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Characterization of amaranth proteins modified by trypsin proteolysis. Structural and functional changes

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ABSTRACT

Amaranth protein isolates were prepared from *Amaranthus hypochondriacus* and hydrolyzed using trypsin. Degrees of hydrolysis varied between 2% and 7% with the time of hydrolysis reaction. The structure, solubility and foaming properties of protein isolates were studied. SDS-PAGE analysis demonstrated that polypeptides from 11S-globulin and P globulin were the main targets for the protease, while a polypeptide of 45 kDa from the 7S globulin was more resistant to trypsin action. The FPLC analysis of hydrolyzed proteins showed that the structure is partially conserved, while an increase of the fraction of lower molecular weight is observed. Such structure exhibits a single endotherm of lower denaturation heat than the protein isolate.

The solubility and foaming properties of protein isolates and hydrolysates were analyzed, in the later case at two protein concentrations (1 mg and 2.5 mg of solid matter per ml). Protein solubility increased markedly with hydrolysis, while changes in foaming properties were less dramatic. Nevertheless, foams obtained with amaranth protein hydrolysates were more dense and stable than those prepared with non-digested proteins, specially for foams produced with the protein hydrolysate of higher concentration.

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1. Introduction

Although animal proteins are widely used in food formulation because of their high nutritional value and their versatile functional performance, it is desirable to find alternative protein sources, more economic and with high nutritional quality and bioavailability. Several plant proteins have been studied to achieve this goal, including storage proteins from soy, pea and sunflower (González-Pérez & Vereijken, 2007; Rangel, Domont, Pedrosa, & Ferreira, 2003; Tsumura et al., 2005). More recently the interest has been focused on ancestral crops, such as amaranth, which seeds contain proteins with a well-balanced composition in essential amino-acids and important content of sulfur-containing amino-acids (Bressani, 1989), which add to their well known agronomic advantages (Lehmann, 1996).

To be used in the food industry, the selected proteins should display a wide range of functional properties which are closely related to their structure (Kinsella & Phillips, 1989). Amaranth seeds major protein fractions (albumin, 7S globulin, 11S-globulin, globulin-P and glutelin) appear as good candidates to achieve this end due to their variety in structural and physicochemical properties (Castellani, Martínez, & Añón, 1998; Marcone, Niekamp, LeMaguer, & Yada, 1994; Martínez, Castellani, & Añón, 1997; Segura-Nieto, Barba de la Rosa, & Paredes-López, 1994).

Several physical, chemical and enzymatic treatments have been used to modify the functional properties of seeds proteins. Enzymatic hydrolysis is frequently used to improve the functional and nutritional properties of natural food proteins. Usually, the enzymatic modification is preferable due to milder processing conditions, easier control of the reaction, and minimal formation of by-products (Mannheim & Cheryan, 1992). While protein hydrolysates with a higher level of hydrolysis (>10%) are used as nutritional supplements, protein hydrolysates with a low degree of hydrolysis (1–10%), are usually produced to improve the functional performance relative to that of the original proteins, mainly foaming and emulsifying properties (Foegeding, Davis, Doucet, & McGuffey, 2002; Rodríguez Patino et al., 2007).

Considering the close relationship between structural and functional properties of proteins, the goal of the present study was to evaluate the structure of an amaranth protein isolate modified by enzymatic hydrolysis, and to determine some of its functional properties. With this aim a partial tryptic hydrolysis of amaranth

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proteins was performed, the effect of hydrolysis on the solubility and foaming properties of proteins was studied, and the relationship between structural changes and functional performance was examined.

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, in Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata. The resulting flour was defatted with hexane at 4 °C for 24 h (100 g l^{-1} suspension) under continuous stirring. After drying at room temperature, the flour was stored at 4 °C until used. The flour composition was determined. The protein content (19.8 ± 0.2 g/100 g of flour) was determined by Kjeldahl (method 954.01 AOAC, 1990), $f = 5.85$ (Segura-Nieto et al., 1994). Other flour components were carbohydrates (54.1 ± 0.9 g/100 g), lipids (10.5 ± 0.6 g/100 g), ashes (6.10 ± 0.04 g/100 g), and water (10.4 ± 0.2 g/100 g). The methods used for measuring these parameters were, respectively, Fehling after complete acid hydrolysis, ethylic ether extraction with the Soxhlet method, heating in a muffle at 550 °C, and drying in a stove at 105 °C until constant weight, according to AOAC (methods 974.06, 920.39, 923.03, and 925.09; 1990).

2.3. Preparation of amaranth protein isolates

Amaranth protein isolates (*I*) used in this study were prepared according to Martínez and Añón (1996). Briefly, defatted flour was suspended in water (100 g l^{-1}) and the pH was adjusted to 9.0 with 2 mol equi/L NaOH. The suspension was stirred for 60 min at room temperature and then centrifuged for 20 min at 9000×*g* at 15 °C. The supernatant was adjusted to pH 5.0 with 2 mol equi/L HCl and then centrifuged at 9000×*g* for 20 min at 4 °C. The pellet was resuspended in water, neutralized with 0.1 mol equi/L NaOH and freeze-dried. The protein isolate obtained was stored at 4 °C until used. Percent composition of the protein isolate was determined using the same methods mentioned above for flour composition.

Two protein isolates were used as controls. One of them was a protein isolate without any treatment (*I*) and the other, heated amaranth protein isolate (HI), was prepared by heating a suspension of *I* in buffer B (33.3 mmol/L K_2HPO_4, 1.7 mmol/L KH_2PO_4, pH 8.5) for 10 min at 85 °C, followed by freeze-drying.

2.4. Preparation of amaranth hydrolyzed protein isolates

Hydrolyzed protein isolates were prepared as follows: 25 g l^{-1} *I* suspensions were prepared in buffer B. The suspensions were stirred for 60 min at 37 °C and then hydrolyzed with trypsin (Trypsin powder from porcine pancreas; Sigma catalog number T1426, 10600 BAEE units/mg solid). The enzyme/isolate mass ratio in the reaction mixtures was 1/62.5. The reaction was stopped by heating in a bath for 10 min at 85 °C; the whole suspension, was freeze-dried and stored at 4 °C until used. The hydrolyzed protein isolates were labeled TH and time of hydrolysis, in minutes, was indicated by a subscript number. A sample of protein isolate subjected to the stopping thermal treatment immediately after the addition of the enzyme was labeled as trypsin hydrolyzed at time zero (TH_0).

2.5. Degree of hydrolysis

The degree of hydrolysis (DH) was determined by a TNBS method, which is based on the reaction of primary amino groups with trinitro-benzene-sulfonic acid (TNBS) reagent (Adler-Nissen, 1979).

DH values were calculated using the following equation:

$$\mathrm{DH}\% = \frac{100(\mathrm{AN}_2 - \mathrm{AN}_1)}{\mathrm{Npb}}$$

where AN_1 and AN_2 are the amino nitrogen content of the protein substrate before and after hydrolysis (mg g^{-1} protein), respectively, and Npb the nitrogen content of the peptide bonds in the protein substrate (mg g^{-1} protein). The values of AN_1 and AN_2 were obtained by reference to a standard curve of optical density at 340 nm versus mg l^{-1} amino nitrogen (generated with L-leucine). These values were then divided by the protein content of the test samples to give mg amino nitrogen per gram of protein (Adler-Nissen, 1979). At least two determinations were performed for each condition.

2.6. Molecular exclusion chromatography

Protein isolates and hydrolyzed protein isolates were analyzed by gel filtration chromatography using a molecular exclusion column. A Superose 6 N° 3 Amersham Biosciences column and a Pharmacia LKB, FPLC System (Uppsala, Sweden) were used. 200 μl of samples dissolved in buffer B (15 mg ml^{-1}) were injected and eluted with the same buffer at 0.2 ml min^{-1}. Absorbance at 280 nm was monitored and 0.5 ml fractions were collected. The column was calibrated with blue dextran (for void volume, $V_0 = 7.64$ ml), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and cytochrome C (12.4 kDa). The molecular masses of the fractions were calculated using the equation:

$$\log \mathrm{MM} = 4.5 - 5.1K_{\mathrm{AV}}$$

where $K_{\mathrm{AV}} = (V_e - V_0)/(V_T - V_0)$, V_e is the elution volume of the resolved species, V_0 is the void volume, and V_T is the total volume of the column ($V_T = 25.09$ ml).

Every determination was performed at least twice.

2.7. Electrophoresis

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

This analysis was performed using the procedure described by Martínez et al. (1997). Runs were carried out with 40 g l^{-1} and 120 g l^{-1} acrylamide gels (stacking and separating, respectively). The following continuous buffer system was used: 0.375 mol/L Tris-HCl, pH 8.8, 1 g l^{-1} SDS for the separating gel; 0.025 mol/L Tris-HCl, 0.192 mol/L glycine and 1 g l^{-1} SDS, pH 8.3 for the running buffer, and 0.125 mol/L Tris-HCl, pH 6.8, 200 ml l^{-1} glycerol, 10 g l^{-1} SDS, and 0.5 g l^{-1} bromophenol blue as sample buffer. For runs under reducing conditions the sample buffer contained 50 ml l^{-1} 2-mercaptoethanol (2-ME) and samples were heated for 60 s in a boiling water bath. The following protein molecular mass standards were used: phosphorylase b (94 kDa); bovine serum albumin (67 kDa); ovalbumin (45 kDa); carbonic

anhydrase (30 kDa); trypsin inhibitor (20.1 kDa); α-lactalbumin (14.4 kDa). Gels were fixed and stained with Coomassie Brilliant Blue Stain. Gels images were acquired with the Gel Doc 1000 Image Analysis System (Bio-Rad, Richmond, CA, U.S.A.) and analyzed with the Molecular Analyst Software (Bio-Rad) in order to determine the molecular masses of the polypeptides and the relative intensity of the bands.

2.7.2. Native gel electrophoresis (PAGE)

This analysis was performed using an adaptation of the method of Laemmli (1970), in which SDS was replaced with distilled water. Polyacrylamide gels were prepared at 80 g l^{-1}.

2.7.3. Two-dimensional gel electrophoresis

Two kinds of bidimensional electrophoresis were carried out. In both cases PAGE was carried out in the first dimension; followed in one case by SDS-PAGE with 2-ME in the second dimension, and in the other case by SDS-PAGE without reducing agent. Each lane of the first dimension slab gel was treated with 10 volumes of treatment buffer (62.5 mmol/L Tris-HCl pH 6.8, 10 g l^{-1} SDS and 200 g l^{-1} sucrose, 0.2 mol/L 2-ME) at 55 °C for 30 min. The procedure was repeated twice, changing the treatment buffer. Immediately, this portion of the gel was placed on top of the gel used for the second dimension. Runs were performed under the same conditions mentioned for the one-dimensional gel.

Gels were fixed and stained with Coomassie Brilliant Blue Stain. Gels images were acquired with the Gel Doc 1000 Image Analysis System (Bio-Rad).

2.8. Differential scanning calorimetry (DSC)

Protein fractions were analyzed by DSC according to the method of Martínez and Añón (1996). Samples were prepared in distilled water. Aluminum hermetic capsules (Perkin-Elmer No. 0219-0062) were loaded with 10–15 mg of the 200 g l^{-1} dispersions and allowed to stabilize for at least 30 min at room temperature (25 °C) before testing. Runs were performed in a DSC Polymer Laboratories (Rheometric Scientific) device, using a heating rate of 2 °C min^{-1} between 25 °C and 28 °C, and then a heating rate of 10 °C min^{-1} between 28 °C and 130 °C. A sealed double empty capsule was used as a reference. All experiments were conducted in triplicate.

2.9. Solubility

Solubility of protein isolates and protein hydrolysates was analyzed by preparing 10 g l^{-1} suspensions in water or buffer B. Samples were incubated 1 h at room temperature (25 °C) and vortexed every 15 min. Samples were then centrifuged at 10 000×*g* for 15 min at room temperature. Protein content in the supernatant (*s*) was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) and solubility (*S*) was expressed as follows:

$$\%S = s \times 100/\text{total protein}$$

Total protein content was determined for Kjeldahl method ($f = 5.85$). Determinations were performed in duplicate.

2.10. Foaming properties

The foams studied in this work were obtained according the method of Guillerme, Loisel, Bertrand, and Popineau (1993). Sample dispersions at 1.0 mg ml^{-1} or 2.5 mg ml^{-1} in buffer B were placed in the sparging chamber at the base of an acrylic column (length: 27.5 cm, internal and external diameters: 2.4 and 3.0 cm, respectively). Foam was generated by sparging nitrogen through a porous glass disk 2 mm average pore size, G4 type at a rate of 102 ml min^{-1} into 6 ml of the protein solution during 1 min. The volume of initial or residual liquid under the foam was measured by conductivity through two electrodes located in the sparging chamber. Conductivity measurements, as a function of time (C_t) and with reference to the conductivity of the buffered test solution (C_{init}), were used to calculate the volume of liquid in the foam (V_L): $V_L = V_{init}\,[1-(C_t/C_{init})]$, where V_{init} is the volume of sample solution (6 ml) introduced into the sparging chamber (Loisel, Guéguen, & Popineau, 1993).

Foaming capacity was estimated from the initial rate of foam formation (v_o), maximum volume of liquid in the foam, (V_L), and maximal density of foam, D_{max} (with D_{max} = maximum volume of liquid in the foam/maximum volume of foam).

Foam stability was estimated from the time of half drainage ($t_{1/2}$). Measurements were performed in triplicate.

2.11. Statistical analysis

The least significant difference (LSD) test (after analysis of variance, ANOVA) was used to identify pairwise differences between means. Significance was determined at $P < 0.05$.

3. Results and discussion

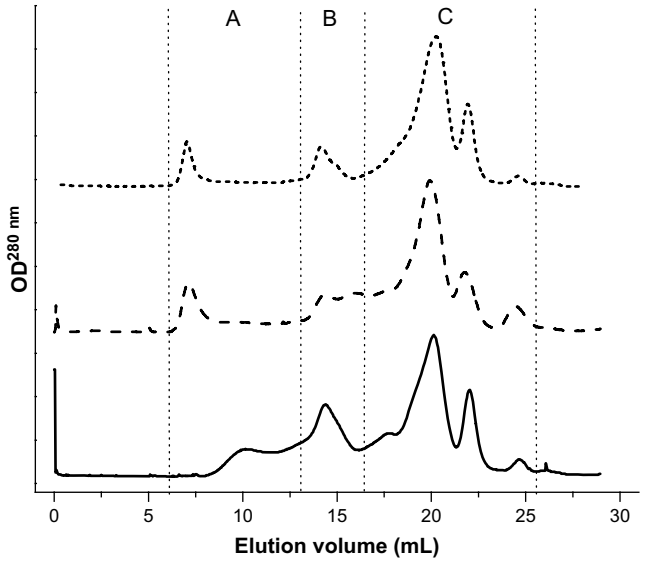
3.1. Protein isolate composition

The percent composition of the protein isolate, *I*, was determined. The content of proteins, water, ashes and carbohydrates was 75.4 ± 0.5 g/100 g of protein isolate ($f = 5.85$), 3.87 ± 0.05 g/100 g of protein isolate, 5.0 ± 0.3 g/100 g of isolate, and 10.0 ± 0.4 g/100 g of isolate, respectively. The fiber content, estimated by difference, was 5 g/100 g of protein isolate.

The amaranth protein isolate (*I*) contained albumins, 7S and 11S-globulin and globulin-P as major fractions (Martínez et al., 1996). As reported previously, globulin-P is a legumine-like protein composed by unitary molecules similar to the 11S hexamers and large polymers. The protein composition of the isolate was confirmed by FPLC (Fig. 1, *I*). The profile showed the presence of globulin-P polymers (component A, with MM ranging from 500 to 30 000 kDa), globulin-P and 11S-globulin molecules of 280 ± 7 kDa, which were described elsewhere (Martínez et al., 1997), and 7S globulin (component B) and species of MM < 100 kDa (component C, mainly composed by albumins). The heated protein isolate was also analyzed by FPLC (Fig. 1, HI). The thermal treatment resulted in dissociation and aggregation of unitary molecules, which is evidenced by the diminished B/A areas ratio and the increased C/B ratio (Fig. 1). The chromatographic profiles of both control protein isolates (*I* and HI) exhibited similar elution volumes, the only difference being the volume of the zone *I* peak. Such volume was lower after thermal treatment, confirming the aggregation induced by heating.

3.2. Protein isolate hydrolysis

The amaranth protein isolate was subjected to enzymatic hydrolysis with trypsin. Different reaction times were assayed and the degree of hydrolysis (DH) reached was determined in each case (Table 1). The DH increased with reaction time until 30 min of hydrolysis, with no significant differences thereafter ($P < 0.05$). Therefore, the product obtained after 30 min of hydrolysis was chosen for studying its characteristics and functional properties.



Sample	% area FPLC zone			Relation of areas		
	zone A	zone B	zone C	r B/A	r C/A	r C/B
I	14.2	21.6	64.2	1.5	4.5	3.0
HI	14.2	16.8	69.0	1.2	4.8	4.1
TH_{30}	10.6	11.8	77.6	1.1	7.3	6.6

Fig. 1. Gel filtration FPLC profile of amaranth protein isolate (*I*, ——), heated protein isolate (HI, – – –) and trypsin hydrolyzed isolate (TH_{30}, ·····) in buffer B (33.3 mmol/L K_2HPO_4, 1.7 mmol/L KH_2PO_4, pH 8.5). **A:** components eluting between 6 and 13 ml; **B:** components eluting between 13 and 16.5 ml; **C:** components eluting between 16.5 and 25.5 ml.

The DH in the protein isolate at TH_0 (stopping thermal treatment applied immediately after enzyme addition) was 2.03 ± 0.05%, showing that the enzyme can degrade the substrate even after a very short contact time, such as the 4 min necessary to reach 85 °C.

3.3. Characterization of amaranth protein isolates and hydrolyzed

Protein isolates and protein hydrolysates were analyzed by native, denaturing, and reducing denaturing electrophoresis (Fig. 2 A, C, and D, respectively). In the native electrophoresis amaranth protein isolates (*I*), exhibited two species of different electrophoretic mobility (Rf 0.13, band 1, and Rf 0.19, band 2, arrows in Fig. 2 A). The electrophoretic pattern of the thermally treated protein isolate (HI) did not differ from the unheated control, *I* (results not shown). Two species were also observed in protein hydrolysates, with mobilities that increased with hydrolysis time (TH_0 to TH_{60}, bands 1 and 2). However, the larger mobility increase was observed between TH_0 and the protein isolate (*I*) (Fig. 2 B).

Table 1
Degree of hydrolysis.

	Samples				
	TH_0	TH_{15}	TH_{30}	TH_{45}	TH_{60}
Time of hydrolysis (min)	0	15	30	45	60
DH%	2.03 (0.05)[a]	4.32 (0.12)[b]	6.36 (0.42)[c]	7.39 (0.29)[c]	7.28 (0.15)[c]

Means and standard deviations (in parenthesis) of duplicate analyses are given. Different superscript letters (a, b, and c) indicate significant difference ($P < 0.05$).

As reported previously (Martínez et al., 1997) the electrophoretic pattern of amaranth protein isolates under denaturing conditions exhibited several polypeptides, including two bands of similar MM (56–54 kDa) corresponding to the AB subunits and the P-54 polypeptide (Fig. 2 C and D). Amaranth 11S-globulin is formed by a hexamer of AB subunits, whereas both, P-54 and AB subunits, constitute globulin-P (Castellani, Martínez, & Añón, 2000). In addition to these two bands, a well defined band of 45 kDa, probably a constituent of 7S globulin (Marcone, 1999; Quiroga, 2008), was also observed. The electrophoretic pattern also exhibited some bands of MM between 32 and 38 kDa, corresponding to type A polypeptides naturally free in the amaranth seed, and other bands of MM between 20 and 18 kDa that may correspond to type B polypeptides. The possibility that some bands may correspond to the albumin fraction present in the protein isolate cannot be ruled out (Marcone et al., 1994; Segura-Nieto et al., 1992). The electrophoretic pattern of the thermally treated protein isolate (HI) did not differ from the unheated control, *I* (results not shown). Electrophoretic patterns changed after enzymatic treatment. Several polypeptides were substrates of the enzyme, with some disappearing from the pattern very quickly and others exhibiting a progressive diminution of intensity as the hydrolysis time increased (Fig. 2 C and D). One of these was the 45 kDa polypeptide that would constitute the 7S globulin. The intensity of this band diminished with hydrolysis time; if the intensity of the band in the protein isolate (*I*) is given a value of 100, a value of 80 is recorded at TH_0, 27 at TH_{15}, and 17 at TH_{30}, with no changes recorded thereafter (Fig. 2 D).

The results of two-dimensional electrophoresis are shown in Fig. 3. In every case the first dimension was run under native conditions. The analysis of the amaranth protein isolate revealed a differential polypeptide composition of bands 1 and 2 (Fig. 3 A). Band 1 was formed by P-54, A polypeptides of approximately 32 kDa, and B polypeptides of approximately 20 and 18 kDa. Band 2 was constituted mainly by the 45 kDa polypeptide. This polypeptide and a second one of 67 kDa were also included in band 1, although with lower intensity. In the absence of 2-ME (Fig. 3 B) two protein species of high MM were observed, while both disappeared after 2-ME treatment. Band 1 in TH_0 (Rf = 0.31) was constituted by polypeptides of 67, 45, 25, 22 and 20 kDa, while band 2 (Rf = 0.47) was formed by 45 kDa polypeptides, although the presence of 67 kDa species could not be ruled out (Fig. 3 C and D). The two-dimensional analysis of the protein hydrolysate obtained after 30 min of proteolysis (TH_{30}) revealed that band 1 (Rf = 0.43) was formed by polypeptides of low MM (24, 22, 18 and 14 kDa, Fig. 3 E), while band 2 (Rf = 0.58) retained some 45 kDa polypeptide together with hydrolysis products of 25 and 16 kDa (Fig. 3 E). Polypeptides present in band 1 were linked by disulfide bonds, yielding mainly molecules of approximately 40 kDa and a lower proportion of 67 kDa species (Fig. 3 F). Therefore, 30 min of trypsin treatment was enough to attack all the peptides present in the protein isolate. P-54 and A polypeptides were attacked first, although the susceptibility of B polypeptides to enzymatic hydrolysis cannot be ruled out. The 54 kDa polypeptide, a constituent of the P globulin fraction, is also an early substrate of seed proteases during germination (Aphalo, Molina Ortiz, Martínez, Scilingo, & Añón, 2005) and also a substrate of other plant proteases characterized in previous studies (Scilingo, Molina Ortiz, Martínez, & Añón, 2002). The 45 kDa polypeptide suffered a delayed and only partial hydrolysis.

Changes produced by the hydrolytic reaction were also evidenced by size exclusion chromatography-FPLC (Fig. 1, TH_{30}). While the percent area of zone C increased, that of zones B and A diminished as compared to controls. The molecules of low MM

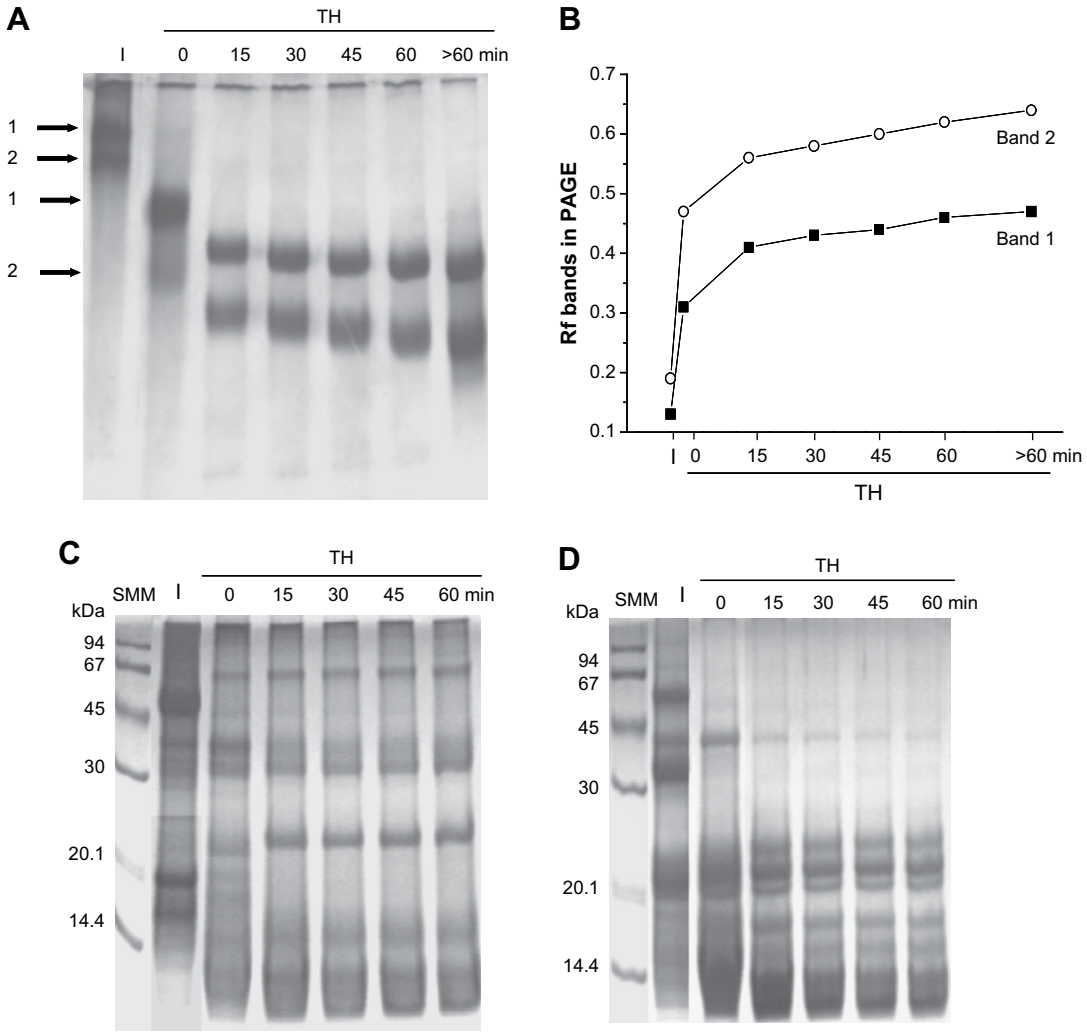


Fig. 2. Electrophoresis profiles of native (*I*) and hydrolyzed (TH) amaranth protein isolates. Panel A: electrophoresis profiles in non denaturing conditions (PAGE); the arrows indicate bands 1 and 2 of *I* and TH_0. Panel B: Relative migration (Rf) of bands 1 and 2 vs. time of hydrolysis. Panel C: electrophoretic pattern under denaturing conditions (SDS-PAGE). Panel D: electrophoresis under reducing and denaturing conditions (SDS-PAGE + ME). SMM: standard molecular mass.

produced by hydrolysis contribute to the area increase of zone C. The area reduction of zones A and B as a consequence of the enzymatic reaction indicate that both the molecules of 280 kDa that constitute globulins P, 11S and 7S (zone B) and the aggregates (zone A) are substrates for trypsin (Fig. 1).

The amaranth protein isolate exhibited two denaturation endotherms with peak temperatures of 71.7 ± 0.2 and 98.3 ± 1.1 °C, respectively (Fig. 4). The enthalpy associated to the process was 10.71 ± 0.04 J g^{-1} of protein. These values agree with those obtained previously by Martínez et al. (1996). The thermal treatment modified the isolate proteins as evidenced by the presence of a single endotherm of lower denaturation heat (3.3 ± 0.9 J g^{-1} of protein). In this thermogram, corresponding to heated protein isolate (HI), the peak temperature was 101.2 ± 0.1 °C, indicating that the fraction that remained in native state had a higher thermal stability than those present in the protein isolate (*I*). The thermogram of the protein hydrolysate during 30 min (TH_{30}) also exhibited a single endotherm. The peak temperature for this transition was 98.6 ± 0.6 °C, and the associated enthalpy was 5.0 ± 0.9 J g^{-1} of protein, suggesting that although the hydrolysis process introduces conformational changes some of the structure is conserved.

The results indicate that the thermal treatment of the protein isolate (HI) produced a partial denaturation. A partial denaturation would favor aggregation during the calorimetric study, leading to a reduction of ΔH. On the contrary, when thermal treatment is applied to proteins previously digested with trypsin, the residual structure is less prone to aggregation during calorimetry, yielding a higher ΔH. These results are in line with those shown above for FPLC and electrophoresis, which show that although both thermal treatment and hydrolysis modify the proteins, the molecular structure is partially conserved.

3.4. Functional characterization of native and hydrolyzed amaranth protein isolates

3.4.1. Solubility

As shown in Table 2, the amaranth protein isolate had a low solubility in water. This may be explained by the presence of an important amount of globulin-P and 11S-globulin which are insoluble in water (Konishi, Fumita, Ikeda, Okuno, & Fuwa, 1985; Martínez et al., 1997). The protein isolate solubility was higher in buffer B than in water (Table 2) because globulin-P increases its solubility in alkaline medium (Castellani et al., 1998).

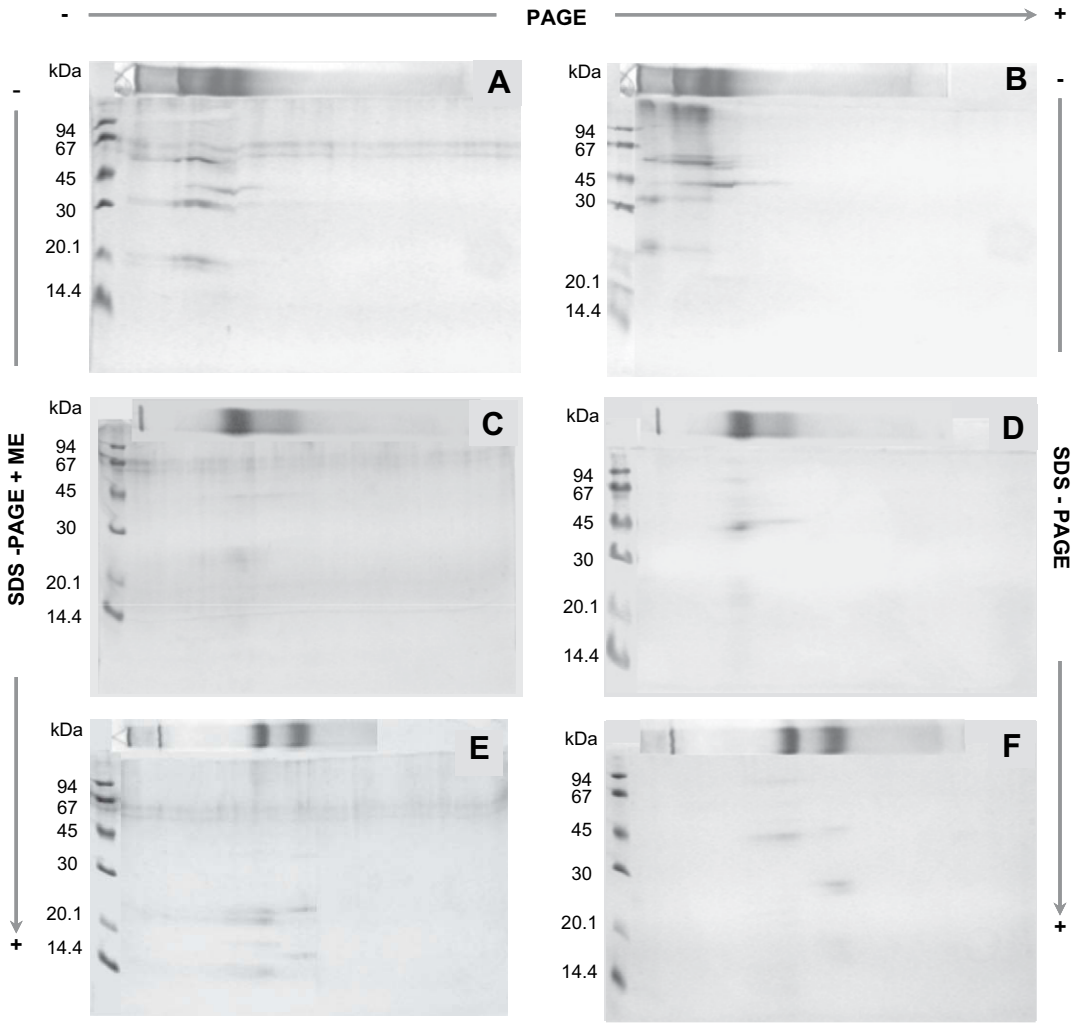


Fig. 3. Two-dimensional electrophoresis of amaranth protein isolate (*I*, panels A and B), isolate at time 0 of hydrolysis (TH_0, panels C and D), and isolate hydrolyzed during 30 min (TH_{30}, panels E and F). First dimension: PAGE. Second dimension: SDS-PAGE in the presence of 2-ME: A, C, and E; SDS-PAGE: B, D, and F.

The thermal treatment applied (HI) does not reduce water solubility as compared to the values determined for the amaranth protein isolate (*I*). In buffer B, however, in which globulin-P polymers are more soluble (*I*, buffer B), heating results in a partial reduction of solubility (Table 2, HI, buffer B).

Comparing the trypsin hydrolyzed isolate with the amaranth protein isolate, TH_{30} was 1.7 and 1.4 fold more soluble than *I* in water and buffer B, respectively (Table 2). These results may be explained by a reduction of molecular size, as evidenced by FPLC analysis.

3.4.2. Foaming properties

Foams were prepared at 1.0 and 2.5 mg ml^{-1} dispersions of protein isolate and hydrolysate, as indicated (Fig. 5, A, B, C and D). Foam formation initial rate, maximum volume of liquid in the foam, and foam density were used as a measure of foam formation ability.

Foam formation initial rate was similar for the protein isolate and the protein hydrolysate at the two concentrations used (Fig. 5 A). Regarding the maximum volume of liquid in the foam (Fig. 5 B), both the protein isolate and the protein hydrolysate had better foaming performance when the solid concentration used in the assay was increased. The protein hydrolysate was more efficient than protein isolate to form foam only when 1 mg ml^{-1} of solid

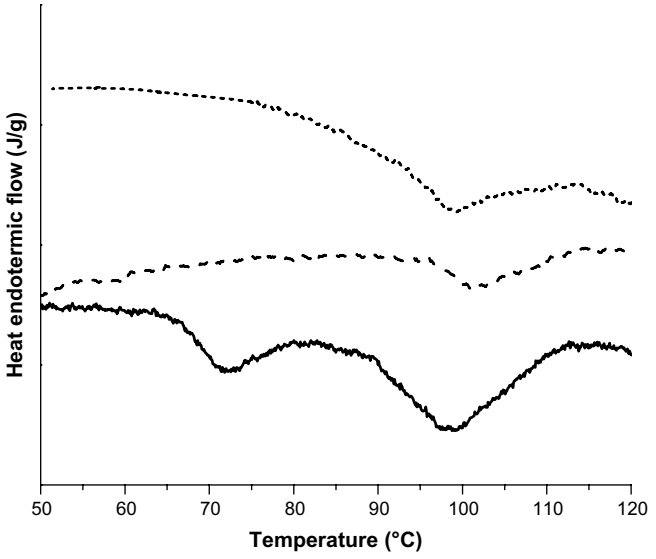


Fig. 4. Differential scanning calorimetry (DSC) of amaranth protein isolate (——), heated protein isolate (– –), and trypsin hydrolyzed isolate TH_{30} (·····).

Table 2
Solubility of amaranth isolates and hydrolysates.

		% Solubility	
		Water	Buffer B*
Isolate	***I***	32.6 (0.2)[a]	67.7 (1.1)[b]
Heated isolate	**HI**	37.8 (2.8)[c]	34.4 (0.9)[a,c]
Trypsin hydrolyzed	**TH_{30}**	55.8 (1.6)[d]	98.3 (0.2)[e]

Means and standard deviations (in parenthesis) of duplicate analyses are given. Different superscript letters (a, b, c, d, and e) indicate significant difference ($P < 0.05$).
*Buffer B: 33.3 mmol/L K_2HPO_4, 1.7 mmol/L KH_2PO_4, pH 8.5.

was used. These results can be related to the increased solubility of the protein hydrolysate in buffer B, which would facilitate the proteins arrival to the interface at this concentration.

The foam density (maximum liquid volume in the foam/foam maximum volume) estimates the ability to incorporate liquid in the foam, and its relation with the final foam volume. In the present study, foam density (Fig. 5 C) increased with protein concentration, for both protein isolate and protein hydrolysate. In addition, this parameter improved with hydrolysis at both concentrations. Then, to form more dense foams it was necessary a greater protein concentration and species of smaller molecular size.

Time of half drainage was studied to evaluate the ability of proteins to stabilize foams. This measure indicates the time necessary to drain half of the volume of liquid incorporated into the foam. The results obtained are shown in Fig. 5 D. Longer half drainage times were obtained for foams prepared with protein hydrolysate dispersions, and for higher concentrations (TH_{30}, 2.5 mg ml^{-1} of solid).

The protein permanence in the interface is greater if the proteins used to form the foams have a molecular size that hampers protein desorption or allows the formation of stable structures in the interface (Walstra, 1989). The tryptic hydrolysis would generate species of molecular size similar to those in the protein isolate, but that are more efficient than the latter to remain in the interface.

According to the results of the present study, the best foam formation and stability properties are obtained with the protein hydrolysate dispersion at the highest solid concentration.

In summary, this study shows that trypsin treatment induces changes in amaranth proteins, and that such proteins exhibit a differential resistance to hydrolysis. Polypeptides from 11S and P globulins are early substrates of the enzyme, while some polypeptides from the 7S globulin are not affected. Results from gel filtration chromatography and DSC indicate that the enzymatic action does not produce a complete structural unfolding of amaranth globulins. The hydrolytic process modifies the functional performance of amaranth proteins, improving their solubility and the characteristics of the foams obtained. These results suggest that a partially hydrolyzed proteinaceous product can be used as functional ingredient in food formulation.

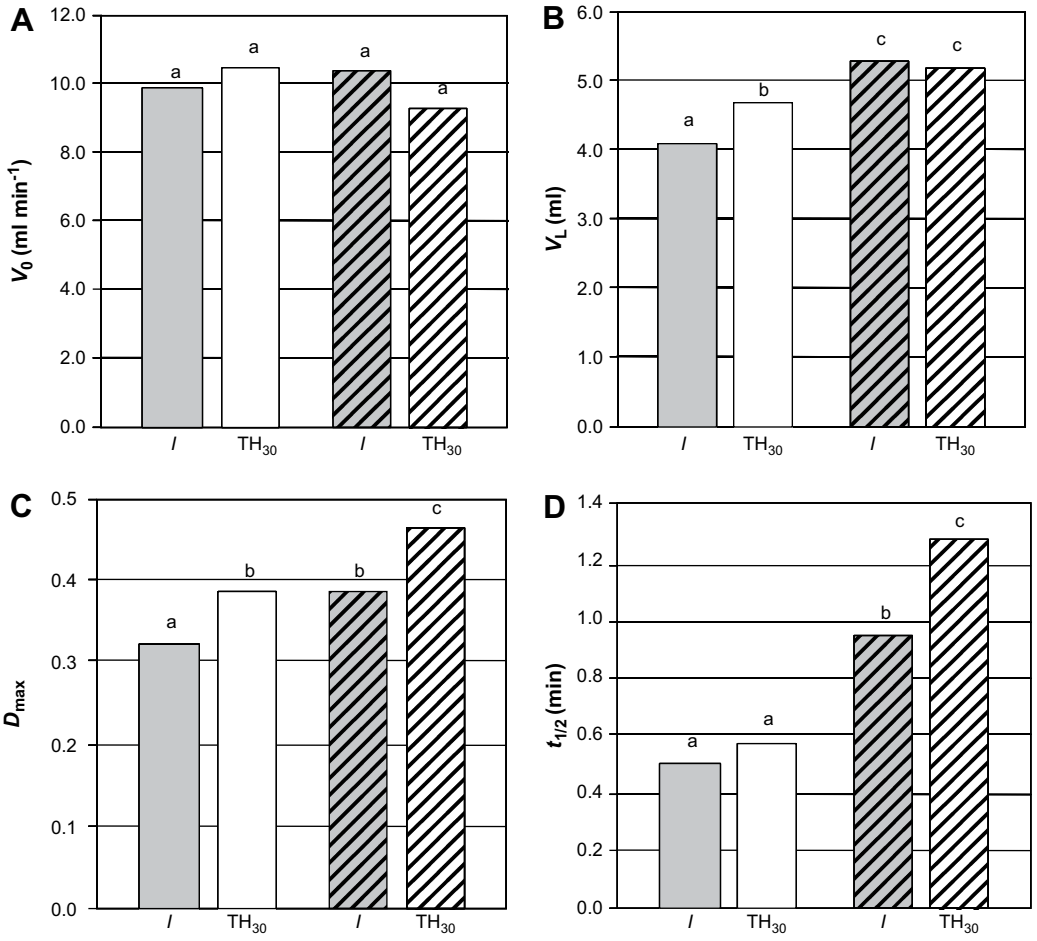


Fig. 5. A: foam formation initial rate, v_0; **B:** maximun volume of liquid in the foam, V_L; **C:** foam density, D_{max}; **D:** half drainage time, $t_{1/2}$. Solid concentration: 1.0 mg ml^{-1} (empty white and grey bars), 2.5 mg ml^{-1} (hatched white and grey bars). ***I*:** amaranth protein isolate; **TH_{30}:** protein isolate at 30 min of trypsin hydrolysis. Different letters on bars indicate significant differences ($P < 0.05$).

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