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Amaranth protein isolates modified by hydrolytic and thermal treatments. Relationship between structure and solubility

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Abstract

Structure and solubility of modified amaranth isolates were studied. Isolates were obtained from proteolytic (cucurbita or papain) and/or thermal treatments of an amaranth isolate. Results showed that 11S-globulin and globulin-P, the main targets for the proteases, were hydrolyzed more efficiently by papain than by cucurbita. Thermal treatment induced both aggregation and dissociation of proteins. Dissociation increased with hydrolysis. It was also shown that polymers and globulin molecules were responsible for the insolubility of a non-treated isolate and that their hydrolytic modification made the isolate more soluble. While a thermal treatment decreased the isolate solubility, a previous hydrolysis with papain increased the solubility. Consequently, the amaranth isolate hydrolyzed with papain is a suitable ingredient in foods submitted to thermal treatment considering that it keeps a high solubility after heating.

Keywords: Amaranth; Protein isolates; Proteolysis; Protein solubility; Thermal treatment

1. Introduction

Proteins are commonly employed as food ingredients on the basis of their importance in the human diet. The best proteins belong to animal sources since they meet human nutritional requirements and because they have a suitable functionality. However, the high cost of animal proteins makes vegetable proteins the main dietary component for most of the world's population. Legume (e.g. soybean) proteins are widely available and of good quality but they are deficient in sulfur-containing amino-acids. Amaranth proteins appear as an excellent alternative or complement due to their well-balanced composition in essential amino-acids and enough content of sulfur-containing amino-acids (Bressani, 1989).

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, 11S-globulin, globulin-P and glutelin) show up as good candidates to achieve this end due to their variety in structural and physicochemical properties (Castellani, Martínez, & Añón, 1998; Marcone, Niekamp, LaMaguer, & Yada, 1994; Martínez, Castellani, & Añón, 1997; Segura-Nieto, Barba de la Rosa, & Paredes López, 1994). Preparation of protein isolates containing these fractions in different proportions have been previously reported by Martínez and Añón (1996). These isolates may show an important functional versatility because of the structural diversity in its composition. However, amaranth proteins display a rather low solubility in aqueous solvents limiting their use in the food industry (Konishi & Yoshimoto, 1989; Segura-Nieto et al., 1994).

Enzymatic hydrolysis is one of the chemical approaches used to alter protein functionality improving their solubility (Kim, Park, & Rhee, 1990; Shih & Campbell, 1993; Sijtsma, Tezera, Hustins, & Vereijken, 1998). The induced modifications strongly depend on the proteolytic enzyme and the hydrolysis conditions. These conditions should be carefully selected for applicability in food formulation.

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Thermal treatment is a physical procedure frequently used in food industry modifying protein functionality (Boye, Ma, & Halwarkar, 1997). Depending on the thermal stability of proteins and the heating conditions, proteins may be either partially or completely denatured and sometimes may aggregate changing their functional behavior (Petruccelli & Añón 1995; Plumb, Mills, Tatton, D'Ursel, Lambert, & Morgan, 1994).

The aim of this work was to improve the solubility of an amaranth protein isolate. Consequently, the isolate was modified by proteolytic treatments with two plant proteases (cucurbita and papain) and proteolysis was stopped either by freezing or by thermal treatment. The solubility and structural characteristics of all the modified isolates were analyzed to establish their structure–function relationship.

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 10XX mesh. The resulting flour was defatted with hexane for 24 h (10% w/v suspension) under continuous stirring. After drying at room temperature, the flour was stored at 4 °C until used. The protein content [18.69 ± 0.03% (w/w)] was determined by Kjeldahl (AOAC, 1984), factor 5.85 (Becker et al., 1981; Segura Nieto et al., 1994).

2.3. Preparation of protein isolates

The amaranth isolate (AI) used in this study was prepared according to Martínez and Añón (1996). Briefly, flour was suspended in water (10% w/v) and pH adjusted to 9 with 2 N NaOH. The suspension was stirred for 30 min at room temperature and then centrifuged 20 min at 9000 ***g***. The supernatant was adjusted to pH 5 with 2 N HCl and then centrifuged at 9000 ***g*** for 20 min at 4 °C. The pellet was resuspended in water, neutralized with 0.1 N NaOH and freeze-dried. This isolate was resuspended in buffer A (32.5 mM K_2HPO_4–2.6 m M KH_2PO_4, pH 7.5, 0.4 M NaCl) or in buffer B (33.3 m M K_2HPO_4–1.7 mM KH_2PO_4, pH 8.5) for further analysis. Buffer A is the best solvent for 11S globulins and albumins but not for globulin-P, this fraction is soluble in buffer B.

Hydrolyzed isolates were prepared as follows: 3% w/v AI suspensions were prepared in buffer B and then hydrolyzed either with cucurbita (Curotto, González, O'Reilly, & Tapia, 1989) or papain (Sigma Chem. Co., St. Louis, MO) for 16 h at 40 °C. The enzyme/protein mass ratio in both reaction mixtures was 1:300 corresponding to 0.032 AU/ml (AU: Anson units) for cucurbita and 0.019 AU/ml for papain. The enzyme concentration was chosen to reach the highest velocity with the established isolate concentration. The condition of hydrolysis (16 h at 40 °C) ensured the maximum degree of hydrolysis. For each sample the reaction was stopped in three different ways: in one aliquot the reaction was stopped with trichloroacetic acid (TCA, 19% w/v) to determine the degree of hydrolysis (DH), in the second aliquot the reaction was stopped by placing test tubes in an ice-bath for 5 min, frozen to −20 °C and lyophilized. The lyophilized aliquots were labeled: frozen papain isolate (FP) and frozen cucurbita isolate (FC) for the mixtures with papain and cucurbita, respectively. In the third aliquot the reaction was stopped by heating the samples for 10 min at 90 °C and then lyophilized. This heating treatment caused the enzyme inactivation, whose denaturation temperatures were Td = 70 °C for cucurbita and Td = 89 °C for papain (Molina Ortiz, 1997). The aliquots were labeled: heated papain isolate (HP) and heated cucurbita isolate (HC). The chance of a residual activity of papain and cucurbita in the hydrolyzed isolates was investigated. Results showed no further proteolysis beyond 15 h of incubation at 40 °C in buffer B.

Three more isolates were prepared as controls. The heated amaranth isolate (HAI) was prepared by heating for 10 min at 90 °C a suspension of AI in buffer B and then lyophilized. To obtain the control heated isolate (HI) and the control frozen isolate (FI), a suspension of AI was prepared in buffer B. After an incubation of 16 h at 40 °C, the mixture was divided in three aliquots, one was used to determine the degree of hydrolysis, another was treated for 10 min at 90 °C (HI) and the last was frozen (FI). HI and FI were then lyophilized. It is important to point out that the frozen isolate FI and the heated isolate HI displayed a low degree of hydrolysis which may be ascribed to a residual activity of an endogenous protease.

2.4. Protein determination

Protein content of isolates was determined by Kjeldahl (AOAC, 1984), factor 5.85. To determine solubility and degree of hydrolysis, protein content was determined by Lowry (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin (BSA) as standard protein. At least, two replicates were done for all determinations.

In order to compare the results obtained by Kjeldahl and Lowry, soluble protein content in the isolates was determined in several samples by both methods. The

ratio mg protein Kjeldahl/mg protein Lowry showed a low correlation between the two methods (0.84). On the basis that Lowry values may be overestimated due to the different composition between the amaranth and standard proteins and since the Kjeldahl method also considers non-protein nitrogen, protein content was expressed according to the values obtained by Kjeldahl.

2.5. Degree of hydrolysis

The degree of hydrolysis was expressed as TCA soluble protein according to Dulley (1976). Hydrolysis reactions were stopped with trichloroacetic acid (19% w/v) and then the amples were centrifuged at room temperature for 15 min at 1150 ***g***. The supernatant was neutralized with $NaHCO_{3(s)}$ and TCA soluble protein (S_{TCA}) was determined by Lowry. The data was corrected by the factor 0.84 in order to be expressed as values obtained by Kjeldahl. S_{TCA} was also determined for AI before the incubation ($S_{TCA}AI$). The protein content in the reaction mixture (total protein) was determined by Kjeldahl. DH% was calculated according to the following equation:

$$\%DH = 100 \times (S_{TCA} - S_{TCA}AI)/\text{total protein} \quad (1)$$

2.6. Chromatography

Isolates in buffer B and in buffer A were analyzed by gel filtration chromatography at room temperature. A Superose 6B HR 10/30 column was employed using a Pharmacia LKB, FPLC System (Uppsala, Sweden). Samples (4 mg of protein) were eluted with the same buffer in which they were prepared. The optical density at 280 nm was recorded. The column was calibrated with blue dextran (V_0), α-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), cytochrome C (12.4 kDa) and aprotinin (6.5 kDa). The calibration curve obtained from duplicate measurements was:

$$\ln MM = 17.11 - 0.52 \times Ve \quad (r = 0.97) \quad (2)$$

where *Ve* is the elution volume in ml and MM is the molecular mass in kDa. Curves were processed and data evaluated using Pharmacia AB, FPLC director and FPLC assistant software.

2.7. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the procedure previously described (Martínez et al., 1997). Runs were carried out with 5–15% (w/v) acrylamide gels and the following continuous buffer system: 0.375 M Tris–HCl, pH 8.8, 0.1% (w/v) SDS for the separating gel; 0.025 M Tris–HCl, 0.192 M glycine and 0.1% (w/v) SDS, pH 8.3 for the running buffer and 0.125 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 1% (w/v) SDS, and 0.05% (w/v) bromophenol blue as sample buffer. For runs in reducing conditions the sample buffer contained 5% 2-mercaptoethanol (2-ME) and samples were heated for 1 min in a boiling-water bath. Samples of 40–50 μg of protein were loaded. 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 Coomasie Brilliant Blue Stain or Silver Stain as indicated in the figures. Gels images were acquired with the Gel Doc 1000 Image Analysis System (Bio-Rad, Richmond, CA, USA) and analyzed with the Molecular Analyst Software (Bio-Rad Richmond, CA, USA).

2.8. Solubility

Solubility of isolates and hydrolysates was analyzed preparing 1% w/v suspensions in water, buffer A or buffer B. Samples were incubated 1 h at room temperature vortexing every 15 min. Samples were then centrifuged at 10 000 ***g*** for 10 min at room temperature. Protein content in the supernatant (s_p) was determined by Lowry and solubility was expressed as follows:

$$\%\boldsymbol{S} = (0.84 \times 100 \times mg\ s_p)/\text{mg total protein} \quad (3)$$

Kjeldahl method was not suitable to determine protein content in solubility assays due to the small size of the samples. Determinations were performed in duplicate. 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. FPLC analyses of isolates dissolved in buffer B

The amaranth isolate AI containing 80.0±0.5% (w/w) of protein was composed by albumins,11S globulin and globulin-P as major fractions (Martínez & Añón, 1996). As already informed globulin-P is a legumine-like protein composed by unitary molecules similar to the 11S hexamers and large polymers. The protein composition was confirmed by FPLC (Fig. 1, AI). The profile showed the presence of globulin-P polymers (component I, with MM ranging from 500 to 30 000 kDa), globulin-P and 11S-globulin molecules of 280±7 kDa (component II), which were described elsewhere (Martínez

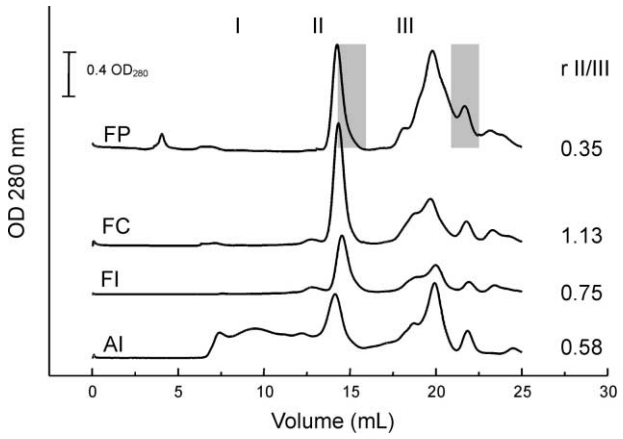

Fig. 1. Gel filtration FPLC of isolates in buffer B. Amaranth isolate, **AI**; incubation control isolate, **FI**; cucurbita hydrolysate, **FC** and papain hydrolysate, **FP**. Shadowed zone corresponds to 0.5 ml fractions from 14 to 16 ml and from 20 to 22 ml. r II/III: peak II area/peak III area ratio. Components eluting between 6 and 13 ml: **I**; component with elution volume 14.2 ml: **II**; components eluting from 15 to 25 ml: **III**.

et al., 1997) and species of MM < 100 kDa (component III, mainly composed by albumins). The isolate, submitted to enzymatic hydrolysis and a thermal treatment, resulted in the four hydrolyzed isolates: frozen papain isolate (FP), heated papain isolate HP, frozen cucurbita isolate FC and the heated cucurbita isolate (HC) as depicted in Table 1. The higher degree of hydrolysis was achieved with papain even though the activity of papain was lower than that of cucurbita (Materials and Methods). This result could be ascribed to a difference in the enzymatic specificity. The amaranth isolate seemed to be a better substrate for papain than for cucurbita. Previous results working with soybean as substrate showed that papain yielded higher degree of hydrolysis than cucurbita (Molina Ortiz, 1997), suggesting some kind of structural similarity between the main target proteins.

Globulin-P polymers (component I) were absent in the frozen papain isolate (FP) and the frozen cucurbita isolate (FC) dissolved in buffer B (Fig. 1), suggesting that the enzymes may have cut some polypeptides involved in globulin polymerization. It was already shown that papain incubated with partially purified globulin-P cleaves the M polypeptide inducing the polymers disruption (Castellani, Martínez, & Añón, 2000). However, component I was not present in the protein profile of the control frozen isolate (FI) which was not incubated with enzyme, indicating that another factor than cucurbita or papain proteolysis may be participating in the rupture of the polymer. To check this speculation, a partially purified globulin-P fraction was incubated for 12 h at 40 °C. Since globulin-P polymers did not disappear after the treatment (data not shown) we concluded that a factor present in the isolate and absent in the globulin-P fraction would have broken the polymers during the incubation.

The processing of FPLC curves of the control frozen isolate (FI) and the frozen cucurbita isolate (FC) showed that the ratio of component II to component III (monomers/small molecules: r II/III) were 0.75 and 1.13, respectively (Fig. 1), and that they were higher than in the amaranth isolate (AI) profile (0.58, Fig. 1) indicating that polymers may have been converted to unitary molecules. On the other hand, in the frozen papain isolate (FP), the amount of small molecules (component III) was larger than component II (r II/III 0.35), suggesting that most of the molecules may have been disassembled.

FPLC profiles of the isolates treated 10 min at 90 °C are shown in Fig. 2. In the control heated amaranth isolate, component III predominated over component II (r II/III = 0.24) and component I was absent, suggesting that the treatment provoked the insolubilization and/or dissociation of both polymers and some globulins. On the basis that the denaturation temperature of amaranth globulins is 94 °C (Marcone, Kakuda, & Yada, 1998; Martínez & Añón, 1996), it could be inferred that a partial denaturation may be occurring after 10 min at 90 °C. The control heated isolate contained component III together with three peaks of low elution volumes. These peaks may be formed by soluble aggregates (peaks 1 and 2) and globulin molecules in a more open conformation (peak 3) as a result of the incubation followed by the thermal treatment. The heated cucurbita isolate (HC) lacked peaks 1 and 2 whereas in the heated papain isolate profile peaks 1, 2 and 3 were not present. These results may indicate that when isolates are hydrolyzed the effect of the heat treatment on the proteins is mainly dissociation rather than aggregation.

3.2. Electrophoretic analyses of isolates dissolved in buffer B

Fig. 3 depicts the SDS-PAGE patterns of all isolates. As previously reported (Martínez & Añón, 1996), in non-reducing conditions (Fig. 3a), the AI contains P aggregates, ascribed to globulin-P polymers, dimeric subunits (D) integrating 11S globulin and globulin-P molecules, and several polypeptides of molecular masses lower than 40 kDa (S in Fig. 3a) most of them coming from albumin. Monomeric polypeptides of 56 kDa (M), of approximately 30 kDa (A) and of near 20 kDa (B) are present in the amaranth isolate (AI) in reducing conditions (Fig. 3b). They come from dissociation of P-aggregates and dimeric polypeptides D (Martínez et al., 1997). Monomeric polypeptides from albumin are also shown in this profile.

Fig. 3a shows that in the control frozen isolate and in the frozen cucurbita isolate profiles there is a decrease in the intensity of some bands of P-aggregates and the

Table 1
Amaranth isolates, treatments and degree of hydrolysis

Isolate	AI	HAI	FI	HI	FP	FC	HP	HC
Protease	–	–	–	–	Papain	Cucurbita	Papain	Cucurbita
10 min 90 °C	–	+	–	+	–	–	+	+
DH[a]	0	0	0.60 (0.01)[b]	0.60 (0.01)[b]	8.2 (0.2)[b]	3.3 (0.1)[b]	8.2 (0.2)[b]	3.3 (0.1)[b]

AI, amaranth isolates; HAI, amaranth heated isolate; FI, incubation control isolate; HI, heated control isolate; FP, papain hydrolysate; FC, cucurbita hydrolysate; HP, heated papain hydrolysate; HC, heated cucurbita hydrolysate.

[a] Degree of hydrolysis.

[b] Numbers in parenthesis are standard deviations.

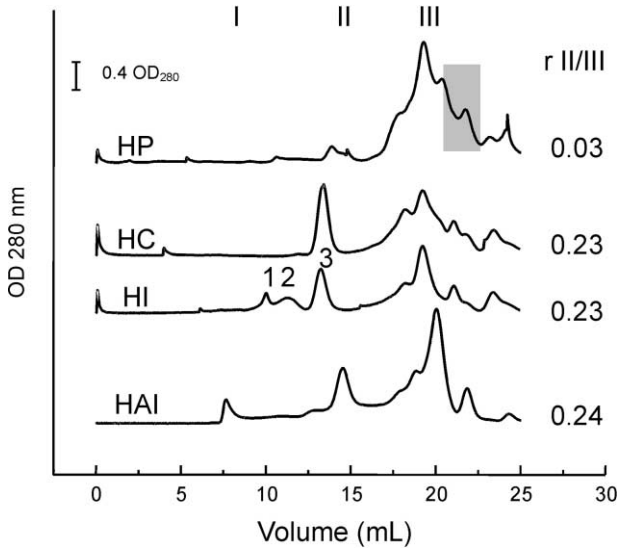

Fig. 2. Gel filtration FPLC of isolates in buffer B. Amaranth heated isolate, **HAI**; heated control isolate, **HI**; heated cucurbita hydrolysate, **HC**; heated papain hydrolysate, **HP**. Shadowed zone corresponds to 0.5 ml fractions from 20 to 22 ml. r II/III: peak II area/ peak III area ratio. Components eluting between 6 and 13 ml: **I**; component with elution volume 14.2 ml: **II**; components eluting from 15 to 25 ml: **III**.

presence of new bands corresponding to polypeptides of low molecular mass. The pattern of the frozen papain isolate shows a higher decrease in the intensity of P-aggregates whereas in the lower molecular weight zone, many bands appeared while others decreased or even disappear. The absence of polymers may be the origin of the decrease of P-aggregates in the hydrolyzed isolates.

A densitometric analysis of the reducing SDS-PAGE profiles of Fig. 3b showed that the 56 kDa polypeptide M was 25% less intense in the frozen isolate than in the amaranth isolate. On the other hand, this polypeptide was absent in the frozen cucurbita isolate and in the frozen papain isolate. In this isolate, bands corresponding to the A polypeptides were less intense than in the amaranth isolate. Besides, new bands appeared in the low molecular mass zone. This result confirms that M polypeptide is one of the targets for papain and shows that it is also the target for cucurbita and probably for another seed protease.

The SDS-PAGE patterns of the heat-treated isolates were similar to the corresponding non-heated isolates (Fig. 3a and b), although the heated isolates profiles

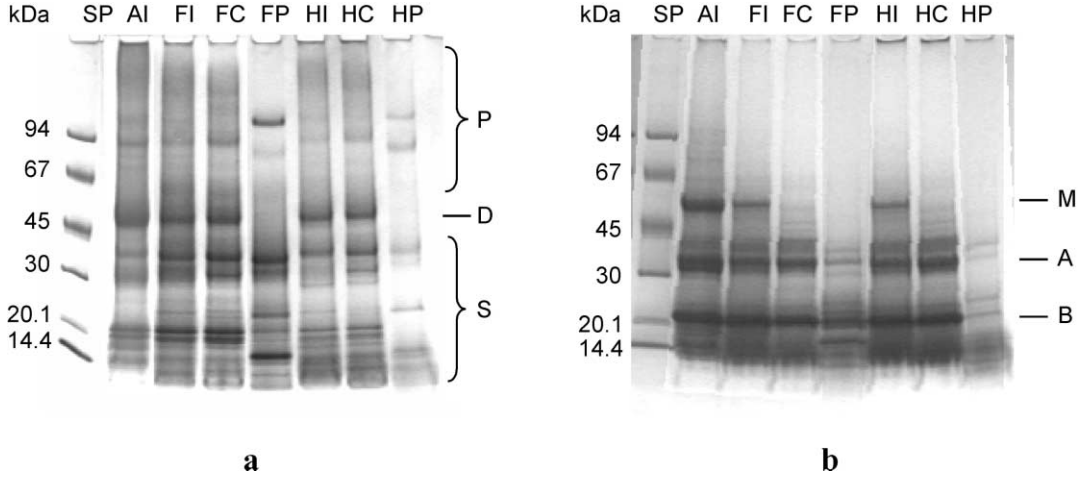

Fig. 3. SDS-PAGE of isolates in buffer B. Standard molecular mass proteins, **SP**; amaranth isolate, **AI**; incubation control isolate, **FI**; cucurbita hydrolysate, **FC**; papain hydrolysate, **FP**; heated control isolate, **HI**; heated cucurbita hydrolysate, **HC**; heated papain hydrolysate, **HP**. (a) Non-reducing condition. Aggregated polypeptides: P; globulin dimeric subunits: D; polypeptides of molecular masses lower than 45 kDa: S. (b) Reducing conditions, samples +2-ME. Monomeric polypeptide of 56 kDa: M; globulin dissociated polypeptides: A and B. Gels were stained with Coomasie Blue.

presented a slight increase in the intensity of the faster bands.

The polypeptide composition of component II (globulin 280 kDa molecules) and component III (small molecules) of the frozen papain isolate (FP) (Fig. 1, shadowed fractions) was analyzed by SDS-PAGE in non-reducing conditions (Fig. 4a). The patterns of component II contained intense bands ascribed to P-aggregates as well as other bands of low molecular mass (<45 kDa). Fig. 4b shows SDS-PAGE patterns of the small molecules (component III) of the heated papain isolate HP. They contain the same P-polypeptides bands as those present in the globulin molecules (component II) of the frozen papain isolate (Fig. 4a). The presence of the same P polypeptides bands in the frozen papain isolate component II (Fig. 4a) and in heated papain isolate component III (Fig. 4b) and its absence in the frozen papain isolate component III indicated that the frozen papain isolate component II (globulin molecules) have turned into component III after heating suggesting a dissociation of globulin molecules hydrolyzed by papain. These results agree with those obtained by Achouri, Zhang, and Shiying (1998) working with a soy isolate.

Patterns obtained by FPLC and electrophoresis indicate that papain produced stronger modifications to the proteins of the isolate. The main differences between hydrolyzed isolates (FP and the frozen cucurbita isolate FC) and the non-hydrolyzed amaranth isolate come from the modifications of globulins. Albumins may have also been hydrolyzed turning into molecules of smaller size. Since their molecular masses were similar to the hydrolysis products obtained from globulins (Segura-Nieto et al., 1994), it was not possible to differentiate between these two hydrolysis products.

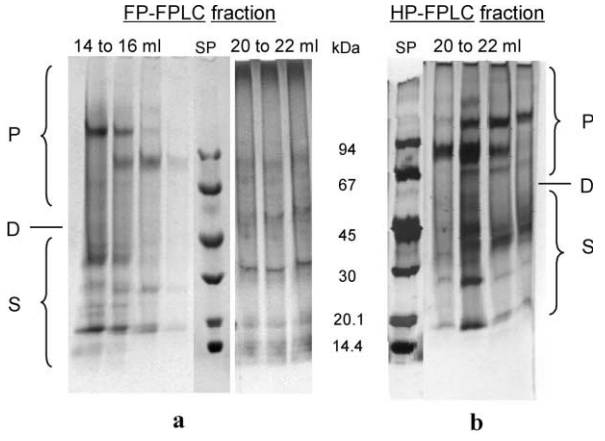

Fig. 4. SDS-PAGE of FPLC fractions. Standard molecular mass proteins, **SP**. (a) Fractions from 14 to 16 ml and 20 to 22 ml of the frozen papain isolate (FP) in buffer B. (b) Fractions from 20 to 22 ml of heated papain isolate (**HP**) in buffer B. Gels were stained with Silver Stain.

3.3. FPLC analyses of isolates dissolved in buffer A

Isolates in buffer A (a solvent of higher ionic strength) were also analyzed and their FPLC profiles are shown in Fig. 5. The absence of polymers in the amaranth isolate profile may be a consequence of their insolubilization due to the low solubility of globulin-P in a medium of high ionic strength (Castellani et al., 1998; Martínez, 1997). The profile of the amaranth isolate in buffer A also showed a slight decrease in component II compared with amaranth isolate in buffer B reflected by the lower value of r (Figs. 1a and 5a). As regards the other isolates, their profiles in buffer A were similar to those obtained in buffer B excepting for the heated isolate. The heated isolate profile in buffer A did not show the two low elution volume peaks ascribed to aggregates, suggesting that these aggregates were insolubilized due to the high ionic strength. SDS-PAGE patterns of the isolates in buffer A were similar to the corresponding in buffer B differing only in the amount of bands of low mobility (data not shown).

Results obtained with the isolates in buffer A are related with their solubility in this solvent, therefore these data will be analyzed together with solubility data.

3.4. Solubility

As shown in Table 2, amaranth isolate had a low solubility in water. It was 1.6 times more soluble in buffer A and even more soluble in buffer B. This may be explained by the presence in the isolate of an important amount of globulins (11S-globulin and globulin-P) which are insoluble in water (Konishi, Fumita, Ikeda, Okuno, & Fuwa, 1985; Martínez, 1997). The 11S-globulin is more soluble in buffer A than in water (Segura-Nieto et al., 1994) while globulin-P increase its solubility in alkaline medium (buffer B, Castellani et al., 1998). The control frozen isolate was more soluble in water and in buffer A than the amaranth isolate. Both hydrolyzed isolates, the frozen cucurbita isolate and the frozen papain isolate, have also a higher solubility in buffer A than the amaranth isolate and a higher solubility in water than the amaranth isolate and the control frozen isolate ($P \leqslant 0.05$, Table 2). All the earlier isolates did not show significant differences in their solubility in buffer B ($P \leqslant 0.05$). These results may be explained on the basis that globulin-P polymers are insoluble in water and buffer A and after hydrolysis they turned into smaller and consequently more soluble molecules. On the other hand, polymers were soluble in buffer B so their change in size after hydrolysis may not produce an increase in the solubility in that solvent.

After the thermal treatment, the amaranth isolate became less soluble in buffer A and buffer B ($P \leqslant 0.05$, Table 2) indicating that some insoluble aggregates were formed. FPLC of heated isolates reflect the absence of

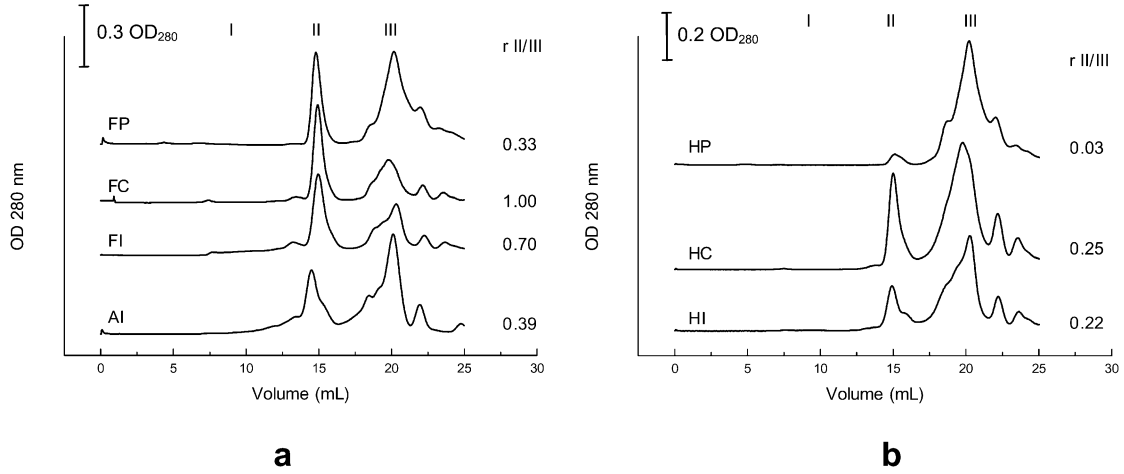

Fig. 5. Gel filtration FPLC of isolates in buffer A. (a) Amaranth isolate, **AI**; control isolate: **FI**; cucurbita hydrolysate: **FC**; papain hydrolysate **FP.** (b) Heated control isolate: **HI**; heated cucurbita hydrolysate: **HC**; heated papain hydrolysate, **HP**. r II/III: peak II area/ peak III area ratio. Components eluting between 6 and 13 ml: **I**; component with elution volume 14.2 ml: **II**; components eluting from 15 to 25 ml: **III**.

Table 2
Solubility of isolates in different media[a]

Isolate	% Solubility		
	H_2O	Buffer A	Buffer B
AI	38.96 a	61.05	74.77 b,d
FI	56.22 c	68.67 c,d	80.10 b
FC	72.83 b,d	72.55 d	73.57 b,d
FP	78.28 b,d	75.85 b,d	77.16 b,d
HAI	35.08 a	37.68 a	42.61 e,a
HI	36.88 a	43.17 e,a	42.49 e,a
HC	46.22 e	52.48 c	53.33 c
HP	74.29 b,d	75.85 b,d	72.69 b,d

AI, amaranth isolates; HAI, amaranth heated isolate; FI, incubation control isolate; HI, heated control isolate; FP, papain hydrolysate; FC, cucurbita hydrolysate; HP, heated papain hydrolysate; HC, heated cucurbita hydrolysate.

polymers (Figs. 2 and 5b) suggesting that those polymers may have turned into aggregated, insoluble species. The control heated isolate was also less soluble than the frozen isolate in the three solvents ($P \leqslant 0.05$), indicating that together with polymers, other molecules were aggregated and unsolubilized. The thermal treatment not only made polymers insoluble but also globulin molecules became insoluble in both buffers as reflected by the lower II/III ratio in the heated isolate than in the frozen isolate (Figs. 1, 2, 5a and 5b).

The higher solubility of the heated cucurbita isolate over the heated amaranth isolate in the three solvents indicated that cucurbita hydrolysis partially prevented the unsolubilizing effect of the heat treatment. However, the heated cucurbita isolate was less soluble than the amaranth isolate and the frozen cucurbita isolate, implying that the heat treatment still influenced the solubility after proteolysis with cucurbita. Papain hydrolysis was more effective preventing heat insolubilization and therefore the heated papain isolate had the same high solubility as the frozen papain isolate in the three solvents. In this case, the unsolubilizing effect of the thermal treatment on the amaranth isolate was not visible because of the papain action.

4. Conclusion

The results obtained in this study show that among amaranth isolate proteins, globulins (11S-globulin and globulin-P) are main targets for cucurbita and papain. Their behavior was similar to the soybean isolate glycinin (Molina Ortiz, 1997). Apparently, a residual proteolytic activity was present in the amaranth isolate that may have eliminated globulin-P polymer after an overnight incubation at 40 °C. However, after the incubation and lyophilization, no proteolytic activity remained in the isolate. From structure and solubility data, it can be concluded that polymers and globulin molecules are the main proteins responsible for the isolate insolubility and that their hydrolytic modification increased the solubility of the isolate. Solubility has a significant influence on the other functional properties of proteins, consequently the increment in solubility is of primary importance.

After the thermal treatment, the solubility of the amaranth isolate decreased as a consequence of protein (mainly globulin) aggregation, whereas the hydrolysis with papain before the heating treatment allowed to obtain an isolate of higher solubility than the original. Therefore, the amaranth isolate obtained after papain hydrolysis could be used in foodstuffs which are to be submitted to thermal treatment on the basis that its proteins maintain a high degree of solubility after heating.

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