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Amaranth protein films prepared with high-pressure treated proteins

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ABSTRACT

This work studies the effect of using high-pressure modified amaranth proteins in the preparation of edible film and compares the efficiency of high pressure and thermal treatment on the functionality of amaranth protein films. This films were prepared by casting using glycerol as plasticizer from protein dispersions submitted to high pressure treatments of different intensity (0.1, 200, 400 and 600 MPa). Protein dispersions treated with high-pressure were able to form uniform films with better mechanical properties, lower water solubility and water vapor permeability than those prepared from non-treated protein dispersions without modifying its thickness, color and water content, but somewhat more opaque. This could be attributed to structural changes by high-pressure treatment, which favored protein unfolding, increasing protein surface hydrophobicity and the amount of free SH, that were re-associated during film formation producing a higher crosslinking of matrixes that was denoted in a better functionality of films. These films also showed better properties than those prepared with amaranth protein isolates thermally treated.

1. Introduction

In the last decades the technology based on the use of hydrostatic high pressure (HP) has been shown to constitute an adequate option for satisfying the high demand of high quality and minimally processed foods, free of additives and microbiologically safe (Gould, 1995). HP treatment (100–1000 MPa) is known to modify the functional proper- ties of alimentary macromolecules (Farr, 1990; Hayashi, 1995; O'Reilly et al., 2001). In particular HP produces a variable degree of protein denaturation that depends mainly on the pressure level used, leading to aggregation and dissociation of polypeptides, and modifying their surface hydrophobicity, solubility, etc. These effects depend on extrinsic factors such as pH, temperature and ionic strength of the medium (Puppo et al., 2004; Speroni et al., 2010), as well as on intrinsic factors such as the nature and the concentration of the protein. In this way, the effect of high-pressure treatment (100–1000 MPa) on the structure of globular proteins has been widely studied for soy, lupin, wheat, milk and egg albumin proteins (Bouaouina et al., 2006; Chapleau and de Lamballerie-Anton, 2003; Puppo et al., 2005; Van der Plancken et al., 2007; Zhang et al., 2005). Furthermore it has been reported that some of these modified proteins, showed a remarkable improvement in their functionality for example, in its ability to form and stabilize foams, emulsions and gels (Bouaouina et al., 2006; Speroni et al., 2009; Puppo et al., 2005).

Condés et al. (2012) has reported that amaranth isolate proteins were very sensitive to HP treatment, more than others such as soybean proteins, since they suffered a higher degree of denaturation at 200 and 400 MPa, which was accompanied by a decrease in protein solubility and protein dissociation and aggregation via hydrophobic interactions and disulfide bonds.

In a previous work we reported that protein films from native amaranth protein isolates had interesting water vapor permeability but poor mechanical properties, but these last properties could be improved by denaturing proteins partially or totally by thermal treatments prior film formation. The resulting films showed higher tensile strength and lower water solubility but also higher WVP, due to the higher crosslinking of these proteins through hydrogen and disulfides bonds (Condés et al., 2013).

Considering that amaranth protein didn't gel during high-pressure treatment at protein concentrations of filmogenic dispersions (≈5% w/v) (Condés et al., 2012) and that large volumes of sample could be handled by HP, protein modification may be carried out on film forming dispersions, avoiding having to lyophilize and redisolve proteins (as was necessary with heat-treated proteins), what would be a major advantage in processing.

There is no information in the literature on edible film preparation from amaranth protein previously treated with high-pressure.

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Therefore, the aim of this work was to study the effect of using high-pressure modified amaranth protein in the preparation of edible film and to compare HP treated protein films functionality with those of thermally treated ones.

2. Materials and methods

2.1. Plant materials

Seeds of *Amaranthus 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 at 4 °C until used.

2.3. Preparation of amaranth protein isolates

Amaranth protein isolate 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 mol/L 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 mol/L HCl and then centrifuged at 9000*g* for 20 min at 4 °C. The pellet was suspended in water, neutralized with 0.1 mol/L NaOH and lyophilized. Amaranth protein isolate was stored in hermetic containers in a chamber at 4 °C until used. Its protein content, determined by Kjeldahl (AOAC 920.53, 1995), $N = 5.85$ (Segura-Nieto et al., 1994) was 91.1 ± 0.2% w/w of proteins (d.b.), and process yield was 14.1 (2.0 g isolate per 100 g of defatted flour.

2.4. High pressure treatment (HP)

Aqueous dispersions of amaranth protein isolate at 5% w/v were vacuum conditioned in a polyethylene bag and subjected to high-pressure treatment at 200, 400, and 600 ± 5 MPa for 5 min in a 2.0 L reactor unit model FPG 9400:922 (Stansted Fluid Power Ltd, UK) equipped with temperature and pressure regulation. A mixture of propylene glycol and water (30:70) was used as pressure-transmitting medium. The target pressure was reached at 6.5 MPa/s and released at 20 MPa/s. The adiabatic heating was manifested as an increase in temperature that was maximal for 600 MPa. In that case, a transient increase was verified up to 33.5 °C. Conditions of HP processing were chosen in accordance to Chapleau and de Lamballerie-Anton (2003).

2.5. Characterization of high-pressure treated isolates

2.5.1. 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 were 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 20–120 °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 ($\Delta \underline{H}_d$) 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).

2.5.2. Determination of protein solubility

Non-treated and HP-treated isolate dispersions were centrifuged at 10000*g* for 20 min at 20 °C. Protein solubility was calculated as:

$$\text{Solubility} = (P/P_{\text{total}}) \times 100 \quad (1)$$

where P is the protein content (mg/mL) of supernatants determined by the Bradford procedure (Bradford, 1976) using bovine serum albumin (p.a., Sigma Chemical Co., St. Louis, MO) as standard; and P_{total} is the total protein content determined by Kjeldahl method (AOAC 920.53, 1995), $N = 5.85$.

2.5.3. Free sulfhydryls

Free SH groups were determined according to the procedure described by Beveridge et al. (1974). Non-treated and HP-treated isolates dispersions were dissolved in a specific buffer (0.086 mol/L Tris buffer, 0.09 mol/L glycine, 0.004 mol/L EDTA, and 8 mol/L urea, pH 8.0), and after 30 min the samples were centrifuged for 20 min at 10,000*g* at 20 °C. Forty μL of Ellman's reagent (4 mg of 5,5′-dithio-bis(2-nitrobenzoic acid)/mL in methanol) (p.a., Sigma Chemical Co.) were added to 1 mL aliquots of the supernatant. Absorbance at 412 nm was determined at different times until the maximum absorbance was reached. A molar extinction coefficient of 13,600 mol/L cm was used. Protein concentration was determined by the Bradford method. Determinations were performed at least twice. The concentration of SH groups was expressed as μmol SH/g of protein.

2.5.4. Surface hydrophobicity

Surface hydrophobicity (H_0) of non-treated and HP-treated isolates dispersions was measured according to Kato and Nakai (1980) using 0.008 mol/L 1-anilino-8-naphthalene-sulfonate (ANS, p.a., Aldrich Chemical Co.) as probe. Protein dispersions were diluted (0.1 g/L) in water. Fluorescence intensity (FI) was measured with an Aminco-Bowman SPF 100 fluorescence spectrometer (Sylver Spring, Maryland, EEUU) at 450 nm (excitation) and 540 nm (emission) wavelengths. The initial slope of the fluorescence intensity versus protein concentration plot was used as an index of H_0. Measurements were performed in duplicate.

2.6. Film formation

Films were prepared by dispersing amaranth protein isolate (5% w/v) and glycerol (1.25% w/v, Anedra, Argentina) in distilled water, or directly adding the same concentration of glycerol to amaranth protein dispersions treated at 200, 400 or 600 MPa. All 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:

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

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^{-1} H_2O) divided by the cell area (m^2). WVP (g Pa^{-1} s^{-1} m^{-1}) was calculated as:

$$WVP = \left[(WVTR) \times d\right] \Big/ \left[P_v^{H_2O} \times (RHd - RHc) \times A\right] \quad (3)$$

where P_V^{H2O} = vapor pressure of water at saturation (Pa) at the test temperature (20 °C), RH_d = RH in the desiccator, RH_c = RH in the permeation cell, A = permeation area (m^2), and d = 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 analyser 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. Small pieces of films (≅4 mm^2) were weighted (≅20 mg) 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 9000g 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 HP treatment on structural properties of filmogenic protein dispersions

Fig. 1 depicts the denaturation temperatures and enthalpies (T_d and ΔH_d) of amaranth protein dispersions non-treated (0.1 MPa) and treated at 200, 400 and 600 MPa, obtained by DSC.

The native amaranth protein isolate dispersion exhibited the two characteristic endotherms, one at 70.7 °C with an enthalpy of 4.1 J/g that can be attributed to the denaturation of albumins and a minor globulin fraction (globulin-7S), and a second one at 98.6 °C with an enthalpy of 5.7 J/g corresponding to the denaturation of globulin-11S, globulin-P and glutelins (Avanza and Añón, 2007; Martínez et al., 1997).

Protein fractions of lower thermal stability (endotherm 1) exhibited a great sensitivity to HP treatment since they were denatured almost completely (less than 6% of these protein fractions retained their native structure when comparing to the control sample) regardless the intensity of the pressure applied. Protein fractions of higher thermal stability (endotherm 2) were partially denatured after treatment at 200 MPa (42% of the protein fractions conserved their native structure when comparing to the control). However, these protein fractions were denatured by at least 94% upon treatment at higher pressures ($\geqslant$400 MPa).

Td values showed that after HP treatment the resistant structures from albumins and globulin-7S exhibited a smooth decreased in thermal stability while those from glutenins, globulin-11S and globulin-P exhibited a smooth increased in thermal stability, not observing significant differences in Td with the intensity of the treatment. This results agree with those reported by Wang et al. (2008) and Speroni et al. (2010) for soybean proteins. These effects on the thermal stability of protein molecules may be due to the tendency of each fraction to aggregate or dissociate after unfolding, like has been demonstrated by Condés et al. (2013) when analysing the effect of HP treatment on amaranth polypeptide composition by SDS–PAGE and FPLC.

Table 1 shows protein solubility, surface hydrophobicity and free sulfhydryls groups (SH) concentration of amaranth protein dispersions non-treated (0.1 MPa) and treated at 200, 400 and 600 MPa. Treatment at 200 MPa did not affect protein solubility in water probably due to the formation of soluble macro aggregates (Condés et al., 2013), but at 400 and 600 MPa, solubility decreased more than 50%, with proteins almost totally denatured. Protein solubility correlates with protein surface hydrophobicity, which increased about twice for proteins treated at 400 and 600 MPa, possibly as the result of the average between dissociation and aggregation processes of denatured proteins. The maximal content of SH was detected in samples treated at 200 MPa. It seems that at this pressure intensity, proteins are partially unfolded and more free SH are detected due to conformational changes, since free SH measurement often give an underestimation of the real value simply because some free SH groups are buried inside the protein structure. With increasing pressures and unfolding degrees of protein molecules, protein aggregation becomes more evident due to an increase of hydrophobic interactions, which favors the generation of new S-S bonds (Wang et al., 2008).

Table 1
Protein solubility, surface hydrophobicity and free sulfhydryls groups concentration of amaranth protein dispersions non-treated (0.1 MPa) and treated at 200, 400 and 600 MPa.

Pressure intensity (MPa)	Protein solubility%	Surface hydrophobicity	Free sulfhydryls (μmoles/g protein)
0.1	42.5 ± 0.1[a]	6827 ± 32[a]	8.5 ± 0.4[a]
200	41.3 ± 0.5[a]	7005 ± 15[a]	12.3 ± 0.7[b]
400	20.6 ± 0.2[b]	14,718 ± 58[b]	10.4 ± 0.3[c]
600	17.9 ± 0.9[b]	14,702 ± 52[b]	9.6 ± 0.5[c]

All values were average ± SD of three values. Means of protein solubility, surface hydrophobicity and free sulfhydryls within a column with same superscripts (a, b and c) are not significantly different ($P < 0.05$).

3.2. Effect of high-pressure treatment on protein films properties

3.2.1. Appearance and moisture content

Films prepared with amaranth proteins dispersions non-treated (0.1 MPa) and treated at 200, 400 and 600 MPa were found to be homogeneous and flexible. Their thickness and moisture content are shown in Table 2. Those protein films obtained from HP treated protein dispersions presented the same water content and thickness as the control film. Furthermore any color parameters was modified (Table 2), but the opacity of the films increased smoothly with high-pressure treatment intensity.

3.2.2. Mechanical properties

Fig. 2 shows the mechanical properties (measured in tensile test) of the studied films. Films prepared with high-pressure treated amaranth protein dispersions showed better mechanical properties than those of non-treated proteins films. The tensile strength increased progressively with the high-pressure treatment intensity: 26%, 101% and 165% for films prepared from protein dispersions treated at 200, 400 and 600 MPa, respect to the ones prepared with untreated proteins. The elastic modulus also increased, but only after treatment at 400 MPa, reaching 74% improvement for the films formed with proteins treated at

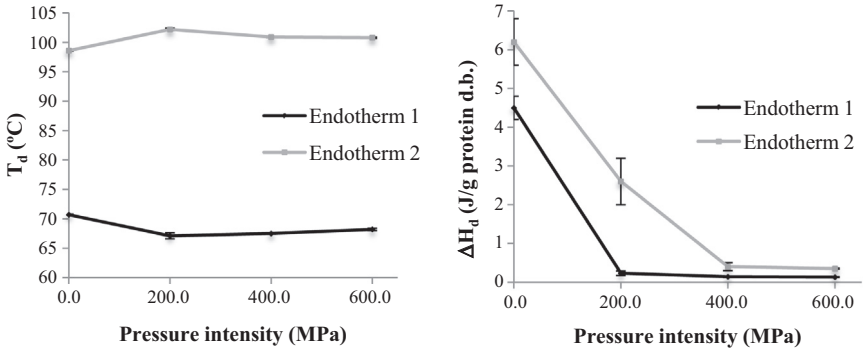

Fig. 1. Denaturation temperatures (T_d) and enthalpies (ΔH_d) of amaranth protein dispersions non-treated (0.1 MPa) and treated at 200, 400 and 600 MPa.

Table 2
Thickness, moisture content, Hunter color parameters (a, b and L), total color difference (ΔE) and opacity of films prepared from amaranth protein dispersions non-treated (0.1 MPa) and treated at 200, 400 and 600 MPa.

Pressure intensity (MPa)	Thickness (μm)	Moisture content (%)	Hunter-Lab color parameters				Opacity (UA/mm)
			a	b	L	ΔE	
0.1	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]
200	53.2 ± 9.8[a]	18.5 ± 0.3[a]	−1.3 ± 0.1[a]	25.7 ± 1.3[a]	82.6 ± 1.1[a]	28.2 ± 1.7[a]	1.4 ± 0.1[a,b]
400	58.4 ± 9.0[a]	19.2 ± 0.4[a]	−1.1 ± 0.2[a]	24.5 ± 1.7[a]	82.7 ± 1.2[a]	27.1 ± 2.1[a]	1.7 ± 0.1[b]
600	54.7 ± 9.2[a]	20.9 ± 1.9[a]	−1.2 ± 0.1[a]	23.5 ± 0.8[a]	83.7 ± 0.7[a]	25.7 ± 1.0[a]	2.2 ± 0.2[b]

All values were average ± SD of two values. All parameters reported within a column with same superscripts (a and b) are not significantly different ($P < 0.05$).

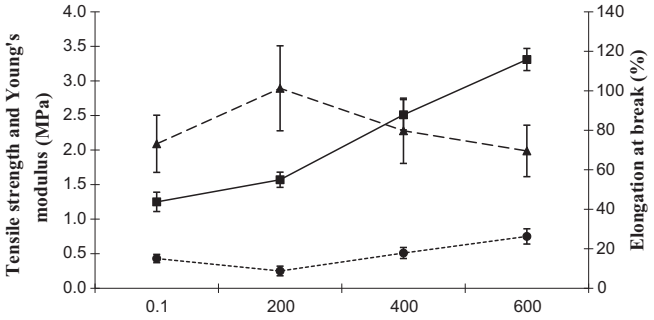

Fig. 2. Tensile strength (■), elongation at break (▲) and Young's modulus (●) of films prepared from amaranth protein dispersions non-treated (0.1 MPa) and treated at 200, 400 and 600 MPa.

600 MPa. These improvements were very interesting because occurred without causing a decrease in the elongation at break of films. By increasing the degree of protein denaturation, especially above 400 MPa, the interaction among protein chains during film formation should be favored, and this resulting higher crosslinking (through covalent and non covalent bonds) was displayed in more resistant films with similar deformation at break. Similar behavior was reported by Zhang et al. (2005), who prepared tofu gel with higher strength from soy milk previously treated by high-pressure, and attributed it to the formation of a more cross-linked network.

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. Water solubility of amaranth protein films decreased 29% and 42% with increasing intensity of HP treatment to 200 and 400 or 600 MPa respectively. Water vapor solubility decreased progressively with the intensity of HP treatment, till a 43% for film prepared with proteins submitted to 600 MPa. This improvements in films water susceptibility may be attributed to the higher crosslinking of films prepared from proteins unfolded by HP, that also showed better mechanical properties, as well to the different type of protein interactions that stabilize the protein matrix, because these films resulted from the interaction of proteins with higher surface hydrophobicity (Table 1). Meanwhile, it could be suggested that amaranth proteins unfolded by HP, could form a more cross linked film protein network than untreated proteins, which retard the passage of water vapor molecules through it, and retain the film structure after being immersed in water for 24 h.

Table 3
Water solubility, water vapor permeability (WVP) and surface hydrophobicity of films prepared from amaranth protein dispersions non−treated (0.1 MPa) and treated at 200, 400 and 600 MPa.

Pressure intensity (MPa)	Water solubility (%)	WVP $*10^{-11}$ (g H_2O/Pa m s)	Contact angle (°)
0.1	79.9 ± 2.1[a]	5.6 ± 0.5[a]	69.5 ± 2.5[a]
200	56.4 ± 5.5[b]	4.8 ± 0.4[a,b]	70.2 ± 6.7[a]
400	46.1 ± 0.5[c]	4.6 ± 0.1[b]	58.8 ± 3.3[b]
600	46.1 ± 2.5[c]	3.2 ± 0.6[c]	54.9 ± 4.9[b]

All values were average ± 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 ($P < 0.05$).

It should be noted that WVP value obtained with non treated amaranth proteins was an order of magnitude lower than those corresponding to soy and sunflower films similarly prepared and processed (1.5×10^{-10} g H_2O/Pa m) (Denavi et al., 2009; Salgado et al., 2010). This result might be attributed to the hydrophobic nature of amaranth protein, particularly that of 11S and P-globulins (Condés et al., 2013).

The contact angle measured by depositing a drop of water on the films prepared from protein dispersions treated at 200 MPa showed no significant differences from the control film. However, films prepared with protein dispersions treated at 400 and 600 MPa showed a lower contact angle that indicates a more hydrophilic surface. Probably films prepared with proteins treated by HP, which have higher surface hydrophobicity should probably interact through their hydrophobic sites with each other, leaving their hydrophilic sites exposed to films surface.

3.2.4. Differential solubility of proteins

In order to further characterize protein crosslinking that stabilized films prepared from amaranth protein dispersions non-treated (0.1 MPa) and treated at 200, 400 and 600 MPa, 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. Fig. 3 shows the differential solubility of film proteins in these buffers systems.

The solubility in water of proteins from film prepared with amaranth protein isolates dispersions was significantly higher than those from HP-treated proteins, suggesting that in the latters 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 all films the solubility in PB was lower than that obtained in water – possibly because this buffer may favor ionic interactions between polypeptide chains, producing a salting out effect (Mauri & Anón, 2006; Salgado et al., 2010) – , it was more evident in the film prepared from non-treated protein dispersions and those treated at 600 MPa, whose solubility was very low. It denotes that electrostatic interactions play a less relevant role in the stabilization of these films.

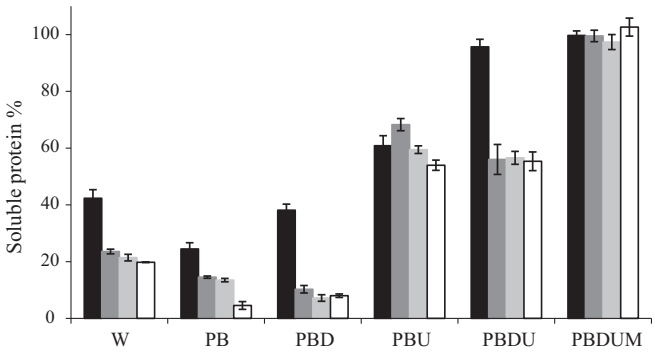

Fig. 3. Differential protein solubility of films prepared from amaranth protein dispersions non-treated (0.1 MPa) (■) and treated at 200 (■), 400 (■) and 600 (□) MPa in solvents with different chemical activity: Water (W), 0.1 M sodium phosphate buffer (PB), PB containing 0.1% w/v SDS (PBD), PB containing 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 average ± standard deviation.

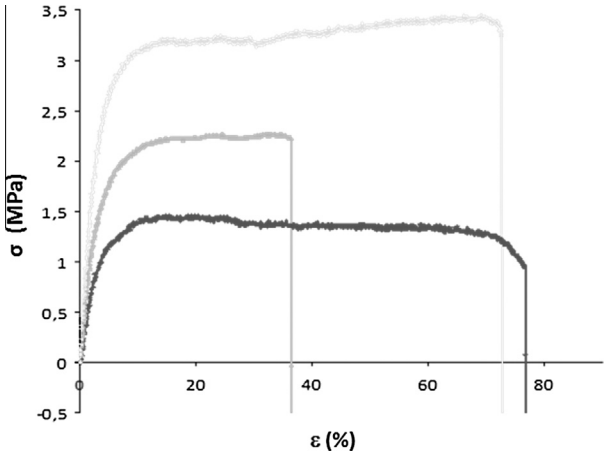

Fig. 4. Stress–strain curves of films prepared from dispersions of amaranth protein isolates dispersions non-treated (0.1 MPa) (—), treated at 70 °C (—) and from protein isolates dispersions treated at 600 MPa (—).

Upon employing PBD, the solubility of proteins in the control film was at least 4 times higher than that prepared from HP treated proteins. This finding would evidence 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. In addition, it is also possible that the hydrophobic interactions would play a less important role in the stabilization of matrixes prepared with proteins unfolded by HP than in the control. Moreover, the important role of these interactions was manifested in films prepared with untreated proteins, and correlated with their low WVP values. In contrast, in the phosphate buffer containing urea (PBU) protein solubility of all films significantly increased, especially for films obtained from isolates dispersions treated with high-pressure that reached a similar solubility value that the control film. These results would indicate the importance of hydrogen bonds in the formation of films and/or the action of the urea, which unstructured water and would facilitate protein solubility. Meanwhile proteins of control films achieved complete solubilization in PBDU, the corresponding protein solubility of films prepared by HP-treated proteins exhibited similar solubilities than those achieved in the PBU.

Finally, proteins of film prepared from HP treated dispersions could be completely solubilized in PBDUM, thus demonstrating the importance of disulfide bonds in the stabilization of these films matrixes, unlike what was observed for untreated films. It is noteworthy that the disulfide bonds might prevent the release of proteins and polypeptides chains that were also stabilized by other interactions, which might be disrupted by PBD or PBDU, leading to an underestimation of the protein concentration and the importance of these interactions in the stabilization of the films. During proteins dispersion and films preparation sulfhydryl–disulfide exchange and oxidation occurred, especially favored by alkaline pH of the dispersions and high temperature, contributing to the formation of disulfide crosslinks. Results suggest that this exchange favored in a higher extent the stabilization of films prepared from unfolded proteins.

In synthesis, this analysis showed the importance of hydrogen and disulfide bonds in the stabilization of the films prepared with high pressure treated isolate dispersions unlike control films mainly stabilized by hydrophobic and hydrogen bonds. Protein unfolding favors the formation of disulfide and hydrogen bonds among protein chain during film formation, making them more resistant and possibly more hydrophobic, as the films were less soluble in water and showed lower WVP. It is possible that the films were also stabilized by hydrophobic interactions between chains that were not achieved by this essay. This could be due to the presence of disulfide bonds that could avoid an increase in the solubility in the corresponding buffer, although SDS breaks these hydrophobic bonds.

3.3. Comparison of the efficiency of high pressure and thermal treatment on the enhanced functionality of amaranth protein films

Finally in this section the mechanical properties, water vapor permeability and solubility of amaranth protein films prepared from dispersions of amaranth protein isolates not treated (control), treated at 70 °C (Condés et al., 2013) and amaranth protein dispersions submitted at 600 MPa were compared. All films had similar

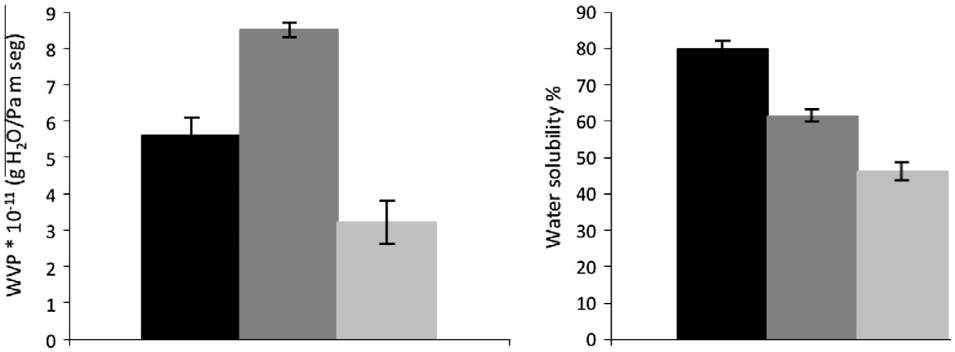

Fig. 5. Water vapor permeability (WVP) and water solubility of films prepared from amaranth protein dispersions non-treated (0.1 MPa) (■), treated at 70 °C (■) and treated at 600 MPa (■).

formulations (same protein and glycerol content and pH) and were likewise processed. Fig. 4 shows the stress–strain curves obtained in tensile tests of these films. Although both protein films prepared with unfolded proteins (treated thermally or by HP) showed higher tensile strength and Young's modulus than those of control films, the ones obtained with protein dispersions treated with high-pressure showed the best mechanical performance. It showed the highest tensile strength and Young's modulus with the same elongation at break than films prepared with non-treated proteins dispersions. This behavior also occurred with films prepared from isolates treated at 400 MPa.

WVP and water solubility for these films are compared in Fig. 5. Films prepared with amaranth protein isolates treated at 70 °C showed higher WVP but lower water solubility than control films. In contrast, high-pressure treatment improved both properties, showing the lowest WVP and water solubility. This behavior also occurred with films prepared from film forming dispersions treated at 200 and 400 MPa.

It is evident that the high-pressure treatment was more effective in the improvement of amaranth protein films functionality than the thermal treatment, since it allowed improving mechanical properties and water susceptibility to a greater extent. With the additional advantage that HP treatment may be performed on the film forming dispersions, while heat treatment was applied to protein isolates dispersions with lower protein concentration in order to avoid gelation. This involves an additional step in the process, because protein isolates thermally treated should be obtained from these dispersions (by spray drying or lyophilization) to be then re-dissolved in the conditions of film forming dispersions.

4. Conclusions

Protein dispersions treated with high-pressure were able to form uniform films with better mechanical properties, lower water solubility and water vapor permeability than those prepared from non-treated protein dispersions without modifying its thickness, color and water content, but somewhat more opaque. This could be attributed to structural changes achieved in proteins by high-pressure treatment, which favored protein unfolding, increasing protein surface hydrophobicity and the amount of free SH, that were re-associated during film formation producing a higher crosslinking of protein matrixes that was denoted in a better functionality of films.

Films prepared from film forming dispersions treated with HP also showed better properties than those studied previously, prepared with amaranth protein isolates thermally treated. This performance together with the additional advantages that this treatment can be made directly on the film forming dispersion and with larger volumes, make to consider high pressure treatment as an interesting strategy to improve the functionality of protein films.

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