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Physicochemical and structural properties of amaranth protein isolates treated with high pressure

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ABSTRACT

The effects of high pressure (HP) (200, 400 and 600 MPa) on physicochemical and structural properties of amaranth proteins treated at different protein concentrations (1, 5 and 10% w/v) were studied. HP provoked denaturation of amaranth proteins which were very sensitive to HP treatment, achieving almost complete denaturation (93%) at 400 MPa. After HP, the resistant structures from glutenins, globulin-11S and globulin-P exhibited an increased thermal stability while those from albumins and globulin-7S exhibited a decrease of thermal stability. Increasing intensities of HP treatments provoked the disappearance of electrophoretic bands with molecular mass higher than 45 kDa, together with changes in the polypeptides fractions of low molecular weight. HP treatments induced the formation of insoluble aggregates and the dissociation of soluble aggregates. Protein concentration modulated the effects of HP on amaranth proteins. These modified proteins could present improved functional properties.

Industrial relevance: The important effects of high pressure on structural properties of amaranth proteins and their consequences in their functionality may be useful in the handling of these proteins as food ingredients or in the formulation of novel foods.

1. Introduction

Proteins are very important as food ingredients due to their role in nutrition and their functional properties. Vegetal proteins appear as an alternative to animal proteins in diet because of their lower cost and their content of associated compounds, which are also responsible for health improving effects. As a disadvantage, in general their functional properties are not as interesting as those of animal proteins. This weakness, however, can be overcome by protein structure modification. Thus, enzymatic, chemical and physical treatments are an interesting way to improve the functionality and physiological properties associated with proteins.

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 properties of alimentary macromolecules (Farr, 1990; Hayashi, 1995, O'Reilly, Kelly, Murphy, & Beresford, 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, Añón, & de Lamballerie, 2010) as well as on intrinsic factors such as the nature and the concentration of the protein.

Protein concentration is an important parameter for the formulation of a functional ingredient because its requirements differ for different uses: drinks, foams, emulsions, and gels. Since the effects of HP involve polypeptide–polypeptide and water–polypeptide interactions, protein concentration may potentially affect pressure effects on proteins. Several studies (Bouaouina, Desrumaux, Loisel, & Legrand, 2006; Chapleau & de Lamballerie-Anton, 2003; Puppo et al., 2005; Van der Plancken, Van Loey, & Hendrickx, 2007; Zhang, Li, Tatsumi, & Isobe, 2005) have addressed the effects of HP on the structure and functional properties of proteins from soybean, lupin, whey, milk and white egg. Only a few ones have considered protein concentration during HP treatments as a variable and have analyzed its effect on the protein structure at the end of the process showing that it has a decisive effect (Alvarez, Ramaswamy, & Ismail, 2008; Merel-Rausch, Kulozik, & Hinrichs, 2007). HP treatments on amaranth proteins have never been evaluated.

Amaranth (*Amaranthus hypochondriacus*) is a naturally resistant ancestral crop that can develop in environments where other cereals or plants cannot, including dry soils, high altitudes, and high temperatures (Omami, Hammes, & Robbertse, 2006). The amaranth seed has

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an important content of proteins with essential amino acids such lysine and methionine, which are scarce in other cereals (Bressani, 1989). The main proteins in the grains are represented by albumins and globulins and to a lesser extent by glutelins (Duarte-Correa, Jokl, & Carlsson, 1986). Our laboratory has studied the structure–function relationship in amaranth protein isolates and its major protein fractions, as well as the effect of thermal and enzymatic treatments on such relationship (Avanza & Añón, 2007; Ventureira, Martínez, & Añón, 2010).

The aim of this work was to analyze the effects of HP on physicochemical and structural properties of amaranth proteins treated at different protein concentrations.

2. Materials and methods

2.1. Preparation of amaranth isolates

Amaranth protein isolates (API) were prepared from defatted flour obtained from *A. hypochondriacus* seeds, grown in La Pampa, Argentina. Briefly, an aqueous alkaline extraction from the defatted flour (pH 11.0), followed by an isoelectric precipitation (pH 5.0) was carried out according to Martínez and Añón (1996). The isoelectric precipitate was dispersed in distilled water, neutralized with 0.1 mol L^{-1} NaOH (p.a. Anedra) and finally lyophilized. Protein content of API determined by the Kjeldhal method was 90.7% w/w (d.b.) (N: 5.85) (Segura-Nieto, Barba de la Rosa, & Paredes-López, 1994).

2.2. High-pressure processing

Aqueous dispersions of amaranth protein isolates at 1% (API-1), 5% (API-5) and 10% w/v (API-10) were 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^{-1} and released at 20 MPa s^{-1}. 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. Prior to pressure processing, protein dispersions were vacuum conditioned in a polyethylene bag. Conditions of HP processing were chosen in accordance to Chapleau and de Lamballerie-Anton (2003). Table 1 shows the nomenclature used and the treatment performed on each sample. Treatments were performed at least in triplicate.

In most characterization studies described below HP-treated dispersions were used lyophilized, but non-lyophilized samples were used in some cases.

Table 1
Summary of the nomenclature used and the treatments performed on each sample.

Nomenclature	Protein isolate concentration during HP treatment (% w/v)	Intensity of HP treatment (MPa)
API	–	–
API-1_{200}	1	200
API-1_{400}	1	400
API-1_{600}	1	600
API-5_{200}	5	200
API-5_{400}	5	400
API-5_{600}	5	600
API-10_{200}	10	200
API-10_{400}	10	400
API-10_{600}	10	600

2.3. Determination of soluble and disperse protein

Non-treated and HP-treated isolate dispersions were centrifuged at 10,000 *g* for 20 min at 20 °C. Protein content in the supernatant that included soluble and disperse protein was calculated as:

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

where P is the protein content (mg mL^{-1}) 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 (f = 5.85).

Dispersed protein content in non-treated isolate dispersions was determined by turbidimetry. Dispersions were centrifuged at 10,000 *g* for 20 min at 20 °C and the turbidity of supernatants was measured at 660 nm.

All determinations were performed at least in triplicate.

2.4. Surface hydrophobicity

Surface hydrophobicity (H_0) of non-treated and HP-treated isolates dispersions were measured according to Kato and Nakai (1980) using 0.008 mol L^{-1} 1-anilino-8-naphthalene-sulfonate (ANS, p.a., Aldrich Chemical Co.) as probe. Protein dispersions were diluted (0.1 g L^{-1}) 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.

2.5. Free sulfhydryls

Free SH groups were determined according to the procedure described by Beveridge, Toma, and Nakai (1974). Non-treated and HP-treated isolates dispersions were dissolved in a specific buffer (0.086 mol L^{-1} Tris buffer, 0.09 mol L^{-1} glycine, 0.004 mol L^{-1} EDTA, and 8 mol L^{-1} urea, pH 8.0), and after 30 min the samples were centrifuged for 20 min at 10,000 *g* at 20 °C. Forty microliters 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^{-1} cm^{-1} 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^{-1} of soluble protein.

2.6. Electrophoresis

2.6.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The polypeptide composition of samples was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a separating gel (12% w/v in polyacrylamide) with a stacking gel (4% w/v in polyacrylamide) in a minislabs system (Bio-Rad Mini-Protean II Model) (Martínez, Castellani, & Añón, 1997). The following continuous buffers system were used: 0.375 mol L^{-1} Tris–HCl, 1 g L^{-1} SDS, pH 8.8, for the separating gel; 0.025 mol L^{-1} Tris–HCl, 0.192 mol L^{-1} glycine and 1 g L^{-1} SDS, pH 8.3, for the running buffer; and 0.125 mol L^{-1} Tris–HCl, 200 ml L^{-1} glycerol, 10 g L^{-1} SDS, 0.5 g L^{-1} 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^{-1} 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.

2.6.2. Tricine-polyacrylamide gel electrophoresis

Tricine-polyacrylamide gel electrophoresis was used to complete the analysis of polypeptide composition of isolates, especially to determine those polypeptides of molecular mass below 30.0 kDa. Samples were analyzed in gels formed by 4% w/v, 10% w/v and 16% w/v acrylamide gels (stacking, spacer and separating, respectively), modifying the technique described by Schägger and von Jagow (1987). The following continuous buffer system was used: 1 mol L^{-1} Tris–HCl, 1 g L^{-1} SDS, pH 8.45 for the gel buffer; 0.15 mol L^{-1} Tris–HCl, 120 g L^{-1} SDS, 300 g L^{-1} glycerol, 0.5 g L^{-1} Coomassie blue G-250 (Serva) pH 7.0, for the sample buffer; and Tris–Tricine (p.a., Sigma Chemical Co.) buffer system as running buffer [0.1 mol L^{-1} Tris–HCl, pH 8.9 as an anode buffer and 0.1 mol L^{-1} Tris, 0.1 mol L^{-1} Tricine, 1 g L^{-1} SDS, pH 8.25 as a cathode buffer (p.a., Sigma Chemical Co.)]. The following protein MW markers were used: triosephospate isomerase (26.6 kDa); myoglobin (16.9 kDa); α-lactalbumin (14.4 kDa); aprotinin (6.5 kDa); insulin β chain, oxidized (3.5 kDa); and bacitracin (1.4 kDa). Gels were fixed with 50% v/v methanol and 10% v/v acetic acid, 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.

2.7. Viscosity measurement

The apparent viscosity of aqueous suspensions was determined by flow measurements through shear stress versus shear strain assays. Measurements were performed in a Controlled Stress Rheometer RS600 (Haake, Germany) using a serrated plate-and-plate geometry (35 mm diameter, 1.0 mm gap). Frequency was swept from 0.01 to 10 Hz at 20 ± 1 °C. Measurements (performed in duplicate) were carried out at a constant strain of 1% which was within the linear region of the plot.

2.8. Differential Scanning Calorimetry (DSC)

A TA Instrument DSC Q100 V9.8 Build 296 (New Castle, Del., USA) was used for these studies. Temperature and heat flow calibrations of the equipment were carried out according to ASTM Standards, using lauric and stearic acids and indium as standards, respectively. Hermetically sealed aluminum pans containing 10–15 mg of samples (20% w/w of lyophilized samples) were prepared and scanned at 10 °C min^{-1} over the range of 20–120 °C. Denaturation enthalpies (ΔH_d) and peak temperatures (T_d in °C) were calculated from the corresponding thermograms (Universal Analysis V4.2E, TA Instruments, New Castle, Del., USA). Enthalpy values were expressed as J g^{-1} 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, Petruccelli, & Añón, 2004). All assays were conducted in duplicate for each replicate.

2.9. Size exclusion chromatography

Non-treated and HP-treated freeze-dried isolates were dissolved in 0.05 mol L^{-1} NaH_2PO_4, 0.15 mol L^{-1} NaCl (p.a., Sigma Chemical Co.) buffer, pH 8.0, filtered by a 0.22 μm cellulose filter, and analyzed by gel-filtration chromatography at room temperature and 0.2 MPa pressure. A Sephacryl S-300 (Amersham Biosciences, UK) column was employed linked to a Pharmacia LKB, FPLC System (Uppsala, Sweden). Samples (5 mg mL^{-1} of isolate) were eluted at 0.5 mL min^{-1} with the same buffer in which they were prepared and the absorbance at 280 nm was recorded. The column was calibrated with gel filtration calibration kits HMW and LMW (GE-Healthcare, Buckinghamshire, UK). Curves were processed and data evaluated through the use of the Pharmacia AB, FPLC director and FPLC assistant software. Every determination was performed at least twice.

2.10. 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 $\alpha = 0.05$, using the Statgraphics Plus version 5.1 software (Statgraphics, USA).

3. Results and discussion

3.1. Protein denaturation induced by HP treatments

Table 2 depicts the denaturation temperatures and enthalpies (T_d y ΔH_d) of amaranth protein isolates in either native state (0.1 MPa) or treated with HP (200, 400 and 600 MPa) at different protein concentrations (1%, 5% and 10%). The native isolate exhibited the two characteristic endotherms, one at 70.7 °C with an enthalpy of 4.1 J g^{-1} 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^{-1} corresponding to the denaturation of the protein fractions globulin-11S, globulin-P and glutenins (Avanza & 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 the native structure remaining compared to endotherm 1 of the control sample) regardless of protein concentration and the magnitude of the pressure applied. Protein fractions of higher thermal stability (endotherm 2) partially conserved their native structure after treatment at 200 MPa, especially in dispersions of higher protein concentration (45% and 37% of conserved native structure compared to endotherm 2 of the controls for 5% and 10% dispersions), the denaturing effect being higher in the dispersion of lower protein concentration (25% of conserved native structure compared to endotherm 2 of the control for 1% dispersion). However, these protein fractions were denatured by at least 93% upon treatment at higher pressures (≥400 MPa).

Table 2
Denaturation temperatures (T_d) and denaturation enthalpies (ΔH_d) of amaranth protein isolate at 1% w/v (API-1), 5% w/v (API-5) and 10% w/v (API-10) in either native state (0.1 MPa) or after high pressure treatment (200, 400 and 600 MPa).

	Endotherm 1		Endotherm 2	
	T_d (°C)	ΔH_d (J/g)	T_d (°C)	ΔH_d (J/g)
API	70.7 ± 0.1^{a}	4.09 ± 0.35^{a}	98.6 ± 0.1^{a}	5.69 ± 0.57^{a}
API-1_{200}	67.4 ± 1.5^{ab}	0.16 ± 0.02^{b}	102.2 ± 0.1^{b}	1.40 ± 0.01^{b}
API-5_{200}	67.1 ± 0.5^{b}	0.23 ± 0.06^{b}	102.2 ± 0.2^{b}	2.58 ± 0.64^{bc}
API-10_{200}	68.3 ± 0.4^{b}	0.18 ± 0.04^{b}	101.7 ± 0.2^{b}	2.12 ± 0.23^{c}
API-1_{400}	67.4 ± 0.8^{b}	0.27 ± 0.13^{b}	101.3 ± 0.5^{b}	0.25 ± 0.02^{b}
API-5_{400}	67.5 ± 0.1^{b}	0.14 ± 0.02^{b}	100.9 ± 0.1^{b}	0.38 ± 0.15^{b}
API-10_{400}	66.5 ± 0.7^{b}	0.07 ± 0.03^{b}	101.2 ± 0.4^{b}	0.21 ± 0.01^{b}
API-1_{600}	67.8 ± 0.1^{bc}	0.16 ± 0.00^{b}	101.3 ± 0.4^{bc}	0.37 ± 0.13^{bc}
API-5_{600}	68.2 ± 0.2^{b}	0.13 ± 0.00^{c}	100.8 ± 0.1^{b}	0.35 ± 0.01^{b}
API-10_{600}	67.3 ± 0.1^{c}	0.09 ± 0.00^{d}	101.4 ± 0.1^{c}	0.22 ± 0.02^{c}

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 ($P<0.05$).

An effect opposite to the one described above was reported by Speroni et al. (2010) in soybean proteins. In this case β-conglycinin (the less thermally stable fraction) conserved approximately 30% of its native structure upon treatment at 600 MPa, whereas glycinin, the fraction with higher thermal stability, was completely denatured at 400 MPa. This differential behavior of amaranth and soybean proteins may be related to structural differences in terms of molecular flexibility and compressibility. Results obtained in the present study suggest that amaranth proteins are more sensitive to HP treatment than other storage proteins, suffering almost complete denaturation at 400 MPa. Regarding denaturation temperatures, species with conserved native structure belonging to the fraction of lower thermal stability presented lower denaturation temperatures after high pressure treatment, while those belonging to fractions of higher thermal stability exhibited an increased T_d compared to those of the native isolate.

These results indicate that after HP, the resistant structures from albumins and globulin-7S exhibited a decreased thermal stability while those from glutenins, globulin-11S and globulin-P exhibited an increased thermal stability. This result agrees with those reported by Wang et al. (2008), and Speroni et al. (2010) in soybean proteins, where the thermal stability of the peak at low temperature (β-conglycinin, 7S) was decreased while the thermal stability of the peak at high temperature (glycinin, 11S) was increased. These effects on the thermal stability of protein molecules may be due to the tendency of each fraction to aggregate or dissociate after unfolding. If the resulting structure after HP treatment is more compact or is stabilized by a higher number of hydrophobic interactions than the non-treated one, the T_d is expected to be higher (Arntfield, Murray, & Ismond, 1986) whereas if dissociation phenomena are present the resulting structure could be less stable to thermal treatment.

3.2. Association–dissociation degree of polypeptidic species

To determine the association–dissociation degree of proteins present in native or HP-treated isolates, these samples were analyzed by denaturing electrophoresis in polyacrylamide gels (Fig. 1A) and tricine-polyacrylamide gels (Fig. 1B), and also by size exclusion chromatography (Fig. 2). Under denaturing conditions (SDS-PAGE) the native isolate exhibited a pattern typical of an amaranth isolate (Martínez et al., 1997) composed of high molecular weight soluble aggregates that did not enter the gel, and polypeptides corresponding to the 7S fraction (67 and 45 kDa), to the 11S fraction [AB subunits (56 kDa), type A polypeptides (between 38 and 32 kDa) and type B polypeptides (between 20 and 18 kDa)], and to the globulin P fraction (54 and 56 kDa) naturally present in amaranth seeds (Castellani, Martínez, & Añón, 2000; Marcone, 1999; Marcone, Niekamp, LeMaguer, & Yada, 1994). It is important to note that the mass of high molecular weight aggregates increased with increasing protein concentrations in the dispersion.

The electrophoretic patterns of HP-treated isolates exhibited changes compared to the pattern described above, including a diminished concentration of soluble aggregates able to enter the gel. Treatment at 200 MPa led to the disappearance of bands corresponding to 7S polypeptides of 45 kDa, AB subunits and P-54 polypeptide, and resulted also in a diminished intensity of bands corresponding to the 7S polypeptide of 67 kDa and to globulin polypeptides A and B. These later species also disappeared or suffered a marked decrease upon pressure increase (400 or 600 MPa).

Taking into account the data from DSC that indicate that both 45 and 67 kDa polypeptides (7S fraction) were almost completely denatured after 200 MPa, and that the band corresponding to the 45 kDa polypeptide disappeared from the gel after such treatment, whereas that of 67 kDa partially remained, we hypothesize that the polypeptide of 45 kDa is more prone to form aggregates after HP-induced denaturation than that of 67 kDa. On the other hand, the intensity

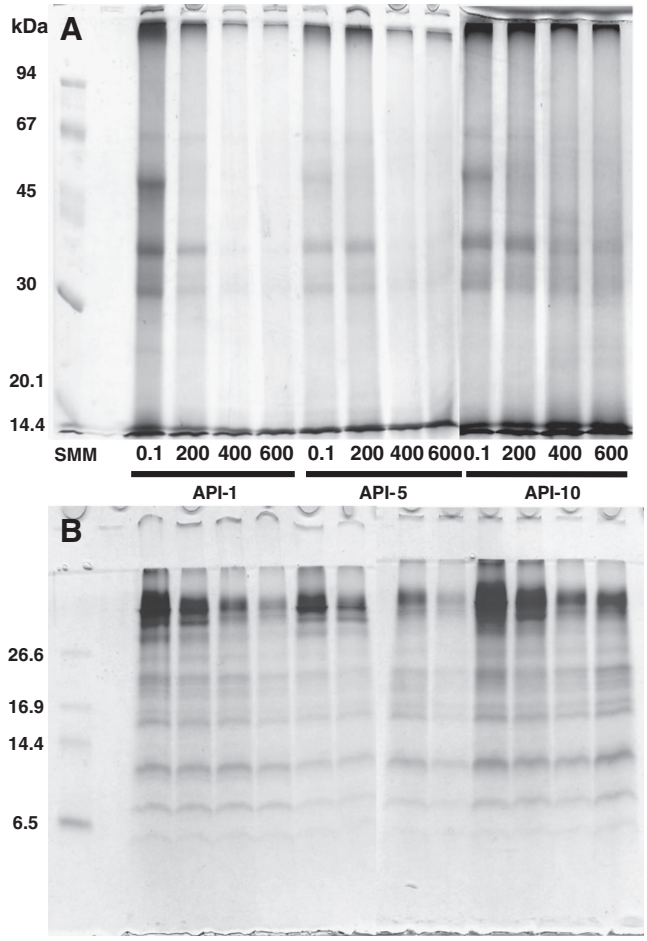

Fig. 1. Polyacrylamide gel electrophoresis profile under denaturing conditions (SDS-PAGE, A) and tricine polyacrylamide gel electrophoresis profile under denaturing conditions (SDS-tricine-PAGE, B) of amaranth protein isolates at 1% w/v (API-1), 5% w/v (API-5) and 10% w/v (API-10) in either native state (0.1 MPa) or after high pressure treatment (200, 400 and 600 MPa).

of the band corresponding to the polypeptide A of 38 kDa partially decreased after 200 MPa in API-1 and API-5, and it seemed to be unaffected in API-10. This result suggests that this polypeptide was less sensitive to HP, and that it was possibly the responsible of the residual endotherm at 102 °C. Taking into account that the same protein mass was loaded in each lane, and that an increased band intensity was observed for each treatment in samples at 10% protein concentration, we consider that the elevated protein concentration led to the formation of aggregates that were not formed in samples at 1% or 5%. No marked differences were detected by tricine-polyacrylamide gel electrophoresis in the pattern of polypeptides smaller than 20 kDa among samples treated at different pressures (Fig. 1B).

It is possible that these small polypeptides are not very compressible and/or do not have internal cavities where water may enter (the filling of those cavities is an important way of HP-induced denaturation), thus these polypeptides are not HP sensitive. These results suggest that HP induced the formation of aggregates that were not soluble in the buffer used for the electrophoresis. These aggregates exhibited different compositions depending on the pressure level.

The size exclusion chromatograms of native and HP-treated isolates at different protein concentrations are shown in Fig. 2. The chromatogram of the native isolate (0.1 MPa) exhibited a peak of high MW (669 kDa, $V_e = 50$ mL), a wider one of higher absorbance with a maximum corresponding approximately to 250 kDa ($V_e = 68.7$ mL), and a third one of 12 kDa ($V_e = 97$ mL),

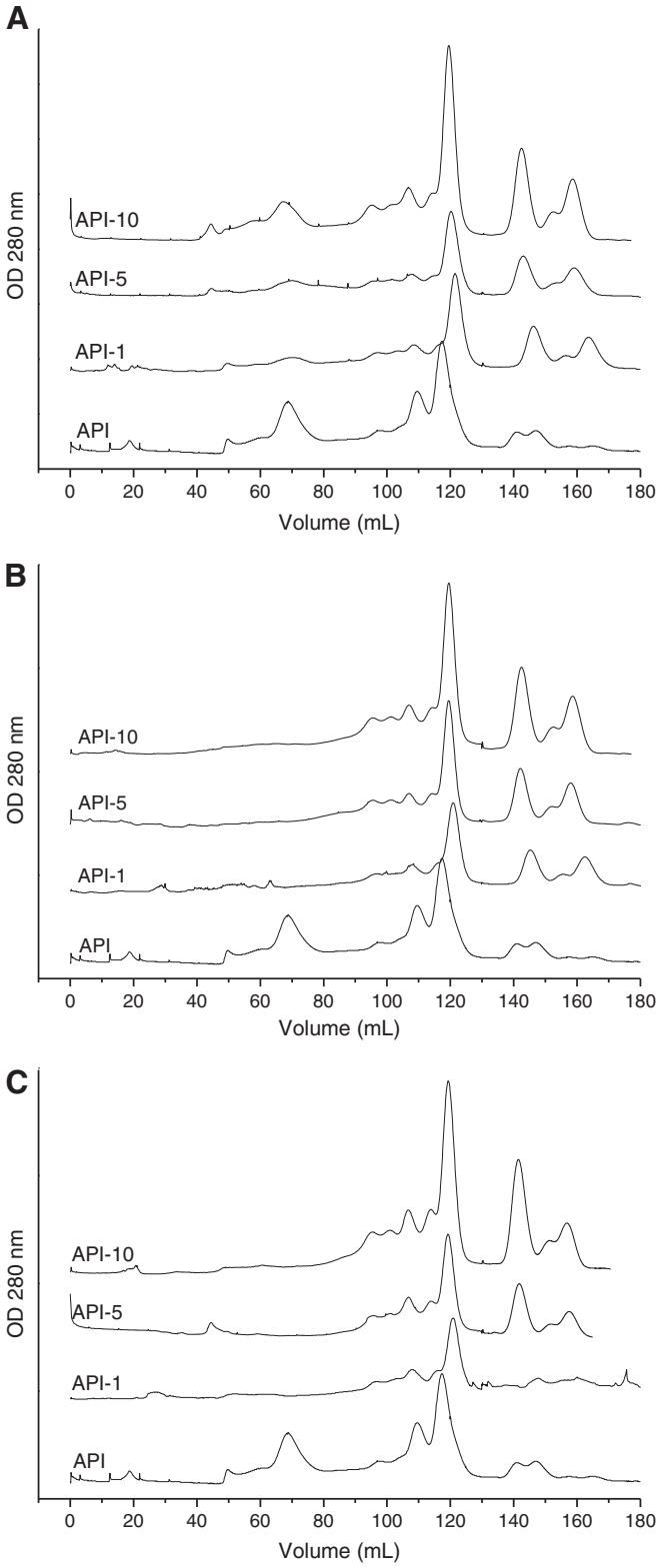

Fig. 2. Size exclusion chromatography of amaranth protein isolates at 1% w/v (API-1), 5% w/v (API-5) and 10% w/v (API-10), in either native state (0.1 MPa) or after high pressure treatment: 200 MPa (A), 400 MPa (B) and 600 MPa (C).

which would comprise the species observed in the electrophoresis gels described above. Well defined peaks of high absorbance were also observed at elution volumes higher than 100 ml, whose MW cannot be assigned because the elution volumes fall outside the calibration range but correspond to species smaller than 9 kDa. It is important to note that the high absorbance of these peaks may be due to the fact that species of lower molecular weight may be enriched in the aromatic compounds responsible of absorption at 280 nm, so that absorbance would not be proportional to the concentration of protein species.

Chromatograms corresponding to HP-treated samples exhibited a modified distribution of polypeptidic species. For isolates treated at 400 and 600 MPa (Fig. 2B and C), and at every assayed concentration, an almost complete diminution of peaks eluting at 50 to 85 mL was observed, together with the shift of already described peaks to higher elution volumes and the possible emergence of new ones.

These results suggest that HP-induced denaturation provoked the dissociation and possibly also the formation of big aggregates, whose size precluded their passage through the 0.22 μm filter. Differences in species of low MW that were evidenced with this technique, but not with SDS-PAGE, suggest the presence of aggregates formed by small polypeptides in the native isolate. These aggregates dissociated in the presence of SDS, possibly because they were stabilized by non-covalent interactions, and also dissociated partially after HP treatment, since an increase in the areas of the peaks with high elution volumes was detected. In addition, HP treatments may compress the molecules, making them to change their hydrodynamic ratio, so that their characteristic peaks would appear at different elution volumes.

When the samples were subjected to 200 MPa (Fig. 2A) a differential effect was found depending on protein concentration: in API-1 and API-5 the behavior was similar to that found at 400 and 600 MPa, but it was different in API-10. Treatment of API-10 at 200 MPa did not seem to affect the distribution of the aggregates with MW between more than 669 and 250 kDa. It is possible that at high protein concentration the partial unfolding did not modify the aggregates present in API because protein was already aggregated. But at 1% and 5% protein concentrations HP promoted the formation of other types of aggregates that were big enough to be excluded by the 0.22 μm filter. Moreover, reinforcing this idea, data from DSC showed that the transition at high temperature was less affected by 200 MPa in API-10 than in API-1 (63% vs 75% of denaturation, respectively).

3.3. Determination of disperse and soluble protein

Since samples were turbid after centrifugation (at 10,000 *g* for 20 min at 20 °C) we concluded that some protein remained in dispersion although in an insoluble state. Turbidity was measured in untreated samples and it was found that this parameter was a function of protein concentration: 0.0175 ± 0.0008; 0.0559 ± 0.0012 and 0.4116 ± 0.0277, for API-1, API-5 and API-10, respectively. This dispersed protein was solubilized once in the presence of Bradford reagents and measured as "soluble protein" that had two contributions: soluble and dispersed protein. "Soluble protein" of untreated samples was 33.8 ± 0.9, 42.5 ± 0.1 and 55.7 ± 2.9% for API-1, API-5 and API-10, respectively. These data suggest that the high protein concentration favored the formation of disperse insoluble aggregates from insoluble proteins, and that the difference of density between aggregates and the continuous phase was not big enough to allow sedimentation and/or the size of these particles was small, avoiding precipitation under the centrifugation conditions used.

The effects of HP on disperse and soluble proteins are shown in Fig. 3 as the percentage of remaining protein with respect to the value measured at 0.1 MPa for each protein concentration. Treatment at 200 MPa produced a decrease in API-1 (25% of the control) but did not affect the solubility in API-5 or API-10. On the other hand, solubility decreased more than 50% at the three assayed concentrations after treatment at 400 and 600 MPa.

Our results indicate the effects of treatment at 200 MPa on protein solubility were dependent on protein concentration. This differential

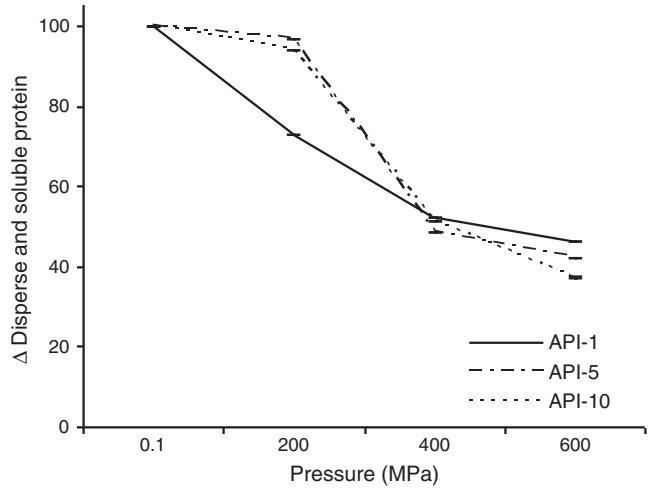

Fig. 3. Disperse and soluble proteins of amaranth protein isolate dispersions at 1% w/v (API-1), 5% w/v (API-5) and 10% w/v (API-10) in either native state (0.1 MPa) or after high pressure treatment (200, 400 and 600 MPa).

effect at 200 MPa may be related with the higher tendency to become denatured under HP that proteins exhibited at low concentration. After treatment at 400 and 600 MPa, the degree of denaturation was similar for all samples, as was also the decrease in remaining protein. It is also possible that in API-5 and API-10 treatment at 200 MPa promoted the formation of soluble macroaggregates, as they were detected by FPLC at 200 MPa. These results differ from those of Puppo et al. (2004), who did not observe changes in the solubility of 1% dispersions of soybean proteins after treatment at 200, 400 or 600 MPa.

3.4. Free sulfhydryls

The content of free sulfhydryl groups in the samples under study is shown in Fig. 4. API-1 exhibited a progressive and significant increase of free SH content with increasing treatment intensities (12%, 52% and 78% for 200, 400 and 600 MPa, respectively), evidencing an increased disruption of S – S bonds in soluble species as a result of HP treatment and/or the exposure of SH groups located in the core of the protein molecule.

In API-5, the maximal content of SH was detected after 200 MPa, whereas in API-10 no changes in the content of SH were detected after any assayed HP treatment. These results suggest that HP-induced unfolding was associated with breakdown of disulfide bonds, thus generating free SH groups that interacted with SH of other molecules when the protein concentration of the dispersion was high enough (API-5 and API-10). It is possible that the formation of these new – S – S – favored the aggregation phenomenon. In the case of API-5, treated at 200 MPa, since proteins are not completely unfolded, free SH groups cannot form new disulfide bonds, whereas in API-10, the higher protein concentration allowed the SH oxidation, even at 200 MPa. 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). Panick, Malessa, and Winter (1999) also reported the formation of aggregates stabilized by the formation of disulfide bonds in HP-treated α-lactoglobulin.

3.5. Surface hydrophobicity

The surface hydrophobicity (H_0) of proteins present in the isolates under study is shown in Fig. 5. Before HP treatment, API-1 exhibited a higher hydrophobicity than API-5 and API-10. It is possible that at high concentration the hydrophobic patches of proteins approach and form the aggregates that were previously described. In addition, H_0 increased in HP-treated isolates, and this effect was dependent on HP level and protein concentration. At 200 MPa the increase was observed in API-1 and API-10, but not in API-5. At 400 MPa H_0 increased for the three concentrations, and particularly for API-5 the highest H_0 value among all assayed conditions was reached at this pressure. In contrast, for API-1 and API-10 the H_0 values registered at 600 MPa were higher than those at 400 MPa. DSC data indicated that proteins exhibit a denaturation degree higher than 93% after treatment at 400 MPa. The ulterior increase in H_0 after treatment at 600 MPa suggests the occurrence of phenomena leading to the exposure of hydrophobic sites, including, for example, the disaggregation of some aggregates formed at 200 or 400 MPa. It is possible that in API-5 be the average between dissociation and aggregation processes.

3.6. Apparent viscosity

The values of apparent viscosity are depicted in Table 3. HP increased this parameter in API-5 and API-10, the increase being greater in API-10 than in API-5, and at 600 MPa than at 400 MPa. Since the effect was observable only at low frequencies, it seems that the HP-induced structure was weak. The increase in apparent viscosity may be related to the formation of the aggregates previously described, which is favored by protein concentration. It is possible that the insoluble nature of aggregates precluded the establishment of a nice network, a fact that was reflected as a small effect on viscosity.

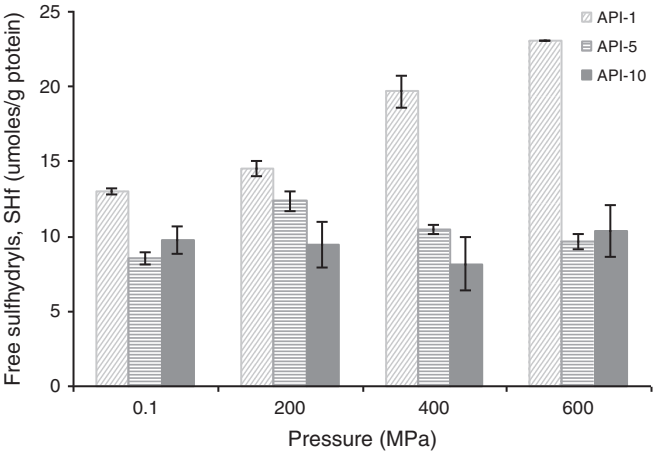

Fig. 4. Free sulfhydryl (SH_f) concentration of amaranth protein isolates at 1% w/v (API-1), 5% w/v (API-5) and 10% w/v (API-10) in either native state (0.1 MPa) or after high pressure treatment (200, 400 and 600 MPa).

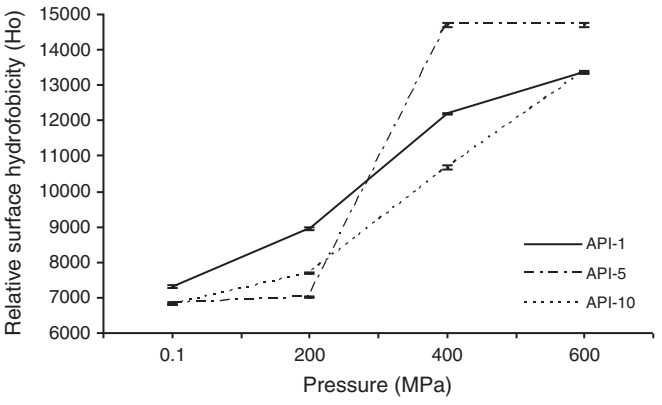

Fig. 5. Surface hydrophobicity (H_0) of amaranth protein isolates at 1% w/v (API-1), 5% w/v (API-5) and 10% w/v (API-10) in either native state (0.1 MPa) or after high pressure treatment (200, 400 and 600 MPa).

Table 3
Apparent viscosity (0.1 and 1 Hz) of amaranth protein isolate at 1% w/v (API-1), 5% w/v (API-5) and 10% w/v (API-10) in either native state (0.1 MPa) or after high pressure treatment (200, 400 and 600 MPa).

	API-1		API-5		API-10	
	0.1 Hz	1 Hz	0.1 Hz	1 Hz	0.1 Hz	1 Hz
0.1 MPa	0.009 ± 0.001^{a}	0.014 ± 0.002^{a}	0.0088 ± 0.0008^{a}	0.008 ± 0.004^{a}	0.004 ± 0.001^{a}	0.0158 ± 0.0007^{a}
200 MPa	0.0169 ± 0.0002^{a}	0.009 ± 0.002^{a}	0.0137 ± 0.0001^{a}	0.13 ± 0.10^{b}	0.008 ± 0.004^{a}	0.06 ± 0.05^{a}
400 MPa	0.020 ± 0.005^{a}	0.007 ± 0.006^{a}	0.008 ± 0.001^{a}	0.014 ± 0.004^{a}	0.12 ± 0.03^{b}	0.03 ± 0.01^{a}
600 MPa	0.021 ± 0.001^{a}	0.008 ± 0.002^{a}	0.33 ± 0.13^{b}	0.021 ± 0.008^{a}	1.51 ± 0.05^{c}	0.198 ± 0.009^{b}

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

4. Conclusions

Proteins from amaranth isolate were very sensitive to HP treatment, more than other protein such as soy bean proteins, since they suffered a high degree of denaturation at 200 and 400 MPa. HP denaturation was accompanied by a decrease in protein solubility, and protein dissociation and aggregation — via hydrophobic interactions and disulfide bonds. Protein concentration modulated the effects of HP on amaranth proteins, as at low concentration (1%) proteins were more sensitive to HP than at high concentration.

The modification of amaranth proteins structure induced by HP treatment may be reflected as an improvement in their functional properties, increasing the added value of this crop. Further studies should be necessary to confirm this hypothesis.

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