (75% RH) was used in the desiccator. The RH inside the cell was always lower than outside, and water vapor transport was determined from the weight gain of the permeation cell. When steady-state conditions were reached (about 1 h), eight weight measurements were made over 5 h. Changes in the weight of the cell were recorded and plotted as a function of time. The slope of each line was calculated by linear regression and the water vapor transmission rate (WVTR) was calculated from the slope (g/s H_2O) divided by the cell area (m^2). WVP (g/Pa s m) was calculated as:

$$WVP = [(WVTR) \times d] / \left[P_v^{H_2O} \times (RH_d - RH_c) \times A\right] \quad (2)$$

where $P_V^{H_2O}$ is the vapor pressure of water at saturation (Pa) at the test temperature (20 °C), RH_d the RH in the desiccator, RH_c the RH in the permeation cell, A the permeation area (m^2), and d is the film thickness (m). Each WVP value represents the mean value of at least three sampling units taken from different films.

2.7.8. Measurement of surface hydrophobicity

Surface hydrophobicity was assessed by measuring contact angle using a goniometer ramé-hart Model 500 (ramé-hart instrument co., USA). A 5 μl drop of demineralized water was placed on the surface of the film with an automatic piston syringe and photographed. An image analyzer was used to measure the angle formed between the base, constituted of the surface of the film in contact with the drop of water, and the tangent to the drop of water. The mean hydrophobicity value for the surface of each film was calculated from six measurements on the film.

2.7.9. Differential solubility of proteins

Protein solubility of the films was determined according to the method described by Mauri and Añón (2006), with some modifications. Pieces of films were weighted and placed into a tube containing 1 ml of water or buffer. Five different buffer systems all at pH 7.5 were used: (a) 0.1 mol/L phosphate buffer (NaH_2PO_4) containing 0.1 mol/L NaCl (PB); (b) PBD buffer: PB with 0.1% sodium dodecyl sulfate (SDS, Anedra, Argentine); (c) PBU buffer: PB with 6 mol/L urea (Riedel-deHaën, Germany); (d) PBDU buffer: PB with 0.1% SDS and 6 M urea, and (e) PBDUM buffer: PB with 0.1% SDS, 6 mol/L urea and 2.5% 2-mercaptoethanol (ME, Sigma–Aldrich, Germany). The tubes were shaken for 24 h at 20 °C. Suspensions were then centrifuged at 9000*g* for 20 min at room temperature and the protein content in the supernatant was determined using a Bradford assay (Bradford, 1976). Standard curves using bovine serum albumin (BSA, Sigma–Aldrich Chemical Co., St. Louis, USA) were constructed for each buffer. For each type of film, at least two samples from four independent film preparations were solubilized. The soluble protein content was expressed as a percentage of the total amount of protein in the film, which was measured by the Kjeldahl method (AOAC 920.53, 1995).

2.8. Statistical analysis

Results were expressed as mean ± standard deviation and were analyzed by analysis of variance (ANOVA). Means were tested with the Fisher's least significant difference test for paired comparison, with a significance level $\alpha = 0.05$.

3. Results and discussion

3.1. Effect of heat treatment on the structure of amaranth proteins

The amaranth protein isolate (API) –91.1 ± 0.2% w/w of proteins (d.b.)- yielded 14.1 ± 2.0 g isolate per 100 g of defatted flour. Its polypeptide composition was analyzed by denaturing electrophoresis (SDS–PAGE) under reducing and non-reducing conditions (Fig. 1). Under non-reducing conditions (Fig. 1, lane 1), the typical profile for these isolates was found (Martínez and Añón, 1996; Abugoch et al., 2010). This profile consisted of two bands of similar molecular mass (56–54 kDa) corresponding to the AB subunits of 11S globulin and to P-54 polypeptide of P-globulin; bands at 67 kDa, probably constituents of 7S globulin (Marcone, 1999); bands between 32 and 38 kDa corresponding to polypeptides A of globulin; other bands of *ca* 21 kDa corresponding to polypeptides B of globulin, or glutelin, and minor bands at 20 kDa, which may correspond to the albumins fraction. High molecular mass

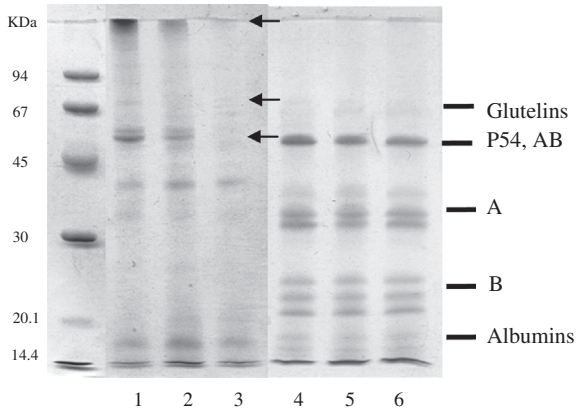

Fig. 1. Polyacrylamide gel electrophoresis profile under denaturing conditions (SDS–PAGE) of amaranth protein isolates in either native state (API: lane 1) or after thermal treatment (API70: lane 2 and API90: lane 3), and under denaturing reducing conditions (API: lane 4, API70: lane 5 and API90: lane 6).

species that could not enter the gel could also be detected, which might belong to the glutelin fraction.

Under reducing conditions (Fig. 1, lane 4), those bands corresponding to high molecular mass species that could not be resolved in the previous gel as well as those bands of 67 kDa, disappeared, while an increase in the intensity was observed for bands of 54–56 kDa, 32–38 kDa and 21 kDa, and bands of lower molecular mass corresponding to 20 kDa, which were linked by disulfide bonds.

By differential scanning calorimetry, the API isolate was found to present two endotherms whose peak temperatures and denaturation enthalpies are shown in Table 1. The endotherm presenting the lowest thermal stability (~70 °C) might be attributed to the denaturation of the glutelins fraction and to a fraction of 7S globulins, whereas the endotherm presenting the highest thermal stability (~98 °C) would correspond to the denaturation of 11S globulins, P-globulin as well as to another glutelins fraction (Avanza and Añón, 2007). Taking into account the thermal stability of amaranth proteins, the temperatures of 70 and 90 °C were selected to perform the thermal treatments. Initially, the thermal treatment was attempted to carry out directly on the film forming protein dispersions so as to simplify the process avoiding the freeze-drying step, but dispersions got gelified under these experimental conditions of temperature and protein concentration (5% w/v). As a consequence, thermal treatments was performed on 1% w/v protein dispersions that then were freeze-drying and the resulting samples were used to prepare thermally treated protein films.

Table 1 also depicts the denaturation enthalpies and temperatures (ΔH_d and T_d) of thermally treated amaranth protein (API70 and API90). The thermal treatment applied at 70 °C induced the disappearance of the first characteristic endotherm of the API due the total denaturation of the protein fractions of low stability (glutelins and 7S globulins). An 87% of the native structure was retained in the fractions of higher thermal stability (11S globulins, P-globulins and the remaining glutelins fraction) which was evidenced by a decrease in the denaturation enthalpy and a shift in the T_d corresponding to the second endotherm. The thermal treatment at 90 °C almost induced the complete denaturation of all the protein fractions. Only a 5% of the native structure of those fractions of higher thermal stability remained. Both API70 and API90 showed a slight increase in the denaturation temperature when compared to the control isolate (API, Table 1), which may indicate the enrichment of the fraction that retains its native structure in thermo stable proteins due to the effect of aggregation or denaturation caused by the heat treatment ($p < 0.05$). Avanza and Añón (2007) have also reported a decrease in the ΔH_d values in thermally treated amaranth protein isolates. These authors provided the same explanation and they also hypothesized about the existence of hydrophobic interactions which would stabilize such aggregates.

Table 1
Denaturation enthalpies (ΔH_d) and temperatures (T_d) of amaranth protein isolate in either native state (API) or after thermal treatment at 70 °C and 90 °C (API70 and API90).

	ΔH_d (J/g isolate d.b.)		T_d (°C)	
	Endotherm 1	Endotherm 2	Endotherm 1	Endotherm 2
API	4.5 ± 0.3	6.2 ± 0.6[a]	70.7 ± 0.1	98.6 ± 0.1[a]
API70	–	5.4 ± 0.2[b]	–	99.8 ± 0.2[b]
API90	–	0.28 ± 0.05[c]	–	100.5 ± 0.2[b]

All values were means ± SD of two values. Means of ΔH_d and T_d within a column with same superscripts (a, b and c) are not significantly different ($p < 0.05$).

To determine the association–dissociation degree of proteins present in thermally treated isolates, these samples were also analyzed by SDS–PAGE (Fig. 1). In both thermally treated isolates (lanes 2 and 3), those bands corresponding to the 67 kDa polypeptides were not observed. Furthermore, the intensity of the band corresponding to the 56 kDa polypeptides was reduced in the API70, and disappeared in the API90. This finding would indicate that these fractions would be involved in the formation of aggregates, as reported previously by Avanza and Añón (2007). High molecular species that had not entered the gel were not disclosed either, especially in the API90. Under reducing conditions, neither qualitative nor quantitative changes were observed in the electrophoretic profile of thermally treated samples (lanes 5 and 6), as compared to the control isolate (lane 4), probably due to the fact that protein aggregates would be stabilized by disulfide bonds.

3.2. Effect of heat treatment of proteins on the functionality of the respective films

3.2.1. Appearance and moisture content of protein films

Protein-based films from API and API70 were found to be homogeneous and flexible, while films obtained from API90 felt rough. Their thickness and moisture content are shown in Table 2. Those protein films obtained from thermally treated protein isolates presented the same amount of water, solids and plasticizers as the control film, however, their thickness increased significantly with the treatment temperature ($p < 0.05$). It is therefore evident that those unfolded protein molecules and aggregates might be able to generate less compact networks when interacting with each other.

Hunter-Lab color parameters and opacity of the studied protein films are also shown in Table 2. Protein films obtained from API70 only presented a minor luminosity (L) than those obtained from untreated isolates, while those films made from API90 displayed a shift in the L, a, b, and ΔE values when compared to those corresponding to the other two films. These films had a lower L value, a more brownish tone (higher a and b values) and a more intense overall color (ΔE) ($p < 0.05$). Hoque et al. (2010) have also observed an increase in the color intensity (higher b value) and a decrease in the luminosity (lower L value) in those films obtained from thermally treated gelatin, a phenomenon that was attributed to the Maillard's reaction. In films prepared with thermally treated soy protein, Kim et al. (2002) have reported the same effect for the b parameter. On the other hand, Choi and Han (2002) have not observed any differences between control films and films obtained from bean protein isolates treated at high temperature.

The opacity of films obtained from thermally treated isolates increased when compared to films obtained from untreated proteins, however, this effect was independent of the treatment temperature ($p < 0.05$). Even though differences in color and opacity were

Table 2
Thickness, moisture content, Hunter color parameters (a, b and L), total color difference (ΔE) and opacity of films prepared from amaranth protein isolate in either native state (API) or after thermal treatment at 70 °C and 90 °C (API70 and API90).

Films	Thickness (μm)	Moisture content (%)	Hunter-Lab color parameters				Opacity (UA/mm)
			a	b	L	ΔE	
API	52.3 ± 13.6[a]	18.9 ± 0.3[a]	−0.8 ± 0.1[a]	23.4 ± 1.3[a]	82.6 ± 0.7[a]	26.2 ± 1.5[a]	1.1 ± 0.2[a]
API70	85.1 ± 12.4[b]	18.0 ± 0.7[a]	1.1 ± 0.2[a]	24.4 ± 4.4[a]	77.3 ± 2.7[b]	30.3 ± 5.1[a]	4.5 ± 0.4[b]
API90	98.2 ± 18.7[b]	18.1 ± 0.2[a]	2.6 ± 0.8[b]	29.0 ± 0.8[b]	70.4 ± 2.3[c]	38.4 ± 2.3[b]	4.6 ± 1.2[b]

All values were means ± SD of two values. Means of thickness and moisture content within a column with same superscripts (a, b and c) are not significantly different ($p < 0.05$).

found between the evaluated films, these differences were not perceptible with the naked eye.

3.2.2. Mechanical properties of protein films

Fig. 2 shows the mechanical properties (measured in tensile test) of the studied films. Values of tensile strength, maximum elongation at break and elastic modulus of the API films proved to be very poor, probably due to the fact that the protein molecules present in the film maintain, to a high extent, their native structure (Mauri and Añón, 2006). The results found in this work were similar to those reported by Denavi et al. (2009a) for films prepared with native soy protein films, but much lower than the values of tensile strength and elastic modulus than those reported by other authors when working with films prepared with soy and sunflower proteins partially or totally denatured, by the same process as the one employed herein (Denavi et al., 2009a; Salgado et al., 2010). Thus, it is evident that the initial conformation of proteins greatly influences the functionality of the resulting films.

Those films prepared with thermally treated isolates presented a higher tensile strength and a lower elongation at break value than controls while keeping the same elastic modulus ($p < 0.05$). The only difference that was found between treatments at different temperature was observed for the elongation at break value, which proved to be higher for API70.

Presumably, the unfolded conformation of thermally treated proteins, when partially or totally denatured, favors the interactions between polypeptide chains during the film formation leading to a greater cross-linking degree, which would confer the film a greater tensile strength. This phenomenon was also observed by Mauri and Añón (2006) when working with films prepared from soy protein isolates dispersions at different pH with different denaturation degrees. These authors reported that films obtained at pH 2 and 11, in which proteins were partially or totally denatured and whose films had denser microstructures and a higher amount of disulfide bonds, showed a higher tensile strength and a higher Young's modulus than the one that had been prepared with native proteins at pH 8. However, films formed at alkaline pH (8 and 11) exhibited a higher deformation than films at pH 2,

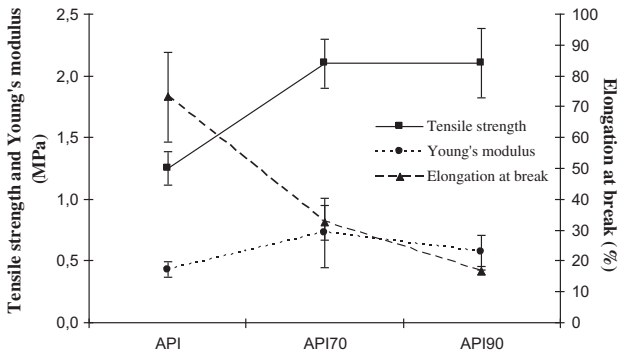

Fig. 2. Mechanical properties (tensile strength, elongation at break and Young's modulus) of films prepared from amaranth protein isolates in either native state (API) or after thermal treatment at 70 °C and 90 °C (API70 and API90).

indicating that the presence of at least a protein fraction in native state allowed macromolecules to unfold during the mechanical test, reaching greater deformation before breaking.

Similar results to the ones presented herein have been found for other proteins films. Pérez-Gago and Krochta (2001) have found similar tendencies in films mechanical properties working with thermal treated wheat proteins that attributed to the disulfide bonds formed after the unfolding of proteins. Choi and Han (2002) have also observed that heat treated bean protein films presented higher tensile strength and elongation, and lower elastic module than the ones prepared without heating probably due to a rearrangement of protein after thermal treatment. Hoque et al. (2010) have demonstrated that squid gelatin films undergo an increase in the tensile strength and a decrease in the elongation when a thermal treatment at 70 °C was applied. However, at higher temperatures, these trends were inverted, most probably due to protein degradation leading to the formation of shorter protein chains.

3.2.3. Susceptibility to water

Water solubility, water vapor permeability (WVP) and contact angle -which reflects the surface hydrophobicity- of the studied films, are shown in Table 3. The solubility in water decreased 23% and 28% for API70 and API90 respectively, with respect to the control film ($p < 0.05$). However, it is worth noting that the films obtained from thermally proteins maintained their integrity after the experiment, while API films were disintegrated during the 24 h of solubilization. It is reasonable to speculate that the higher cross-linking among denatured proteins contributed to the decrease in films solubility as well as to the stabilization of the film structure even after a long time of exposure to water. Pérez-Gago and Krochta (2001) have also observed a significant decrease in the solubility of thermally treated wheat film due to the formation of intermolecular bonds of higher energy among unfolded proteins.

The WVP value obtained with the API (Table 3) was an order of magnitude lower than those corresponding to soy and sunflower films similarly prepared and processed ($\sim 1.5 \times 10^{-10}$ g H_2O/Pa m) (Denavi et al., 2009b; Salgado et al., 2010). This result might be attributed to the hydrophobic nature of amaranth protein, particularly that of 11S and P-globulins. But WVP increased in the case of both films obtained from thermally treated proteins, when com-

Table 3
Water solubility, water vapor permeability (WVP) and surface hydrophobicity of films prepared from amaranth protein isolate in either native state (API) or after thermal treatment at 70 °C and 90 °C (API70 and API90).

Films	Water solubility (%)	WVP $*10^{-11}$ (g H_2O/Pa m s)	Contact angle (°)
API	79.9 ± 2.1[a]	5.6 ± 0.5[a]	69.5 ± 2.5[a]
API70	61.4 ± 1.7[b]	8.5 ± 0.2[b]	77.6 ± 5.8[a]
API90	57.2 ± 3.3[b]	19.9 ± 5.2[c]	67.0 ± 8.4[a]

All values were means ± SD of two values. Means of water solubility, water vapor permeability and contact angle within a column with same superscripts (a, b and c) are not significantly different ($p < 0.05$).

pared to the control one. Moreover, the WVP increase was directly proportional to the increase in the treatment temperature This behavior may be related in part to the increased thickness of API90 and API70 films, taking into account that in general, in hydrophilic films like those prepared by pectins, amylose esters, cellulose, sodium caseinate and soy protein, WVP increases with thickness (McHugh et al., 1993; Ghorpade et al., 1995), and also/or differences in hydrophilic-hydrophobic nature of the films as the result of interactions between proteins with different degree of denaturalization and aggregation. It is evident that those interactions that contribute to the stabilization of protein matrixes can influence this property, since the WVP of films obtained from protein isolates of different origins and with different amino-acid compositions vary differently when compared to films made of untreated proteins. Thus, Hoque et al. (2010) have also observed an increase in the WVP of squid gelatin films treated up to 70 °C, whereas Pérez-Gago et al. (1999) failed to obtain differences in this property when working with thermally treated milk proteins films.

All films analyzed in this work proved to have similar surface hydrophobicities, since no significant differences were observed in the contact angles values when a drop of water was supported on the films. While all values were less than 90°, typical of hydrophilic films, they were higher to those reported for films prepared with soy and whey proteins thermally treated or not (<63°) (Kokoszka et al., 2010a; Kokoszka et al., 2010b; Galus et al., in press). The greater surface hydrophobicity of amaranth protein films gives them better potential to overcome the limitation of hygroscopic property (Tang and Jiang, 2007).

Thermally treated proteins formed films that had higher WVP, similar water content and surface hydrophobicity and less solubility than films obtained from untreated proteins. These properties could be associated with a higher hydrophobicity of heat-treated proteins (not determined) usually associated with a higher degree of aggregation (as it was demonstrated by SDS–PAGE).

3.2.4. Protein solubility

It seemed that thermally treated isolates films present an increased cross linking among protein chains, which is reflected in their modified solubility, higher mechanic resistance and higher water vapor permeability. In order to further analyze the films structure–function relationship, protein interactions involved in the stabilization of films prepared from thermally treated and untreated proteins were studied. In particular, the differential solubility of film proteins in buffer systems with the capacity to disrupt different types of interactions was studied. Such systems were: water (*W*), which can dissolve free polypeptides not strongly linked to the protein matrix; phosphate buffer (PB), which affects protein electrostatic interactions; PBD, which contains SDS and disrupt mainly hydrophobic interactions and also interacts with proteins increasing their charge/mass ratio; PBU, which contains urea and disrupts the water structure affecting hydrogen bonds and also hydrophobic interactions; PBDU, which disrupts all the interactions mentioned above and also modifies protein charge; and PBDUM, which also disrupts disulfide bonds because it contains 2-mercaptoethanol. The results obtained are shown in Fig. 3. The solubility in water of proteins from API film was higher than those from API70 and API90 ones, suggesting that in the latter there were a lower amount of free polypeptides weakly associated with the protein matrix. The same phenomenon was observed upon solubilization of the films in PB, although for the control film, the solubility was lower than that obtained in water, thus demonstrating that electrostatic interactions play a less relevant role in the stabilization of this film. Upon employing PBD, the solubility of proteins in the API film was 4 and 10 times higher than that of API70 and API90, respectively. This finding would evidence that hydrophobic interactions would play a less important role in the

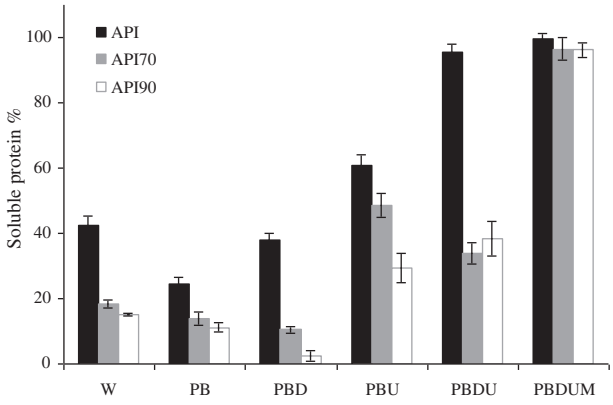

Fig. 3. Differential protein solubility of films prepared with API, API70 and API90 in solvents with different chemical activity: Water (*W*), 0.1 M sodium phosphate buffer (PB), PB containing 0.1% w/v SDS (PBD), PB containing 6 M urea (PBU), PB containing both 0.1% SDS and 6 M urea (PBDU), and PBDU containing 2.5% v/v, 2-mercaptoethanol (PBDUM), All solutions were at pH 7.5. Values for each protein isolate are expressed as means ± standard deviation.

stabilization of matrixes prepared with thermally treated proteins, or that the breakdown of hydrophobic interactions would not be sufficient to release proteins and peptides which in turn would be also stabilized by other sort of interactions. Moreover, the important role of these interactions was manifested in the API films, and correlated with their low WVP values. The addition of urea to the phosphate buffer induced a significant increase in the protein solubility in all the studied films, especially of those obtained from heat treated isolates, indicating the importance of hydrogen bonds in the formation of films. Meanwhile proteins of API films achieved complete solubilization in PBDU, the corresponding protein solubility of films formed by thermally treated proteins exhibited similar solubilities than those achieved in the PBU.

Finally, proteins from API70 and API90 films could be completely solubilized in PBDUM, denoting the importance of disulfide bonds in the stabilization of matrixes prepared from thermally treated proteins. It is noteworthy that the disulfide bonds might prevent the release of protein molecules that were also stabilized by other interactions which might be disrupted by PBD or PBDU, leading to an underestimation of the other type of interactions ($p < 0.05$). Probably it was expected that protein isolates submitted to heat treatment would have less free sulfhydryl groups, which were able to undergo oxidation during film formation, because their SDS–PAGE protein pattern (Fig. 1) showed large aggregates stabilized by disulfide bonds. It is evident that disulfide bonds that stabilized the films were not necessarily formed from free sulfhydryls present in isolates. During proteins dispersion and films preparation sulfhydryl–disulfide exchange occurred, especially favored by alkaline pH of the dispersions.

The study carried out herein demonstrated that in matrices prepared from thermally treated proteins, there exists a greater degree of cross-linking, as was previously suggested to explain films physicochemical properties. This phenomenon is reflected in the lower amount of water-soluble free peptides that are weakly linked to the matrix, and in the relevance of disulfide and hydrogen bonds in the stabilization of API70 and API90. Pérez-Gago and Krochta (2001) have suggested that the loss of the native three-dimensional structure of proteins, due to the thermal treatment, could expose those sulfhydryl groups that were initially in the inner areas of the molecule, thus allowing them to form intermolecular disulfide bonds that rendered the film insoluble in water. It is known that disulfide bonds formation is favored in denatured proteins. In this situation, thiol groups that once properly oriented will react with each other. The latter reaction occurs at a higher veloc-

ity when proteins are unfolded than when they conserve their native conformation (Darby and Creighton, 1995). Moreover, it is also well known that disulfide bonds are the strongest protein–protein interactions and that they are not affected by temperature changes (Bryant and McClements, 1998). The disulfide bonds that are formed after the heat-induced unfolding might be responsible for the improvement of the mechanical properties and for the decrease in the solubility in water, as demonstrated by other authors (Choi and Han, 2002; Pérez-Gago and Krochta, 2001). The major importance of hydrophobic interactions in the stabilization of native films might contribute to the better water vapor barrier properties of these films.

4. Conclusions

It was possible to prepare edible films with interesting barrier properties to water vapor but poor mechanical properties from amaranth native protein isolates.

But when using partially or completely denatured proteins, previously heat treated at different temperatures, it was possible to improve significantly the mechanical resistance and solubility of the films at the expense of their water vapor permeability due to the increased crosslinking of these proteins in films mainly through hydrogen and disulfide bonds. The optimal treatment selection would depend on the application sought for these films.

References

Abugoch, L.E., Martínez, N.E., Añón, M.C., 2010. Influence of pH on structure and function of amaranth (*Amaranthus hypochondriacus*) protein isolates. Cereal Chemistry 87 (5), 448–453.

AOAC, 1990. In: Official Methods of Analysis, 15th ed. Association of Official Analytical Chemists, Washington, DC.

AOAC, 1995. In: Official Methods of Analysis of AOAC International, 16th ed. AOAC International, Gaithersburg, MD.

ASTM, 1989. Standard test methods for water vapor transmission of materials. Designation: E 96-80. Annual Book of ASTM Standards. ASTM, Philadelphia, PA, pp. 745–754.

ASTM, 1991. Standard test methods for tensile properties of thin plastic sheeting. Designation: D882-91. Annual Book of ASTM Standards. ASTM, Philadelphia, PA, pp. 182–190.

ASTM, 1994. Standard test methods for moisture content of paper and paperboard by oven drying. Designation: D644-94. Annual Book of ASTM Standards. ASTM, Philadelphia, PA, pp. 1–2.

Avanza, M.V., Añón, M.C., 2007. Effect of thermal treatment on the proteins of amaranth isolates. Journal of the Science of Food and Agriculture 87, 616–623.

Avanza, M.V., Puppo, M.C., Añón, M.C., 2005. Rheological characterization of amaranth protein gels. Food Hydrocolloids 19, 889–898.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry 72, 248–254.

Bressani, R., 1989. The proteins of grain amaranth. Food Review International 5, 13–38.

Bryant, C.M., McClements, D.J., 1998. Molecular bases of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. Trends Food Science and Technology 9 (4), 143–151.

Cao, N., Fu, Y., He, J., 2007. Preparation and physical properties of soy protein isolate and gelatin composite films. Food Hydrocolloids 21, 1153–1162.

Choi, W.S., Han, J.H., 2002. Film-forming mechanism and heat denaturation effects on the physical and chemical properties of pea-protein-isolate edible films. Journal of Food Science 67 (4), 1399–1406.

Cuq, B., Gontard, N., Guilbert, S., 1998. Proteins as agricultural polymers for packaging production. Cereal Chemistry 75, 1–9.

Darby, N., Creighton, T.E., 1995. Disulfide bonds in protein folding and stability. In: Shirley, B.A. (Ed.), Protein Stability and Folding: Theory and Practice. Humana Press, Inc., Totowa, New Jersey, pp. 219–252.

Denavi, G.A., Perez-Mateos, M., Anon, M.C., Montero, P., Mauri, A.N., Gomez-Guillen, M.C., 2009a. Structural and functional properties of soy protein isolate and cod gelatin blend films. Food Hydrocolloids 23 (8), 2094–2101.

Denavi, G., Tapia Blácido, D.R., Añón, M.C., Sobral, P.J.A., Mauri, A.N., Menegalli, F.C., 2009b. Effects of drying conditions on some physical properties of soy protein films. Journal of Food Engineering 90 (3), 341–349.

Galus, S., Lenart, A., Voilley, A., Debeaufort, F., in press. Effect of potato oxidized starch on the physico-chemical properties of soy protein isolate-based edible films. Food Technology and Biotechnology.

Gennadios, A. (Ed.), 2002. Protein Based Films and Coatings. CRC Press, Boca Raton, FL.

Gennadios, A., Weller, C.L., 1991. Edible films and coatings from soymilk and soy protein. Cereal Foods World 36, 1004–1009.

Gennadios, A., McHugh, T.H., Weller, C.L., Krochta, J.M., 1994. Edible coatings and film based on proteins. In: Krochta, J.M., Baldwin, E.A., Nisperos-Carriedo, M. (Eds.), Edible Coatings and Films to Improve Food Quality. Technomic Publishing Co., Inc., Lancaster, pp. 201–278.

Ghorpade, V.M., Li, H., Gennadios, A., Hanna, M.A., 1995. Chemically modified soy protein films. Transactions of the ASAE 38, 1805–1808.

Gontard, N., Guilbert, S., Cuq, J., 1992. Edible wheat gluten films: influence of the main process variables on films properties using response surface methodology. Journal of Food Science 57, 190–195.

Guilbert, S., 1986. Technology and applications of edible protective films. In: Mathlouthi, M. (Ed.), Food Packaging and Preservation: Theory and Practice. Elsevier Science, New York, pp. 371–394.

Hoque, M.S., Benjakul, S., Prodpran, T., 2010. Effect of heat treatment of film-forming solution on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatine. Journal of Food Engineering 96, 66–73.

Kester, J.J., Fennema, O.R., 1986. Edible films and coatings: a review. Food Technology 40, 47–58.

Kim, K.M., Weller, C.L., Hanna, M.A., Gennadios, A., 2002. Heat curing of soy protein films at selected temperatures and pressures. LWT – Food Science and Technology 35, 140–145.

Kokoszka, S., Debeaufortm, F., Lenart, A., Voilley, A., 2010a. Water vapor permeability, thermal and wetting properties of whey protein isolate based edible films. International Dairy Journal 20, 53–60.

Kokoszka, S., Debeaufort, F., Hambleton, A., Lenart, A., Voilley, A., 2010b. Protein and glycerol contents affect physico-chemical properties of soy protein isolate-based edible films. Innovative Food Science and Emerging Technologies 11 (3), 503–510.

Krochta, J.M., 1997. Edible protein films and coatings. In: Damodaran, S., Paraf, A. (Eds.), Food Proteins and Their Applications. Marcel Dekker, New York, pp. 529–549.

Lehmann, J.W., 1996. Case history of grain amaranth as an alternative crop. Cereal Foods World 41, 399–411.

Marcone, M.F., 1999. Evidence confirming the existence of a 7S globulin-like storage protein in *Amaranthus hypochondriacus* seed. Food Chemistry 65, 533–542.

Martínez, E.N., Añón, M.C., 1996. Composition and structural characterization of amaranth protein isolates. Journal of Agricultural and Food Chemistry 44, 2523–2530.

Martínez, E.N., Castellani, O.F., Añón, M.C., 1997. Common molecular features among amaranth storage proteins. Journal of Agricultural and Food Chemistry 45, 3832–3839.

Mauri, A.N., Añón, M.C., 2006. Effect of the solution pH on solubility and some structural properties of soybean protein isolate films. Journal of the Science of Food and Agriculture 86 (7), 1064–1072.

McHugh, T.H., Avena-Bustillos, R., Krochta, J.M., 1993. Hydrophilic edible films: modified procedure for water vapor permeability and explanation of thickness effects. Journal of the Science of Food and Agriculture 58, 899–903.

Molina, M.I., Petruccelli, S., Añón, M.C., 2004. Effect of pH and ionic strength modifications on thermal denaturation of the 11S globulin of sunflower (*Helianthus annuus*). Journal of Agricultural Food Chemistry 52, 6023–6029.

Pérez-Gago, M.B., Krochta, J.M., 2001. Denaturation time and temperature effects on solubility, tensile properties, and oxygen permeability of whey protein edible films. Journal of Food Science 66 (5), 705–710.

Pérez-Gago, M.B., Nadaud, P., Krochta, J.M., 1999. Water vapor permeability, solubility, and tensile properties of heat-denatured versus native whey protein films. Journal of Food Science 64, 1034–1037.

Petersen, K., Vaeggemose Nielsen, P., Bertelsen, G., Lawther, M., Olsen, M.B., Nilsson, N.H., Mortensen, G., 1999. Potential of biobased materials for food packaging. Trends Food Science Technology 10, 52–68.

Salgado, P.R., Molina Ortiz, S.E., Petruccelli, S., Mauri, A.N., 2010. Biodegradable sunflower protein films naturally activated with antioxidant compounds. Food Hydrocolloids 24 (5), 525–533.

Scilingo, A.A., Molina, S.E., Martínez, E.N., Añón, M.C., 2002. Amaranth protein isolates modified by hydrolytic and thermal treatments. Relationship between structure and solubility. Food Research International 35 (9), 855–862.

Segura-Nieto, M., Barba de la Rosa, A.P., Paredes-López, O., 1994. Biochemistry of amaranth proteins. In: Paredes-López, O. (Ed.), Amaranth: Biology, Chemistry and Technology. CRC Press, Boca Raton, FL, pp. 75–106.

Shih, F.F., 1998. Film forming properties and edible films of plant proteins. Nahrung 42, 254–256.

Stuchell, Y.M., Krochta, J.M., 1994. Enzymatic treatments and thermal effects on edible soy protein films. Journal of Food Science 59, 1332–1337.

Tang, C.H., Jiang, Y., 2007. Modulation of mechanical and surface hydrophobic properties of food protein films by transglutaminase treatment. Food Research International 40, 504–509.

Tapia-Blácido, D., Sobral, P.J.A., Menegalli, F.C., 2005. Development and characterization of biofilms based on amaranth flour (*Amaranthus caudatus*). Journal of Food Engineering 67, 215–223.

Tapia-Blácido, D., Mauri, A.N., Menegalli, F.C., Sobral, P.J.A., Añón, M.C., 2007. Contribution of the starch, protein, and lipid fractions to the physical, thermal, and structural properties of amaranth (*Amaranthus caudatus*) flour films. Journal of Food Science 72 (5), 293–300.