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Amaranth Globulin Polypeptide Heterogeneity

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The polypeptides integrating amaranth globulin-p and 11S-globulin were characterized by two-dimensional electrophoresis, ion-exchange chromatography and RP-HPLC. All polypeptides exhibited charge and hydrophobic heterogeneity. Almost all acid (A, pI 5–7) and basic (B, pI 9–10) polypeptides were present in both globulins, and the same happened with the unprocessed M polypeptides with pI in the range of 7–7.5 which fits well with a sequence containing both the A and B polypeptides. There were other polypeptides only present in 11S-globulin, like some of 41 and 16 kDa, which might come from another precursor or be the products of a different processing of the propolypeptide. These results suggested that, although amaranth subunits from different subfamilies are interchangeable in different oligomers, some structural differences between them might affect the assembly of globulin molecules. Structural differences arising from this behavior could account for the different physicochemical properties of globulin molecules.

KEY WORDS: Amaranth; globulins; polypeptide; heterogeneity.

1. INTRODUCTION

Amaranth proteins are a promising dietary resource due to their well-balanced amino-acid composition. The most abundant proteins of amaranth are storage proteins with legumin-like features. They comprise globulins (11S-globulin, globulin-p) and glutelins, which present different solubility in aqueous solvents. The 11S-globulin is extracted by treating the flour with a saline, neutral buffer (μ = 0.5 *M*) whereas globulin-p (formerly named albumin-2 by Konishi *et al*., 1991) is extracted with water after the saline treatment of the flour. The sequential order of globulin-p extraction is probably due to its location in protein bodies (Konishi *et al*., 1991). On the other hand, glutelin, a storage-aggregated protein, is soluble only in alkaline or acid media (Segura-Nieto *et al*., 1994). Besides the differential solubility, globulin-p molecules show a marked tendency to polymerize (Martínez *et al*., 1997). Glutelins, globulin-p and 11S-globulin may be considered three types of globulins in accordance with the storage protein classification on the basis of their molecular and genetic characteristics, as presented by Fukuyima (1991).

Legumins, or 11–13S globulins, are a major class of seed storage proteins, widely distributed among angiosperms and gymnosperms. They are generally hexameric, and the monomers are composed of a large acidic A-polypeptide and a small basic B-polypeptide. The two polypeptides are derived from a single pre-protein that is co- and post-translationally cleaved to yield a characteristic, disulphide-linked A–B structure. Many

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Abbreviations: DTT, dithiothreitol; IEF, isoelectric focusing; RP-HPLC, reverse phase-high performance liquid chromatography; CHAPS, (3-[(3-Cholamidopropyl)-dimethyl-ammonio]-2-hydroxy-1-propane sulfonate; TFA, trifluoroacetic acid; 2-ME, 2-mercaptoethanol; G11, 11S-globulin molecules; AM, globulin-p aggregates; UM, globulin-p molecules

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well-characterized legumins, like soybean glycinin, sunflower helianthinin, and pea and broad bean legumins, exhibit molecular heterogeneity due to genetic polymorphism (Bacon et al., 1987; Kitamura *et al.*, 1980; Raymond *et al.*, 1995; Utsumi *et al.*, 1981, 1987). Besides the heterogeneity found in a single cultivar, the subunit composition also varies among different soybean cultivars (Mori *et al.*, 1981; Zhang *et al.*, 2002).

Amaranth legumin-like proteins also exhibit heterogeneity in their polypeptide composition, and variation among cultivars (Drzewiecki, 2001; Marcone and Yada, 1998). Three groups of polypeptides have been defined according to their molecular weight: a group in the range of 50–60 kDa, and the A and B polypeptides in the ranges of 31–45 kDa and 16–27 kDa, respectively (Abugoch *et al.*, 2003; Chen and Paredes-López, 1997; Martínez *et al.*, 1997; Romero-Zepeda and Paredes-López, 1996; Segura-Nieto *et al.*, 1994; Vasco-Méndez and Paredes-López, 1995). The polypeptides in the first group (50–60 kDa) are more abundant in globulin-p and glutelin than in 11S-globulin (Abugoch *et al.*, 2003; Martínez *et al.*, 1997; Romero-Zepeda *et al.*, 1996) and are considered to represent unprocessed polypeptides (Barba de la Rosa *et al.*, 1996; Castellani *et al.*, 2000).

Charge heterogeneity among amaranth polypeptides having the same molecular weight was also demonstrated by two-dimensional electrophoresis of non-purified globulins and glutelins, and these two protein fractions displayed different electrophoretic patterns (Segura-Nieto *et al.*, 1994). In those studies globulin-p was not analyzed as an independent fraction, so it was probably included in the glutelin fraction. Six glutelin polypeptides of 32 kDa with pI ranging from 5.7 to 6.3 have been described (Vasco-Méndez and Paredes-López, 1995) which were recognized by an antibody raised against an acidic subfraction of amaranth globulin.

Beyond these findings, a more detailed knowledge of the molecular characteristics of the polypeptides from amaranth legumin-like proteins is not yet available. The purpose of this work was to obtain more information about the size, charge and hydrophobic properties of purified globulin-p and 11S-globulin and to correlate the findings with the physicochemical properties of the oligomeric molecules.

2. MATERIALS AND METHODS

2.1. Plant Material

Seeds from *Amaranthus hypochondriacus* (Mercado variety) were harvested at the Estación Experimental del Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, México, and kindly provided to our laboratory in Argentina.

Flour was obtained by grinding whole seeds in an Udy mill (Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina) with a 1-mm mesh, and was screened using a 0.092 mm mesh. Flour was suspended in hexane 10% (w/v), defatted for 24 h under continuous stirring, air-dried at room temperature, and stored at 4°C until used. The protein content of the flour (17.0% (w/w) on a dry weight basis) was determined by the micro-Kjeldhal method. Finally, ammonia was quantified with the colorimetric method of Nkonge and Ballance (Nkonge and Ballance, 1982). The protein/nitrogen coefficient used was 5.85 (Martínez *et al.*, 1997).

2.2. Extraction of Proteins

Globulins and globulin-p were extracted using a modified Osborne method (Aphalo *et al.*, 2004). The extraction procedure was conducted at room temperature with a meal/solution ratio (p/v) of 1:10. Flour was treated twice with water to extract albumin and then twice with 32.5 m*M* K_2HPO_4, 2.6 m*M* KH_2PO_4, 0.4 *M* NaCl, pH 7.5 (buffer A) to extract 11S-globulin. Globulin-p was extracted by treating the residue twice with water. After each treatment, the extracted residue was separated by centrifugation at $9000\times g$ for 20 min at room temperature. The supernatants containing 11S-globulin and globulin-p (either as soluble or dispersed protein) were each adjusted to pH 6 using 2 N HCl. The resulting precipitates were suspended in water, neutralized with 0.1 N NaOH, and freeze-dried.

2.3. Protein Purification

The 11S-globulin, and the components of globulin-p: molecules of 280 kDa (unitary molecules, UM) and their aggregates of molecular mass > 500 kDa

(aggregated molecules, AM) were prepared by gel filtration using a Sephacryl S-300 HR column (2.5×90 cm) with a fractionation range of 10,000–1,500,000 as previously described by Marcone and Yada (1991) with slight modifications. The column was packed and equilibrated with three bed volumes of buffer B (33.3 m*M* K_2HPO_4, 1.7 m*M* KH_2PO_4, pH 8.5) containing 0.02% (w/v) NaN_3.

A 4-ml sample containing 50 mg of protein was layered onto the gel filtration column. Protein fractions were eluted from the column at room temperature (22°C) at a flow rate of 0.58 ml/min obtained with a Gilson Minipuls 2 peristaltic pump. Fractions (3 ml) were collected with a Buchler (Fractomet e Alpha 400) fraction collector, the absorbance at 280 nm was measured with a UV detector (Econo UV, Bio-Rad, Richmond, Calif., USA), and the protein elution profiles were plotted using a Gilson N2 recorder. Fractions corresponding to 11S-globulin molecules, globulin-p fractions – (UM or AM) – were each pooled and concentrated with an Amicon 8020 (Amicon Inc.) ultrafiltration cell, using a 10,000 Da pore membrane (Amicon, YM10). The resulting fractions were stored at −80°C.

Before bidimensional electrophoresis analysis purified 11S-globulin and globulin-p AM were dissolved in water by exchanging the buffer in the Amicon 8020 ultra filtration cell, and were lyophilized.

Samples for RP-HPLC runs were prepared dissolving purified 11S-globulin or globulin-p AM in 6 N guanidine–HCl, 0.2 *M* trizma pH = 9 and 30 m*M* DTT. Solutions were incubated at 37°C for 1 h and clarified by centrifugation.

2.4. Alkylation of Sulphydryl Groups

Purified globulin-p AM or 11S-globulin were dissolved (20 mg/ml) in 10 m*M* buffer Na_2HPO_4 pH 8.5, and 8 *M* urea, 20 m*M* DTT, was incubated for 60 min at 37°C. Iodoacetamide was added to a final concentration of 40 m*M* and alkylation allowed to proceed for 60 min at 37°C in the darkness.

2.5. High Performance Liquid Chromatography (HPLC)

The runs were performed in a Waters 600 E HPLC Multisolvent Delivery System (pump and controller) equipped with a Water 717 Plus Autosampler and a Waters 996 Photodiode Array Detector (Waters Corporation, Milford, MA, USA).

2.6. Ion Exchange HPLC

It was performed in a Waters AP-Minicolumn 5×100 nm 1000 A 8 μm pre-equilibrated with solvent (A) (80% 10 m*M* Na_2HPO_4 pH = 8.5, 20% acetonitrile). Fifty microliters of reduced/aminoalkylated protein solution were injected and eluted at room temperature at a flow rate of 0.5 ml/min with a linear gradient of solvent B (80% 10 m*M* Na_2HPO_4 pH = 8.5, 1 *M* NaCl, 20% acetonitrile) with holds at 33% of B and 100% of B. Column effluent was monitored at 210 and 280 nm, and peak fractions were collected manually.

Fractions collected from two runs were lyophilized.

2.7. Reverse Phase Chromatography (RP-HPLC)

The runs were carried out in a Sephasil Peptide C8, 5 μm ST 4.6/250 analytical column (Pharmacia Biotech, Upsala, Sweeden) equilibrated with solvent (A) (98% water, 2% acetonitrile, 0.065% (v/v) TFA in water). Fifty microliters of dissociated/reduced protein solutions were injected and eluted at a flow rate of 1 ml/min. After 3 min of elution with solvent A, the column was subjected to an acetonitrile gradient from 100% solvent A at 0 min to 100% solvent B (35% water, 65% acetonitrile/0.065% (v/v) TFA in water) at 55 min. Column effluent was monitored at 210 and 280 nm, and peak fractions were collected manually.

Fractions collected from two runs were lyophilized.

2.8. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Fractions coming from ion-exchange HPLC and RP-HPLC were analyzed by SDS-PAGE. Running gels (12% w/v polyacrylamide) and stacking gel (4% w/v polyacrylamide) were arranged in minislabs (BioRad Mini Protean II Model). Runs were carried out according to the Laemmli method (Laemmli, 1970), as modified by Petruccelli and Añón (1995). Molecular masses of polypeptides were calculated using the following protein standards (Pharmacia Biotech): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), R-lactalbumin (14.4 kDa). At least three analyses were performed for each sample to

obtain the mean value and standard deviation of each molecular mass. The comparison of mean values of molecular masses of globulin-p and 11S-globulin corresponding polypeptides, was done by the least significant difference (LSD) test at a significance level (R) of 0.05.

Fractions coming from RP-HPLC and ion-exchange HPLC were dissolved in sample buffer (0.125 M Tris–HCl, pH 6.8, 20% (v/v) glycerol, 1% (w/v) SDS, and 0.05% (w/v) bromophenol blue). For reducing conditions, 5% (v/v) 2-ME was added and samples were heated (100°C, 3 min). Gels were fixed and stained with Coomassie Brilliant Blue Stain.

2.9. Two-dimensional IEF-SDS-PAGE

The first-dimension IEF was performed using 18-cm linear IPG strips (pH 3–10) in the IPGphor system (GE Healthcare UK Limited, England). All IPG strips were rehydrated with 340 μl of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, 0.5% IPG and 0.002% bromophenol blue) containing 100 μg of purified protein. The voltage settings for IEF were 500 V for 1 h, 1000 V for 1 h, and 8000 V for 4 h until reaching a final condition of 30,000 V/h.

Following IEF, the gel strips were incubated with equilibration buffer (2% SDS, 6 M urea, 50 mM Tris–HCl pH 8.8, 30% glycerol, 30 mM DTT and 0.01% bromophenol blue) for 1 h, followed by equilibration for a further hour with the same solution containing 60 mM iodoacetamide instead of DTT. The strips were cut in two pieces, placed onto two 12% polyacrylamide gel, and run in minislabs (BioRad Mini Protean II Model) with the buffer system described for SDS-PAGE. Strips were overlaid with agarose sealing solution (0.25 M Tris base, 1.92 M glycine, 1% SDS, 0.5% agarose, 0.002% bromophenol blue).

All gels were fixed and stained with Coomassie Brilliant Blue.

The pI ranges of the spots were calculated measuring the distances to the ends of the linear pH gradient.

3. RESULTS AND DISCUSSION

Purified 11S-globulin molecules and globulin-p fractions – aggregates (AM) and 280 kDa molecules (UM) – were analyzed by SDS-PAGE. Results (Fig. 1) showed that these globulins presented some differences in polypeptide composition. In agreement with previous reports (Martínez *et al.*, 1997), globulin-p molecules were composed of a monomeric subunit of 56 ± 1 kDa (named M, Fig. 1b) and two dimeric subunits D_1 (aproximately 60 kDa) and D_2 (aproximately 57 kDa) (Fig. 1a) integrated by polypeptides A (34 ± 1 and 32 ± 1 kDa) and B (22 ± 1 and 20 ± 1 kDa) (Fig. 1b), which were resolved in gels not shown. The only difference between globulin-p aggregates and unitary molecules was that unitary molecules contained a lower proportion of the M subunit (Fig. 1). On the other hand, the 11S-globulin (Fig. 1) contained the polypeptides M, M_2, L and S of 56 ± 1, 52.8 ± 0.6, 41 ± 2 and 15.5 ± 0.6 kDa, respectively, (Fig. 1a and b) and the same D_1 and D_2 subunits as in globulin-p (Fig. 1a). This 11S-globulin composition agrees with the results presented by Romero-Zepeda and Paredes-López (1996) and Marcone *et al.* (1998), and the major difference with globulin-p is the presence of the L (41 ± 2 kDa), S (15.5 ± 0.6 kDa) and M_2 (52.8 ± 0.6 kDa) polypeptides, all of them with molecular masses statistically different from those of globulin-p at a significance level of 0.05.

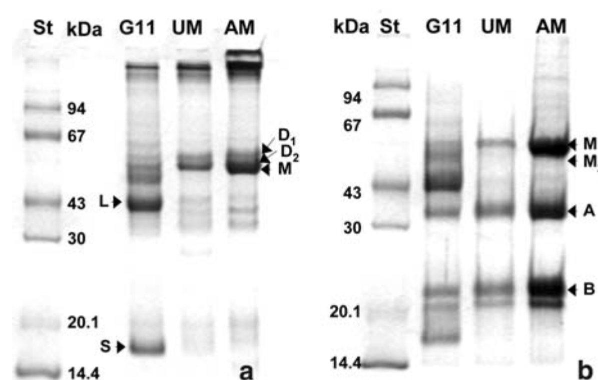

Fig. 1 SDS-PAGE analysis of globulin-p AM and UM, and G11. (a) Non-reducing conditions, (b) reducing conditions. St: Standard proteins; kDa: molecular masses of the standard proteins.

When globulin polypeptides were analyzed by ionic exchange chromatography, globulin-p (Fig. 2a) and 11S-globulin (Fig. 2b) polypeptides of the same molecular weight eluted at the same NaCl concentration. The basic (B) polypeptides of near 20 kDa (Fig. 2a and b, fraction 1 in the chromatograms and electrophoretic profiles) were not

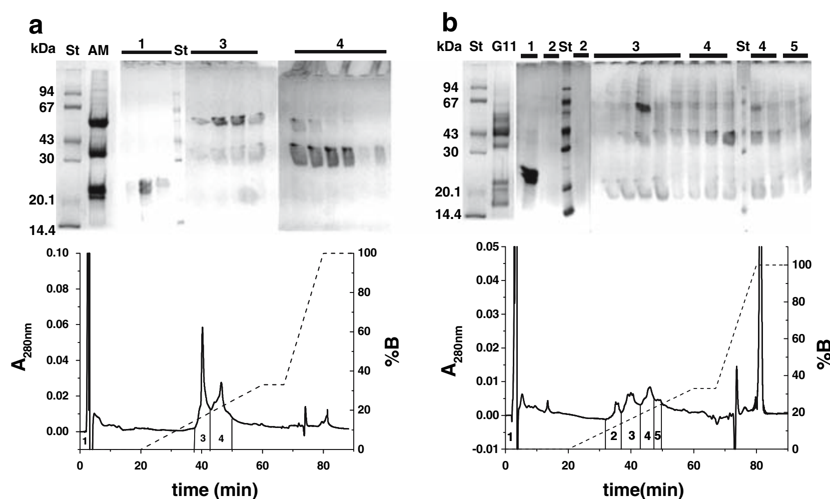

Fig. 2 HPLC ion-exchange profile of reduced/alkylated purified globulins and SDS-PAGE pattern of chromatographic fractions (a) Globulin-p AM, (b) 11S-globulin. St: Standard proteins; kDa: molecular masses of the standard proteins.

retained by the anion exchanger, whereas the M and A polypeptides showed intermediate (Fig. 2a and b, fraction 3) and longer (Fig. 2a and b, fraction 4) retention times, respectively. The 11S-globulin S polypeptides (Fig. 2b) eluted at the same NaCl concentration than M, and the L polypeptide eluted together with A polypeptides (Fig. 2b). This chromatography did not show charge differences among polypeptides of the same MW.

Charge heterogeneity of globulin polypeptides was then investigated by two-dimensional analysis. Globulin-p M polypeptide showed a microheterogeneity with spots in the range of pI 7–7.5 (Fig. 3a). Heterogeneity was most prominent in the A polypeptides which showed four major species with pIs between 5 and 6 (Fig. 3a, spots labeled A_1) and a second one with microheterogeneity in the 6–7 pI range (Fig. 3a, spots labeled A_2). On the other hand, B polypeptides showed pIs from 9 to 10. Two polypeptides with molecular masses similar to B polypeptides exhibited pI values in an acidic range (pH 6–7). Since the pI values of these polypeptides indicate that they are not B polypeptides, we named them A_2' and A_3'. They might integrate a subunit together with another A and B polypeptides, in a way similar to the $A_5A_4B_3$ glycinin subunit (Momma *et al.*, 1985). The two-dimensional pattern of the 11S-globulin (Fig. 3b) was similar to that of globulin-p in that it showed the same A and B polypeptides, but it also included a more acidic 20 kDa polypeptide (A_1' in the figure). The pI range of the M and M_2 polypeptides from the 11S-globulin

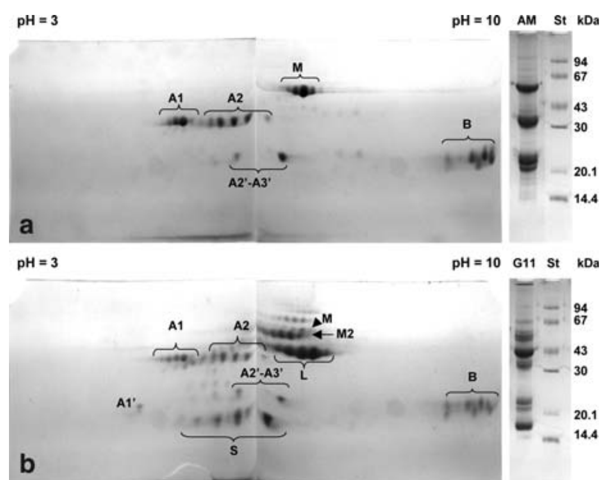

Fig. 3 Bidimensional IEF → SDS-PAGE analysis of (a) globulin-p aggregates (AM) and (b) 11S-globulin (G11). On the right side of each 2D pattern SDS-PAGE of the corresponding samples. St: Standard proteins; kDa: molecular masses of the standard proteins.

(6.5–7) was similar to that of the M polypeptide from globulin-p, and five spots of the L polypeptide with pI between 7 and 7.5 were present. The S polypeptides showed six spots with pI in the 5.5–7 range, whereas, as was observed in globulin-p, the A_2' and A_3' polypeptides were also present.

As shown in the present study, M polypeptides have pIs close to 7, which might include a B sequence corresponding to a pI close to 9 and an A sequence corresponding to a pI close to 5. These findings support the hypothesis that the M

polypeptides are the unprocessed globulin precursors (Valdez-Ortiz *et al.*, 2005). On the other hand the 11S-globulin L polypeptide showed a similar, but more alkaline pI range than the M polypeptides. Considering the presence of both the L polypeptides and the more acidic S polypeptides in the 11S-globulin, it could be interpreted that these polypeptides are the products of the proteolytic cleavage on a precursor different from M. Both the S and L polypeptides might be joined by non-covalent interactions forming 11S-globulin subunits. According to their masses as well as their charges the subunits composed by A and B polypeptides linked by disulfide bridges were present in both globulin-p and 11S globulin.

When globulin-p was reduced and subjected to RP-HPLC separation, the protein was resolved in five peaks (Fig. 4, chromatogram). The SDS-PAGE pattern of the chromatographic fractions (Fig. 4, SDS-PAGE) showed that, according to their hydrophobicity, there were three types of small polypeptides, the most hydrophylic of 23 kDa (peak 1), and those of intermediate and highest hydrophobicity of 20 kDa (peaks 3 and 5). The A polypeptides were also resolved in two groups of different hydrophobicity (peaks 1 and 4) whereas the M polypeptides showed an intermediate behavior (peak 2). When 11S-globulin was subjected to the same analysis (Fig. 5) the results indicated that the two M polypeptides showed the same retention time as in globulin-p (peak 2), though another 11S-globulin M polypeptide eluted later in peak 5. The 11S-globulin A polypeptides eluting in peaks 3 and 3′ presented rather similar behavior than those of globulin-p which eluted in peaks 3 and 4. The hydrophobicity of B polypeptides of 11S-globulin eluting in peaks 3 and 5 was similar to that of the corresponding polypeptides in globulin-p. The 11S-globulin S and L polypeptides presented an intermediate hydrophobicity as they eluted in peaks 3′ and 4, respectively. These analyses added some differences between globulin-p and 11S-globulin polypeptides to those already found regarding their masses and their charge. The main differences between globulin-p and 11S-globulin shown by these chromatographic

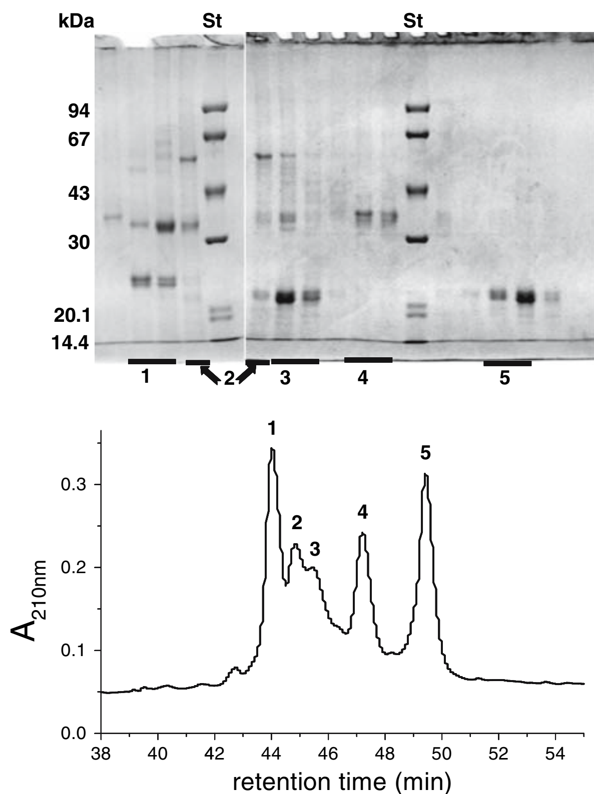

Fig. 4 RP-HPLC profile of dissociated and reduced globulin-p aggregates and SDS-PAGE of peak fractions. St: Standard proteins; kDa: molecular masses of the standard proteins.

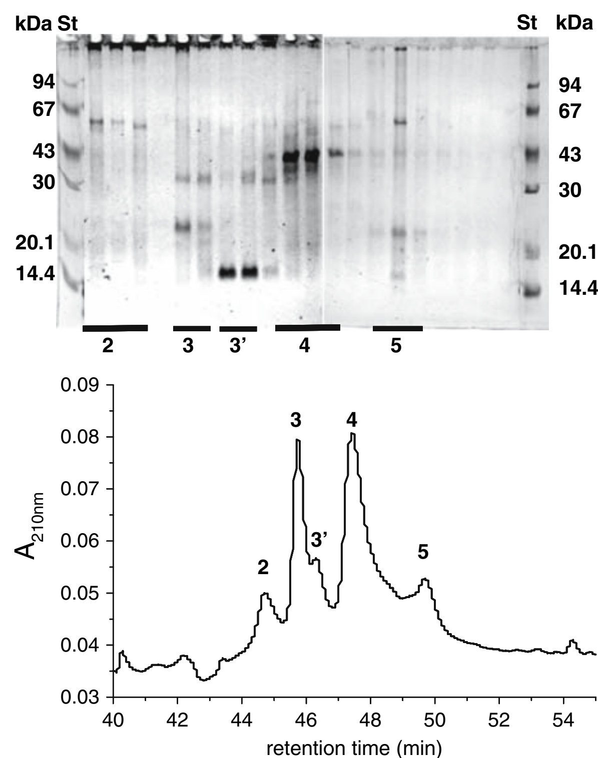

Fig. 5 RP-HPLC profile of dissociated and reduced 11S-globulin and SDS-PAGE of peak fractions. St: Standard proteins; kDa: molecular masses of the standard proteins.

results included the most hydrophilic globulin-p A and 20 kDa polypeptides eluting in peak 1, which were not present in 11S-globulin, and the most hydrophobic 11S-globulin M polypeptide eluting in peak 5, which was not present in globulin-p.

4. CONCLUSION

The polypeptidic heterogeneity shown by these results sustain the existence of genetic polymorphism in amaranth globulins. In agreement with other globulins (Delseny and Raynal, 1999) these results suggested that amaranth subunits from different subfamilies are interchangeable in different oligomers. Nevertheless, the presence of the L and S polypeptides only in 11S-globulin suggested that some structural differences between subunits might affect the assembly of globulin oligomers. The existence of 11S-globulins with different physicochemical properties might be the result of the presence of unprocessed or misprocessed precursor. As was proposed above, the M polypeptides corresponded to unprocessed precursor and the L and S polypeptides might be the products of a different precursor. The different hydrophobicity of M and L polypeptides support the hypothesis of these polypeptides coming from different genes. Concerning the globulin-p and 11S-globulin A and B polypeptides, though they showed similarities regarding charge and pI, the differences revealed by the RP-HPLC results indicated that these polypeptides may be the products of more than one gene.

As shown by these results, those molecules with a large proportion of M polypeptides (globulin-p) showed a trend to aggregation and displayed some particular physicochemical properties whereas those molecules containing a high proportion of processed subunits presented the physicochemical properties of most 11S-globulins.

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