

Structural Modifications of Amaranth Proteins During Germination

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Abstract Amaranth storage proteins begin to be hydrolyzed immediately following the completion of germination. Albumins and globulins (7S globulin, 11S-globulin and globulin-p) were formerly modified, and glutelins, the most aggregated fraction, later. Globulins mobilization starts with the proteolysis of the 7S like-globulin polypeptides and the propolypeptide and acid (A) polypeptides of 11S-globulin and globulin-p. This pattern of 11S-globulin mobilization is accounted by the structural model with propolypeptide and A polypeptides exposed to the outside. Amaranth globulin molecules showed minor changes in their sizes in spite of having some of their polypeptides cleaved. Although globulin-p is more aggregated than 11S-globulin, it showed greater conformational changes. Considering the high susceptibility of the propolypeptide to enzymatic hydrolysis, the higher content of this polypeptide in globulin-p molecules might explain their higher structural changes. According to the results, the order of mobilization of storage proteins depends on the combination of two structural characteristics, the state of aggregation and the presence on the surface of polypeptides susceptible to cleavage.

Keywords Amaranth proteins · Structure modification · Protein mobilization · Proteolytic cleavage · Storage proteins

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Abbreviations

A and B	Globulin-p and 11S-globulin, acid and basic polypeptides, respectively
M	Globulin-p and 11S-globulin propolypeptide
AM	Globulin-p aggregated molecules
L	7S like-globulin 45 kDa polypeptide
CHAPS	3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
hai	Hours after imbibition
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
PMSF	Phenylmethylsulfonyl fluoride
TBS	Saline tris buffer
TCA	Trichloroacetic acid
UM	280 kDa globulin-p unitary molecules

1 Introduction

Amaranth seeds contain globulins as major storage proteins, which includes the legumin type globulins and a minor proportion of a 7S globulin [8, 10]. Legumin polypeptides are synthesized as a unique precursor which is processed during seed maturation [12]. According to their state of aggregation and physicochemical properties, three different legumin type globulins have been described in *Amaranthus hypochondriacus* [10]. These are 11S-globulin, soluble in neutral saline, globulin-p, polymerized and non-soluble in saline solutions and glutelin, the most aggregated globulin, only soluble in alkali.

Globulin-p and glutelin contain a high proportion of a non-processed precursor of near 56 kDa [5, 9] and their aggregates are stabilized by disulphide bridges.

In *A. cruentus*, globulins are located in the storage vacuoles mainly present in embryos [15] whereas, albumins, which comprise near 30% of the amaranth proteins [10], are located outside the protein bodies [15]. Amaranth albumins are supposed to be composed of proteins with several functions; they might include the storage function, same as albumins from other plants [19].

During mobilization, globulins are broken down inside the storage vacuoles, and vicilins are the first globulins to be hydrolyzed [18, 21] while legumins are degraded more slowly. Globulin mobilization begins with a limited proteolysis, rendering some TCA soluble peptides and largely intact modified holoproteins, which become more susceptible to a further unlimited cleavage [11, 20]. The limited proteolysis may be catalyzed by endogenous papain-like enzymes and legumains, which are low-specificity endopeptidases synthesized de novo and transported into the protein bodies during seedling growth [13, 20].

According to experiments in sugar beet [4], vetch [18], field bean, soybean [11] and Arabidopsis [6] legumin degradation starts with the cleavage of the acidic (A) polypeptide while the basic (B) polypeptide remains intact until later in seedling growth. This is attributed to the more exposed position of the A chain in the protein.

There has not been any report about amaranth protein mobilization. Amaranth seeds contain legumin type globulins in different states of aggregation located in a unique type of vacuoles. These structural characteristics may be connected with their function as storage reserves to be utilized during germination.

The purpose of this work is to follow the structural changes of the amaranth proteins during mobilization and correlate these changes with the protein's state of aggregation. This work describes the hydrolysis of proteins from different amaranth fractions during mobilization; it shows which polypeptides are hydrolyzed first and which are the structural modifications of the protein molecules.

2 Materials and Methods

2.1 Plant Material

Seeds from *A. hypochondriacus* (Mercado variety) were harvested at Estación Experimental del Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo, México, and kindly provided to our laboratory in Argentina. The seeds were stored in closed glass flasks at 4 °C. The germination experiments were carried out during the 1 year after harvest.

2.2 Imbibition Procedure of Amaranth Seeds

Amaranth seeds were embedded with 0.02% (g/mL) of sodium hydrochloride solution for 20 min as an antifungal treatment, then they were washed several times with distilled water. Germination assays were carried out at 37 °C on sheets of absorbent paper wetted with distilled water placed in covered plastic boxes. At different (0, 6, 8, 12, 15, 24 and 48) hours after imbibition (hai) seeds were dried for an hour at 42 °C to stop enzymatic activity. Seeds were regarded as germinated when the radicle protruded through the seed coat [7]. At the chosen times of imbibition at least 100 seeds with the same characteristics were selected: control and 6 hai seeds showed no radicle protrusion, 15 hai seeds exhibited a radicle length of less than 0.5 cm while 24 hai and 48 hai seeds showed a radicle length of 0.5 cm and more than 0.5 cm, respectively. Finally, seeds were stored at -80 °C until use. Five independent germination tests were done.

To rule out a possible loss of cellular compartmentation and development of protease activity during the 42 °C treatment, a control experiment was conducted in which the selected seeds at different times of imbibition were immediately frozen under liquid N₂, ground in a mortar and subjected to protein extraction.

2.3 Protein Fractionation

Seeds at different stages of imbibition were frozen under liquid N₂ and ground in a mortar. Sequential extraction of protein fractions was carried out according to the procedure of [10] with minor modifications. Each extraction was performed in one step (60 min) at room temperature at a ratio solvent/meal of 10 mL/g, in the presence of 50 µg/mL PMSF protease inhibitor. The ground seeds were successively treated with water to extract albumin, with buffer A (32.5 mM K₂HPO₄-2.6 mM KH₂PO₄, pH 7.5, 0.4 M NaCl) for globulin extraction, with water for globulin-p extraction, and with 0.1 N NaOH to extract glutelins.

The extraction residue was separated by centrifugation at 9,000×g for 20 min at room temperature. Globulins, globulin-p and glutelins, were isolated from the corresponding supernatants by precipitation at pH 6 and albumins were precipitated at pH 5 using 2 N HCl. The precipitates were suspended in water, neutralized with 0.1 N NaOH, and freeze-dried.

2.4 Sodium-Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gels were prepared in minislabs (Bio Rad Protean II Model). Runs were carried out, using 12% (w/v) poly-

acrylamide gels with stacking gel of 4% (w/v) polyacrylamide [16]. Molecular masses of the polypeptides were calculated using the following protein standards (Pharmacia Biotech, Uppsala-Sweden): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.4 kDa). Protein samples were dissolved in sample buffer (0.125 M Tris-HCl (pH 6.8), 20% (v/v) glycerol, 1% (v/v) sodium dodecyl sulfate, 5% (v/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue and were heated at 100 °C for 3 min. Gels were fixed and stained with Coomassie Brilliant Blue.

Duplicate runs of each sample from five experiments were performed.

2.5 Nondenaturing Polyacrylamide Gel Electrophoresis (ND-PAGE)

These tests were performed in 7% (w/v) acrylamide gels using the same buffer system as that of SDS-PAGE, but without SDS. Protein samples (20 mg/mL) were dissolved in 0.125 M Tris-HCl, (pH 8.8), 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue, and centrifuged at $15,800\times g$ for 5 min. The supernatants were separated and used to load the gel (4–6 μ L/lane). Gels were fixed and stained with Coomassie Brilliant Blue.

Duplicate samples from four experiments were analyzed.

2.6 Two-Dimensional Electrophoresis (IEF/SDS-PAGE)

Globulins at different times of imbibition were analyzed by two-dimensional electrophoresis. The first-dimension IEF was performed using 18 cm linear, immobilized pH gradient (IPG) strips (pH 3–10) in the IPGphor system (GE Healthcare UK Ltd., England). All IPG strips were re-hydrated for 14 h at 22 °C with 340 μ L of re-hydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT, 0.5% (v/v) IPG buffer and 0.002% (w/v) bromophenol blue) containing 200 μ g of protein. The voltage settings for IEF were 500 V for 1 h followed by 1,000 V for 1 h, and then 8,000 V for 4 h to reach a final condition of 30,000 V/h. Following IEF, the gel strips were incubated with equilibration buffer (2% (w/v) SDS, 6 M urea, 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol, 30 mM DTT and 0.01% (w/v) 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% (w/v) 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% (w/v) agarose, 0.002% (w/v) bromophenol blue). Gels were stained with silver nitrate. Duplicate samples from one experiment were analyzed.

2.7 Western Blot Analysis

Anti-globulin-p polyclonal antibodies were prepared as described by [2]. Denaturing gels (SDS-PAGE) were transferred to nitrocellulose membrane at 250 mA for 1 h using buffer containing 20 mM Tris, 0.193 M glycine (pH 8.35), 20% (v/v) methanol, in Transblot System equipment (Bio-Rad, Richmond, CA, USA). Western analyses were performed using the method described by [22]. The nitrocellulose membranes were blocked with a solution of TBS (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) containing 2% (w/v) skim milk. After incubation overnight at 4 °C, a 1:500 dilution of the polyclonal anti-globulin-p antiserum was added. After washing with 0.05% (v/v) Tween/TBS, immunoreactive proteins were detected by the chemiluminescent system. All the the following steps until the fixation of the films were performed in the dark room. Luminescent stock reagents: (a; 40 mg/mL luminol in DMSO) and (b; 15 mg/mL p-coumaric acid in DMSO) were prepared. The chemiluminescent reagent was prepared mixing equal volumes of buffer 1 (5 μ L stock solution a, 2.2 μ L stock solution b, 33 μ L non-denaturant separation buffer and 470 μ L distilled water) and buffer 2 (3.1 μ L 30% (v/v) H₂O₂, 33 μ L non-denaturant separation buffer and 470 μ L distilled water). The chemiluminescent reagent was spread over the blots, protein side up. After 1 min of exposure, excess buffer was drained and an autoradiography film (Kodak, Scientific Imaging Film) was located on top of the nitrocellulose membrane. After an exposure time that varied between a few seconds to a couple of minutes, the film was placed in the developing solution (AGFA G150 diluted 1:4) until a signal was reached. Then the film was soaked in the fixation solution (AGFA G334, diluted 1:4) and finally rinsed with warm water.

Samples from two experiments were analyzed with this method.

2.8 Chromatography

Globulins and globulin-p obtained from seeds at different times of imbibition were analyzed by chromatography at room temperature in a Superose 6B HR 10/30 column using a Pharmacia LKB, FPLC system (Uppsala, Sweden). Samples contained 4 mg of protein in 200 μ L of buffer A for globulins or buffer B [33.3 mM K₂HPO₄, 1.7 mM KH₂PO₄ (pH 8.5)] for globulin-p. Elution was carried out with the same buffer as that used in the sample, at a flow rate of 0.2 mL/min; 0.3 mL fractions were collected, and the elution profile (absorbance at 280 nm) was obtained.

The column was calibrated with Blue dextran (V_0) and the following proteins were used as standards: thyroglobulin (669 kDa), apoferritin (443 kDa), α -amylase (200 kDa), and alcohol dehydrogenase (150 kDa).

Albumins were chromatographed using a Sephacryl S-300 HR column (0.71 cm \times 71.5 cm) with a fractionation range of 10,000–1,500,000. The column was packed and equilibrated with three bed volumes of buffer A. A sample containing 5 mg of protein in 300 μ L of buffer A was layered onto the gel filtration column and was eluted with buffer A at a flow rate of 0.13 mL/min using a Gilson Minipuls 2 peristaltic pump. The absorbance at 280 nm was measured with an UV detector (Econo UV, BioRad,.) and the protein elution profiles were plotted using a Gilson N2 recorder.

Duplicate runs of samples from two experiments were performed.

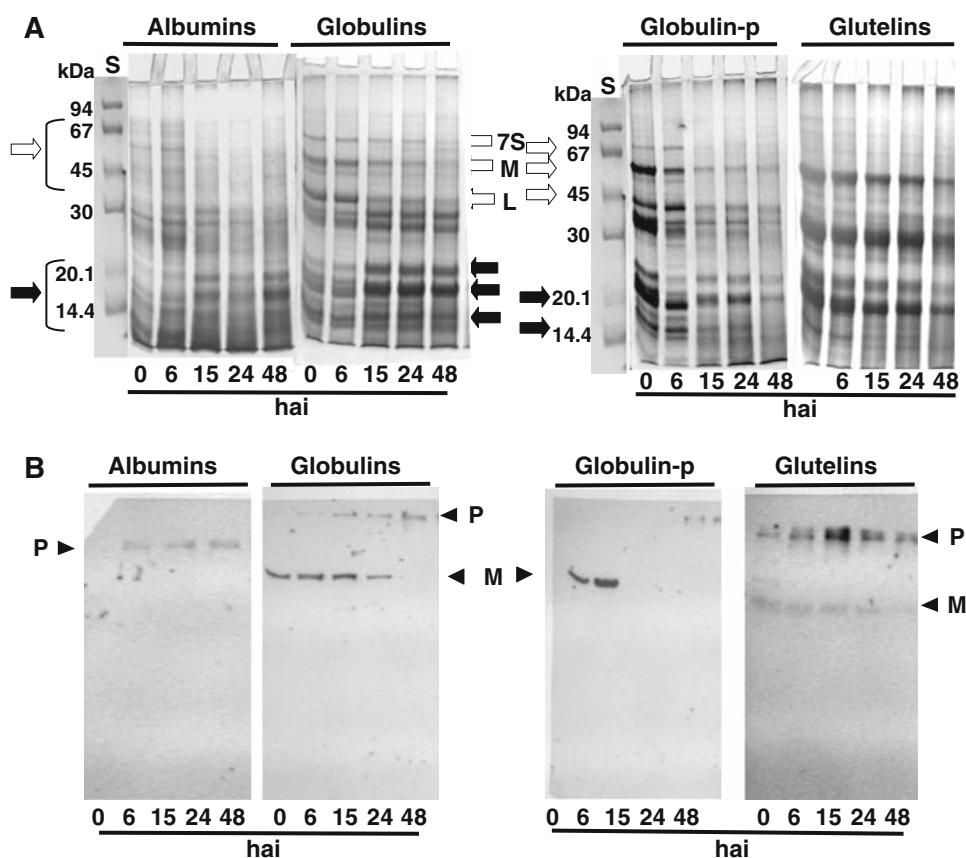
3 Results

3.1 Polypeptide Hydrolysis of the Protein Fractions: Albumins, Globulins and Glutelins

Mobilization of amaranth protein fractions was studied by SDS–PAGE. The profiles shown in Fig. 1 demonstrated that protein hydrolysis began at 15 hai, the beginning of the

post-germination period. This result was confirmed by similar assays, not shown, carried out at 6, 8 and 12 hai. Globulins and albumins showed the greatest changes at early times (Fig. 1) with a decrease of polypeptides of molecular masses higher than 30 kDa (white arrows) and an increase of low MW polypeptides (black arrows). The same result was obtained in an experiment without the 42 °C drying treatment (“Materials and Methods”). Therefore, the degradation of albumins cannot be ascribed to the leakage of protein body proteases during the drying. The non-processed M polypeptide (56 kDa) from globulin-p and 11S-globulin, and the 7S globulin polypeptides 45 kDa (L) and 67 kDa (Quiroga, personal communication) were the first to be mobilized in these protein fractions (Fig. 1a, arrowheads). On the other hand, glutelin polypeptides decreased at a later time. Immunochemical assays (Fig. 1b) using anti-Gp polyclonal antibodies [2] showed that M polypeptide, present in globulins and glutelin, was the most reactive polypeptide with the antibody which is in accordance with previous information [2]. This reactivity gradually decreased along the time of imbibition; in the case of 11S-globulin it was absent at 24 hai, whereas, in globulin-p it was no longer present at 15 hai. No reactive hydrolysis products were detected. As in previous studies [2] albumins from non-treated seeds did not show any reactivity towards the antiserum (Fig. 1b), but in seeds

Fig. 1 Changes in polypeptide profiles of albumins, globulins, globulin-p and glutelins during germination. *Bottom numbers indicate hours of imbibition (hai) a* SDS–PAGE patterns. *Numbers at left indicate molecular masses of standard proteins (S). White arrows indicate polypeptides decreasing with time of imbibition, black arrows indicate polypeptides increasing with time of imbibition. Globulin and globulin-p unprocessed polypeptide, (M); 7S like-globulin 45 kDa polypeptide, (L); 7S like-globulin 67 kDa polypeptide, (7S). b* Western blot using anti-Gp polyclonal antibodies. *Globulin and globulin-p unprocessed polypeptide, (M); aggregated polypeptides not entering the gel, (P). Polyacrylamide gel concentration: 12% (w/v)*



from 15 hai on, albumins formed reactive aggregates (P in Fig. 1b) that did not enter the gel. These reactive proteins may arise from protein body rupture.

3.2 Globulin Polypeptide Mobilization Analyzed by Two-Dimensional Electrophoresis

Figure 2 shows the two-dimensional electrophoresis of globulin fraction at 0, 15 and 48 hai. The zero hai profile shows the legumin type precursor M and the 7S like-globulin L polypeptides [17] at neutral pH, and the A and B 11S-globulin and globulin-p polypeptides at acid and basic pHs, respectively [17]. The 7S large polypeptides are shown at acid pHs (Fig. 2a). At 15 hai, M, L and A polypeptides are found in a lower proportion, hydrolysis products of about

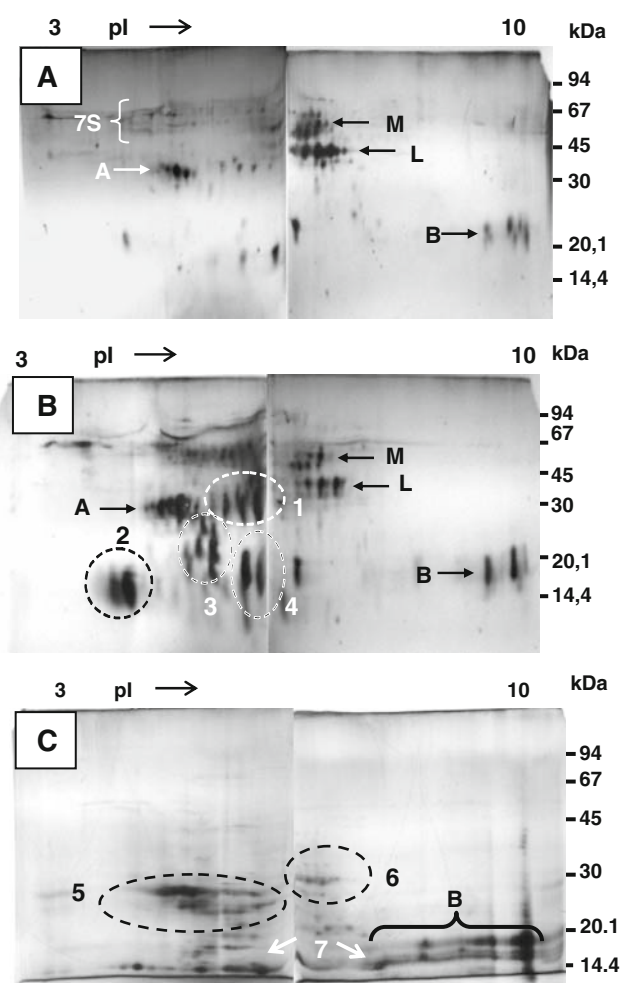


Fig. 2 Two dimensional electrophoretic patterns of globulins at different times of imbibition. (a) control 0 hai; (b) 15 hai, (c) 48 hai. Numbers at right indicate molecular masses in kDa. On top, pI range from pI = 3 at left to pI = 10 at right. Circles 1, 2, 3 and 4 in (b) and 5, 6 and 7, in (c) indicate hydrolysis products. Unprocessed polypeptide, (M); legumin subunits polypeptides, (A) and (B); globulin 45 kDa polypeptide, (L), 7S globulin polypeptide, (7S). Polyacrylamide gel concentration: 12% (w/v)

30 kDa (Fig. 2b, spots in circle 1) and of 14–25 kDa (Fig. 2b, spots in circles 2, 3 and 4) are present at acid pH. The polypeptides in circle 1 may be products of M and/or L hydrolysis, whereas, those in circles 2, 3 and 4 may have the same source or might come from the A polypeptides cleavage. At 48 hai (Fig. 2c) some B polypeptides are still present. The 48 hai profile also showed hydrolysis products of near 30 kDa at acid pH (Fig. 2c, circle 5), neutral pH (Fig. 2c, circle 6) and other products of molecular masses lower than 20 kDa at acid, neutral and basic pHs (Fig. 2c, arrow 7).

These results showed that M, L and A polypeptides were the first to be mobilized. Although the profiles did not show a clear decrease of B polypeptides, the possibility that they were partially hydrolyzed while new B polypeptides from M and/or L cleavage were forming cannot be ruled out. According to these profiles, hydrolysis products coming from M polypeptides were present, but Western blot analyses demonstrated that those peptides have lost the reactivity against the anti-Gp antibodies.

3.3 Structural Modifications of Protein Fractions

Modifications of proteins during mobilization were also analyzed by gel filtration chromatography and ND-PAGE. As glutelins extracted with 1 N NaOH (“Materials and Methods”) were denatured [1], they were not analyzed by chromatography and/or ND-PAGE.

3.3.1 Albumins

Chromatographic analyses of albumins (Fig. 3a) showed minor changes during mobilization. An increase in low molecular weight protein and a decrease of proteins eluting at low volumes was observed (Fig. 3a, filled and void arrows, respectively). The ND-PAGE profiles of albumins (Fig. 3b) showed that as germination progressed there was a decrease in the intensity of bands of low mobility (white arrows) and an increase of the bands of higher mobility (black arrows). These results demonstrate the hydrolysis of proteins not classified as storage proteins, posing the question of their actual role.

3.3.2 Globulins

The major components of the globulin fraction are the 11S-globulin molecules of 280 kDa and the 7S-like-globulin, (Fig. 4a, 0 hai, peak 11S) and a low molecular weight globulin (Fig. 4a, 0 hai, peaks P1 and P2). At 15 hai, there were a larger proportion of 11S and 7S-like molecules, which probably come from solubilization of 11S-globulin aggregates. The 24 hai profile was similar to the 15 hai one, whereas, at 48 hai the amount of 11S and 7S-like molecules decreased and a larger proportion of the

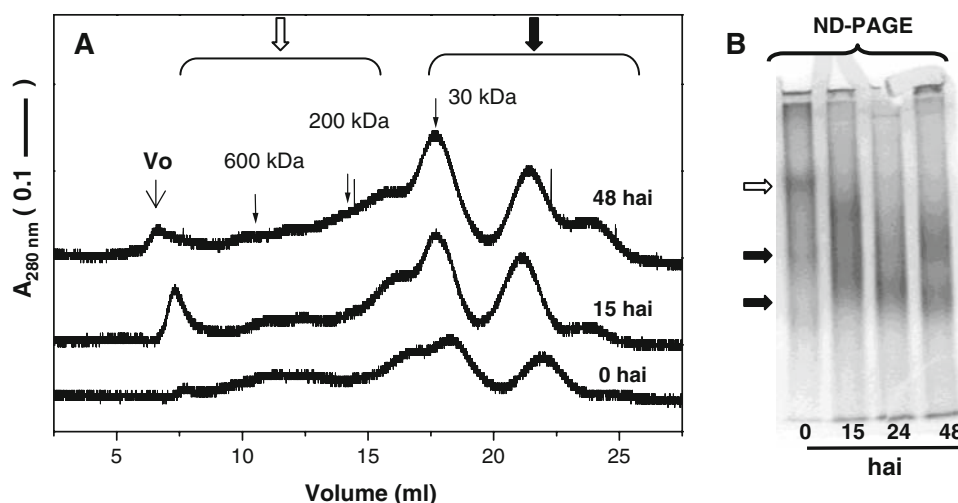
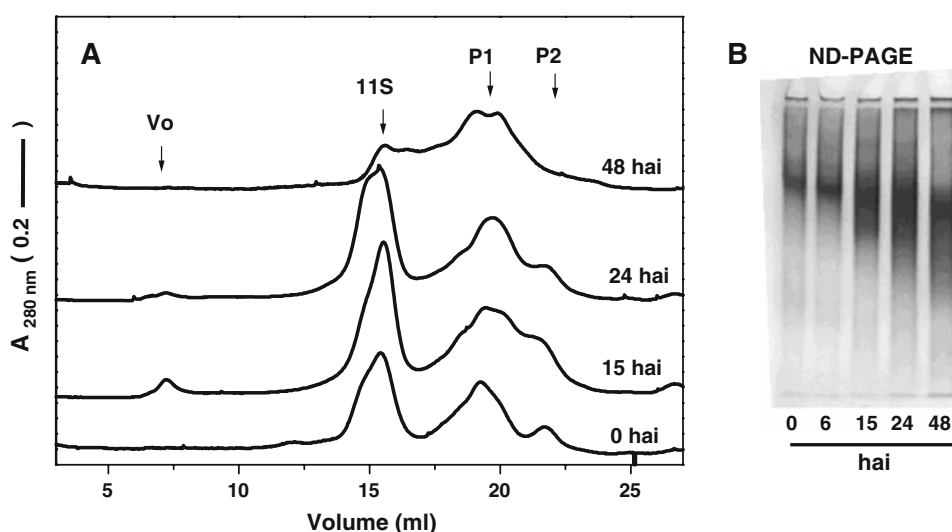


Fig. 3 Modifications of albumins during germination. **a** Chromatographic profiles at 0, 15 and 48 hai. White and black arrows indicate protein species that decrease and increase, respectively. Void volume (V_o) and elution volume of standard molecular mass proteins (kDa) are indicated. **b** ND-PAGE and Western blot profiles at 0, 15, 24 and

48 hai. Hours of imbibition are indicated at the *bottom*. White and black arrows indicate protein species that decrease and increase, respectively. The Western blot was performed with the chloronaphthol system using anti-Gp polyclonal antibodies

Fig. 4 Modifications of globulins during germination. **a** Chromatographic profiles at 0, 15, 24 and 48 hai. Void volume (V_o), 11S, 300 kDa 11S-globulin molecules, P1 and P2 low molecular weight globulin and hydrolysis products. **b** ND-PAGE and Western blot profiles at 0, 15, 24 and 48 hai. The Western blot was performed with the chloronaphthol system using anti-Gp antibodies. Hours of imbibition are indicated at the *bottom*



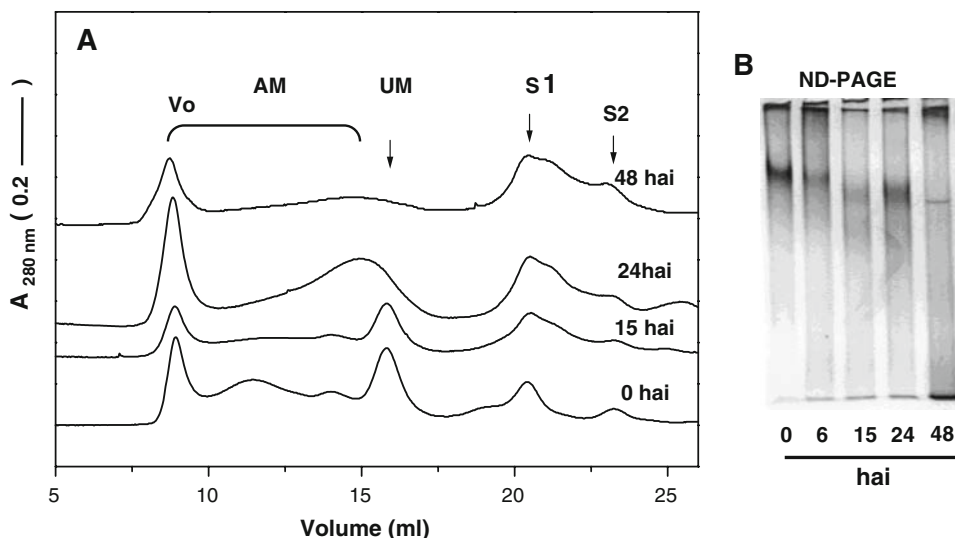
smaller molecules were present. The ND-PAGE profiles of globulins (Fig. 4b) showed that at 15 hai such proteins exhibited a higher mobility than the control at 0 hai. At 24 and 48 hai another band of even higher mobility was also present. Considering the chromatographic results, the higher mobility of proteins at 15 hai cannot be ascribed to a decrease in molecular mass, suggesting that, at that stage of germination, the molecules have changed their charge.

3.3.3 Globulin-p

The chromatogram of globulin-p, the polymerized globulin, (Fig. 5a) revealed the presence of high molecular weight aggregated molecules (AM), the unitary molecules (UM) and low molecular weight protein present in the fraction

[S1, S2; 10]. At 15 hai globulin-p showed a decrease of the amount of aggregates (Fig. 5a, AM) and an increase of the small size protein, probably because of the presence of hydrolysis products. At 24 hai the profile showed a broad peak settled at a rather lower volume than that of the unitary molecules (Fig. 5a, UM), which may correspond to globulin-p molecules with a more open conformation. At 48 hai globulin-p molecules had almost disappeared, the major component being composed of low molecular weight protein (peak S1). ND-PAGE profiles (Fig. 5b) showed that globulin-p of germinating seeds run with bands of higher mobility than the control (0 hai). These results indicated that, at least from 15 hai onwards, globulin-p molecules carrying some hydrolyzed polypeptides had undergone conformational changes, presenting a higher negative

Fig. 5 Modifications of globulin-p during germination. **a** Chromatographic profiles at 0, 15, 24 and 48 hai. Void volume (V_0), AM, aggregated molecules, UM, unitary molecules, S1 and S2, low molecular weight protein. **b** ND-PAGE and Western blot profiles at 0, 15, 24 and 48 hai. The Western blot was performed with the chloronaphthol system using anti-Gp antibodies. Hours of imbibition are indicated at the bottom



charge at pH 8.9. Gel filtration analysis and ND-PAGE profiles showed that globulin-p suffered more severe conformational changes than globulin at 24 and 48 hai.

4 Discussion

Amaranth storage proteins begin to be hydrolyzed, as other seed storage proteins [3], at the onset of the post germination phase. Albumins show a time course of mobilization similar to storage proteins. This is rather unexpected considering that amaranth albumins are located outside the storage vacuoles [15]. In agreement with these results, [18] have informed about the hydrolysis of some lectins from the albumin fraction of *Vicia sativa* at early times of mobilization.

Glutelins, the legumin-like globulins that are in the most aggregated state inside the amaranth seeds [1], were the last to be mobilized. On the other hand, globulin-p, 7S-like and 11S-globulins were cleaved at the same time, though globulin-p is more aggregated than the 11S-globulin [10]. In this sense globulin-p exhibited a rather unexpected behavior during mobilization.

The initial mobilization of globulins preferentially begins with the proteolysis of 7S like-globulin polypeptides, the globulin-p and 11S-globulin, M propolypeptide and acid (A) polypeptide, which is in accordance with studies in other plants [14, 18]. The hydrolysis of the globulin polypeptide is accounted by the structural model proposed by [2] with the M proglobulin and the A polypeptides more exposed to the outside. The presence of new 30 kDa polypeptides at 15 hai that could be hydrolysis products, suggested that M propolypeptides were being cleaved at the processing site. This agrees with the proposal that the legumains, cleaving peptide bonds flanked by Asn, would lead to the processing of the precursor in as much as the molecules keep their

conformation [13]. If the acid polypeptides smaller than 30 kDa shown in the two-dimensional profile of globulins at 15 hai, were hydrolysis products derived from the M propolypeptide, it would be an indication that this polypeptide was also cleaved at the acidic end.

Globulins and globulin-p molecules that have begun their mobilization presented some polypeptides hydrolyzed, small changes in their sizes, and a likely higher negative charge. These findings are in agreement with the hypothesis of the limited proteolysis of globulins during the initial stages of germination [20]. Globulin-p showed greater conformational changes than globulin during 24–48 hai. This finding is not in accordance with the fact that globulin-p contains more disulphide bonds and is more aggregated than 11S-globulin. Considering the high susceptibility of the M propolypeptide to enzymatic hydrolysis [2] the higher content of this polypeptide in globulin-p molecules might be the cause of globulin-p higher susceptibility to mobilization. On the other hand amaranth glutelins, like globulin-p, contain a large amount of M propolypeptide but are in the most aggregated state; they are mobilized after 11S-globulin and globulin-p cleavage.

The present results suggest that the order of mobilization of storage proteins depends on both the state of aggregation and the presence on the surface of polypeptides susceptible to cleavage.

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