Amaranth Globulin Structure Modifications Induced by Enzymatic Proteolysis

Oscar F. Castellani, E. Nora Martínez, and M. Cristina Añón*

Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), 47 y 116 (1900) La Plata, Argentina

Globulin-P was partially hydrolyzed with papain under specific conditions to study the resulting structural modifications. Under mild hydrolytic conditions, globulin-P polymers were cleaved to render their unitary constituents (280 kDa molecules). Under stronger hydrolytic conditions these unitary molecules were 13% smaller than those from nonhydrolyzed globulin. Moreover, these molecules remained assembled even though they contained degraded polypeptides. The monomeric (M) subunit and the A chains were preferentially cleaved under mild and intermediate hydrolytic conditions, whereas B chains remained with the same size. These results suggest that the M and A polypeptides might be located at an exposed site of the molecules resembling the structure of the legumins. The M subunit may be participating in the stabilization of globulin-P polymers, on the basis that these two species disappeared under the same hydrolytic conditions. Similar events such as those described in this paper might be taking place on globulin-P during germination of amaranth grain.

Keywords: *Amaranth; globulin; papain; protein structure; proteolysis*

INTRODUCTION

Cereals and legumes constitute the main source of the world's protein supply. However, many of these proteins, specially those from cereals, have low nutritional value. Amaranth is a dicotyledoneous plant with well-balanced grain proteins and has been proposed as a new alternative source of good quality protein (National Research Council, 1984; Singhal and Kulkarni, 1988).

Globulins, one of the major amaranth protein fractions, are composed of 11S-globulin, globulin-P, and a small amount of 7S globulin (Segura-Nieto et al., 1994; Marcone, 1999; Martínez et al., 1997). Amaranth 11S-globulin has been extensively studied. It was shown to have molecular characteristics similar to those of other legumins (Segura-Nieto et al., 1994; Marcone et al., 1994; Chen and Paredes-López, 1997; Marcone et al., 1997). In addition, globulin-P is composed of unitary molecules of molecular weight and polypeptide composition similar to those of 11S-globulin, but globulin-P tends to polymerize, thus showing different solubility (Martínez et al., 1997; Castellani et al., 1998). Consequently, globulin-P (glb-P) can only be extracted in water after the other globulins by treating the flour with a saline solution (Konishi et al., 1991). Besides, globulin-P contains a higher proportion of monomeric subunits of 54 kDa than 11S-globulin (Martínez et al., 1997). These differences suggest a difference in protein conformation between globulin-P (glb-P) and other legumins and also differences between accumulation and mobilization in the seed.

Controlled enzymatic hydrolysis has been widely used to improve functional properties of food proteins (Adler-Nissen, 1976; Krause and Schwenke, 1995; Schwenke et al., 1995; Sijtsma et al., 1998). After hydrolysis, proteins decrease in size, together with changes in conformation and strength of both intra and intermolecular bonds (Kim et al., 1990; Kamata et al., 1991; Henning et al., 1998). The effect that proteolysis has on protein structure provides a useful tool with which to study food protein structure. Partial proteolysis of legumins with several proteases has similar patterns both in vitro and in germinating seeds (Müntz, 1996). As a result, there is a loss of surface peptides and a subsequent formation of intermediate products of high molecular mass (Shutov et al., 1991; Shwenke et al., 1995; Kamata and Shibasaki, 1978). Analysis of digestion patterns of globulin-P would allow comparison of its behavior with that of the legumins. Moreover, it would be useful for a further understanding of the structural and conformational characteristics of globulin-P, as well as its fate during germination.

The present study was conducted to obtain information about changes in the structure of globulin-P during partial proteolysis with papain. The new knowledge was expected to provide a better structural characterization of the unhydrolyzed globulin-P.

MATERIALS AND METHODS

Materials. Seeds of *Amaranthus hypochondriacus* (commercial cultivar) were harvested at 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 the whole seeds in a Udy mill (Facultad de Agronomía, Universidad Nacional de La Plata, Argentina), 1 mm mesh, and screened by 10-xx mesh. Flour was defatted for 24 h with hexane in a 10% (w/v) suspension under continuous stirring, air-dried at room temperature, and stored at 4 °C until used. Protein content of the flour, as determined by Kjeldhal (AOAC, 1984), was 17.0% (w/w) on dry weight basis (N x 5.85) (Becker et al., 1981; Segura Nieto et al., 1994).

* To whom correspondence should be addressed. Fax: +54−221−4249287/4254853. E-mail: mca@nahuel.biol.unlp.edu.ar.

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Table 1. Conditions (Enzyme Concentration, Incubation Time, and Temperature) of Globulin-P Papain Hydrolysis

hydrolytic condition	C1	C2	C3	C4	C5	C6	C7	C8
enzyme, U/mL	-	0.02	0.2	2	2	2	2	2
time of incubation	-	0	0	0	15 min	20 min	120 min	18 h
temperature, °C	-	20	20	20	25	40	40	40

Protein Isolation. Globulin-P was extracted according to the method previously described for albumin-2 preparation (Martínez et al., 1997). Briefly, flour was treated three times with water for albumin extraction (albumin-1) and then three times with 32.5 mM K_2HPO_4−2.6 mM KH_2PO_4−0.4 M NaCl, pH 7.5 (buffer A) for globulin extraction. Finally, globulin-P was extracted by treating the last residue three times with water in a ratio of 10 mL of water to 1 g of meal. After each treatment, the extraction residue was separated by centrifugation at 9000*g* for 20 min. The extraction procedure was conducted at room temperature. The supernatants containing globulin-P (either as soluble or dispersed protein) were adjusted to pH 6 with 2 N HCl. The resulting precipitate was resuspended in water, neutralized with 0.1 N NaOH, and freeze-dried.

Globulin-P Proteolysis. Globulin-P fraction was suspended in buffer B (33.3 mM K_2HPO_4−1.7 mM KH_2PO_4, pH 8.5) (35 mg/mL) and submitted to papain hydrolysis. Samples were incubated with different amounts of enzyme (*Papaya latex*, Sigma) under the conditions depicted in Table 1. After incubation, samples were centrifuged at 10 000*g* for 20 min at 20 °C, filtered through a 0.2-μm pore nylon membrane, and injected in the FPLC column. A suspension of globulin-P in buffer B (35 mg/mL), centrifuged and filtered in the same manner as the incubation mixtures, was used as a control (nonhydrolyzed globulin-P).

Chromatography. Globulin-P and hydrolyzed globulin-P were analyzed by chromatography at room temperature using a Pharmacia LKB, FPLC System. The samples (200-μL) were loaded in a Superose 6B HR 10/30 column and eluted with buffer B at a flow rate of 0.2 mL/min. Fractions (0.5 mL) were collected and elution profile (absorbance at 280 nm) was obtained. The column was calibrated with dextran blue (V_0 = void volume) and standard proteins: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), and alcohol dehydrogenase (150 kDa). The equation that fitted the calibration curve obtained from duplicate measurements was

$$\log \mathrm{MM} = A - (B \times K_{\mathrm{AV}})$$

where $K_{\mathrm{AV}} = (Ve - V_0)/(V_T - V_0)$; MM is the molecular mass in kDa; $A = 4.5 \pm 0.2$; $B = 5.1 \pm 0.3$; Ve = elution volume of the sample (in mL); $V_0 = 7.64 \pm 0.01$ mL; and, as indicated by Pharmacia Biotech, the total volume of the column $V_T = 25.00$ mL. The lineal correlation coefficient (r) was −0.98. Curves were processed and data were evaluated using a Pharmacia AB, FPLC director and FPLC assistant software. Fractions corresponding to each peak were lyophilized and dissolved in SDS−PAGE sample buffer for a further analysis by electrophoresis.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Gels were prepared in minislabs (BioRad Mini Protean II Model). Runs were carried out according to the method of Laemmli (1970).

One-Dimensional Gel Electrophoresis. Separating gels containing 12% (w/v) of polyacrylamide were used. Molecular masses of the polypeptides were calculated using protein standards from Pharmacia: 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 without heating 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-mercaptoethanol (2-ME) was added and samples were heated (100 °C, 3 min). Gels were fixed and stained with Coomassie Brilliant Blue stain.

Bi-Dimensional Gel Electrophoresis (SDS → SDS + 2-ME). The first dimension was performed in nonreducing denaturing conditions with 6−12% (w/v) polyacrylamide linear gradient separating gels or 12% (w/v) polyacrylamide separating gels as indicated in the figures. The samples were prepared in the same manner as those subjected to one-dimensional electrophoresis. After the run, the first-dimension gel portion was treated with 10 vol of treatment buffer (62.5 mM Tris−HCl, pH 6.8, 1% SDS, 20% sucrose, 0.2M 2-ME) for 30 min at 55 °C, with the solution being changed at least two times. The second dimension was performed in a 12% polyacrylamide gel. All gels were fixed and stained with Coomassie Brilliant Blue Stain.

RESULTS

A partially purified globulin-P fraction was submitted to several papain hydrolytic treatments. Table 1 shows the treatment conditions grouped by enzyme concentration, time, and temperature. Results from FPLC analyses of the samples are shown in Figures 1 and 2. Figure 1 includes the most representative FPLC profiles and Figure 2 illustrates the percentage of protein species for each treatment.

As previously reported (Martínez et al., 1997) the nonhydrolyzed globulin-P fraction (Figure 1, profile C1) contains primarily polymers with molecular masses (MM) ranging from 600 to 30 000 kDa (S1), unitary molecules with MM 280 kDa (S2), and dissociated subunits of lower MM (S3). Under mild hydrolytic conditions (C2, Figures 1 and 2) globulin-P polymers (S1) decreased turning into lower MM species (S1′) together with a major increase of S2 and a minor increase of S3. The increment of papain concentration (C3, Figure 2) led to the disappearance of large polymers but S1′ oligomers were still present. At the same time, S2 and S3 increased with the same tendency as before. In the following treatment conditions C4, C5, and C6 (Figure 2 and C5 in Figure 1) the proportion of S2 molecules was the same as in C3. Besides, a new species of lower MM (S4) appeared as long as S1′ and S3 decreased. Profiles corresponding to the more intense hydrolytic conditions (C7 and C8, Figure 2; C8 Figure 1) showed a decrease in the proportion of S2 molecules and a major increase of S4 which may be hydrolytic products.

As a consequence of a mild hydrolysis on glb-P, polymers were converted into lower molecular mass oligomers and finally into 280 kDa unitary molecules. Under prolonged incubations the latter high molecular mass molecules (S2) were transformed into low molecular mass hydrolytic products (S4).

No significant differences ($p \leq 0.05$) were found between molecular masses of unitary molecules of globulin-P treated with papain under mild conditions (C2 and C3, 270 ± 6 kDa) and those corresponding to nonhydrolyzed glb-P (C1, 277 ± 6kDa). Under intermediate (C4, C5, and C6) and extreme (C7 and C8) conditions, the molecular masses of glb-P unitary molecules were 241 ± 5 kDa and 240 ± 6 kDa, respectively. These molecules were smaller ($p \leq 0.05$) than the nonhydrolyzed molecules and similar to one another.

Figure 3 shows the reducing SDS−PAGE patterns of S1 and S2 components from nonhydrolyzed and mildly hydrolyzed glb-P. Samples analyzed were the FPLC fractions corresponding to C1, C2, and C3 profiles. As

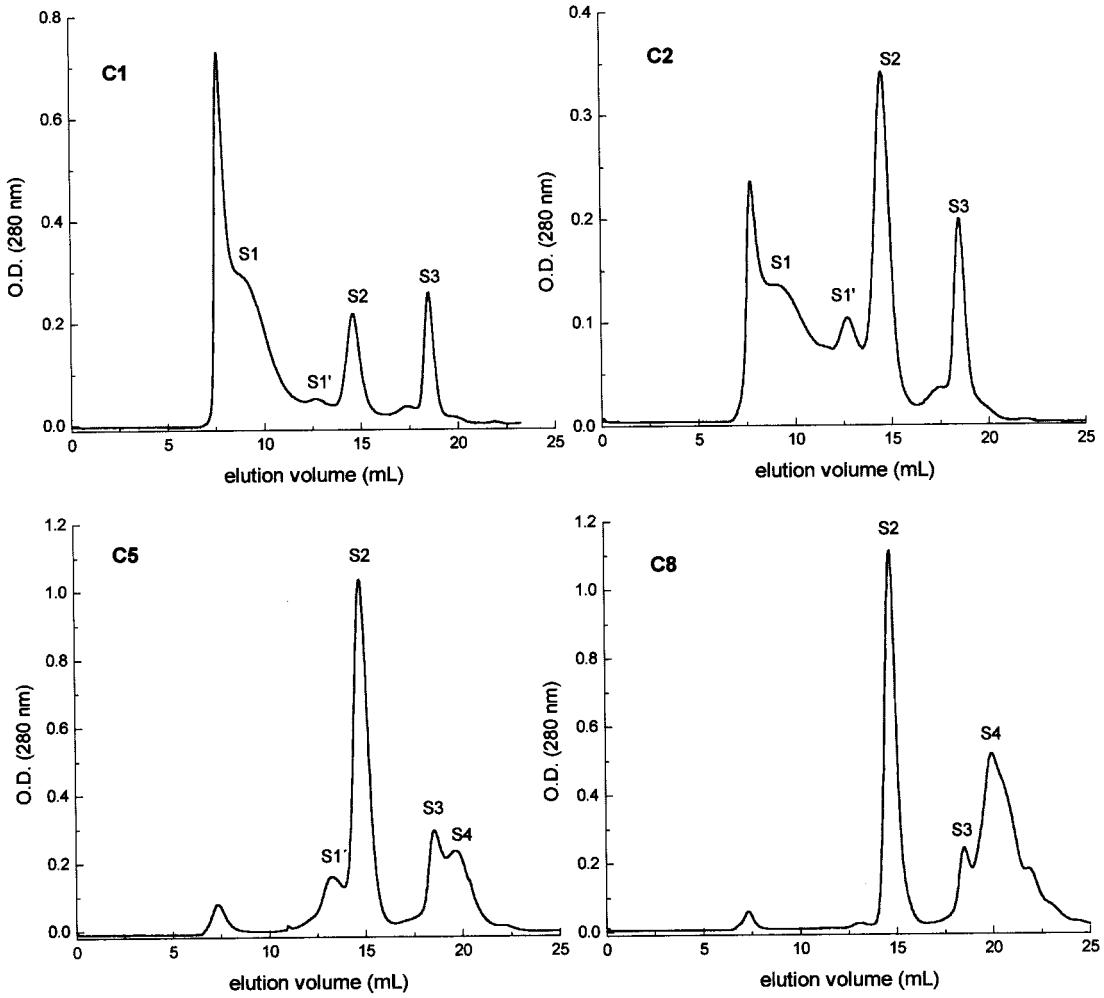

Figure 1. FPLC gel-filtration profiles of crude and hydrolyzed globulin-P. S1, polymers; S1′, oligomers; S2, unitary molecules; S3 and S4, low molecular masses polypeptides. C1, C2, C5, and C8 indicate different conditions of the previous hydrolytic treatment (see Table 1 in materials and methods).

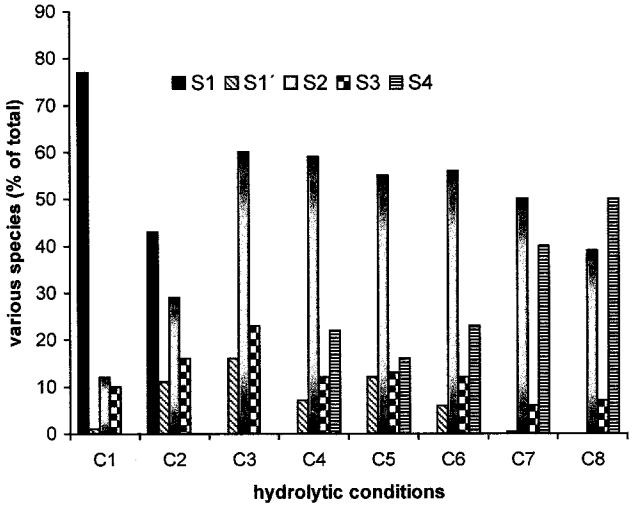

Figure 2. Percentage of globulin-P species (S1, S1′, S2, S3, and S4) present in the different hydrolytic conditions (C1 to C8, see Table 1, materials and methods). Percentages were calculated from FPLC profile areas.

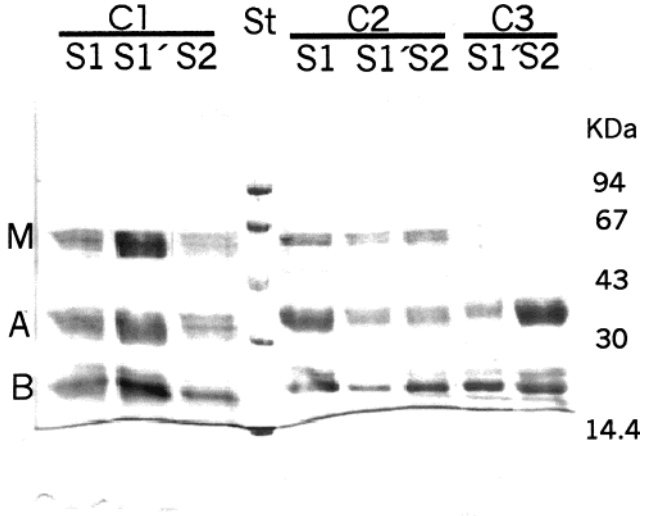

Figure 3. SDS−PAGE in reducing conditions of fractions corresponding to S1, S1′ and S2 species from C1, C2, and C3 gel filtration chromatographies. The acrylamide concentration was 12% (w/v). St, standard proteins (molecular weights are indicated on the right side). Main polypeptides are shown on the left side.

already reported by Martínez et al., 1997, the nonhydrolyzed glb-P pattern contained bands in the 20 kDa zone (B polypeptides) and in the 30 kDa region (A polypeptides), and a band corresponding to a 54 kDa polypeptidic subunit (M). This subunit was present in the S1, S1′, and S2 components of nonhydrolyzed glb-P (C1) and also in glb-P hydrolyzed with C2 treatment. On the contrary, M polypeptide was absent from the S1′

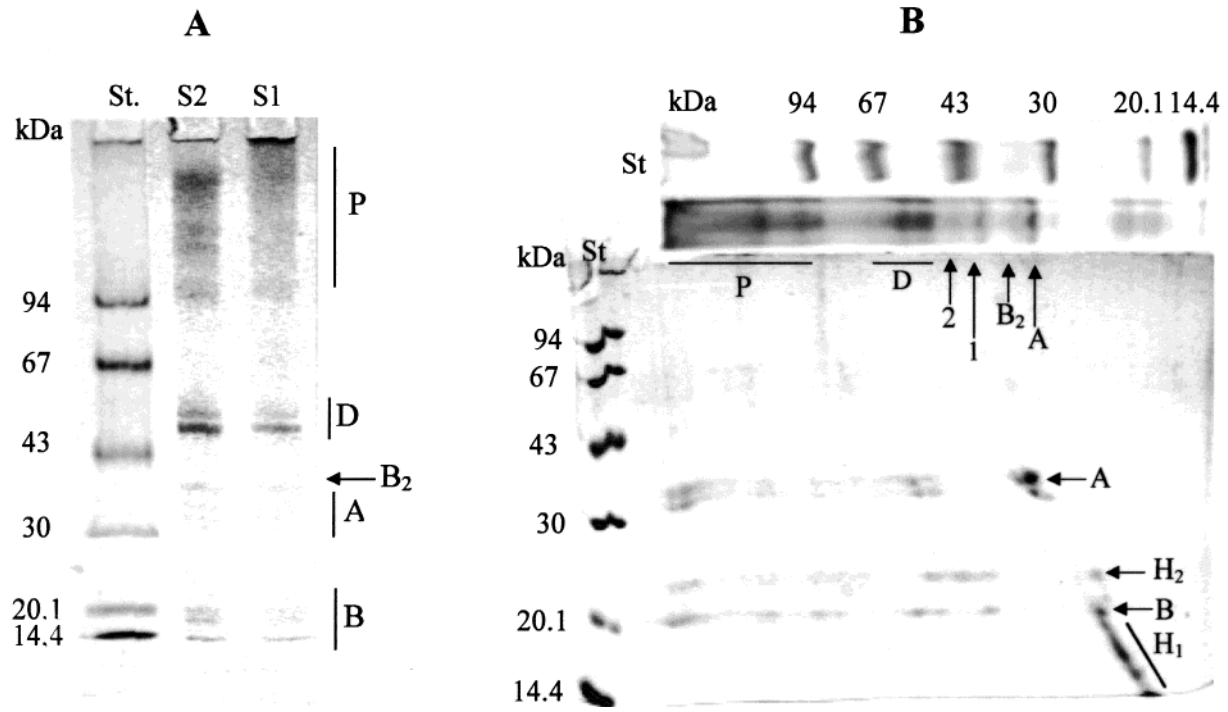

Figure 4. (A) SDS−PAGE patterns of the S1 and S2 species of unhydrolyzed globulin-P (C1). Main polypeptides are shown on the right. St, standard molecular mass proteins. (B) bi-dimensional (SDS → SDS + 2-ME) electrophoretic pattern of globulin-P treated under condition C6 (see Table 1). St, standard molecular mass proteins. The first-dimension gel was 6−12% polyacrylamide and the second-dimension gel was 12% polyacrylamide.

and S2 components of glb-P hydrolyzed in condition C3 (Figure 3). SDS−PAGE patterns of components of glb-P submitted to C4 to C8 treatments also lacked the M subunit band (C6, C7, and C8 are shown in Figures 4, 6, and 7, respectively).

These results show that under the hydrolytic conditions where polymers were absent, the subunit M is not present. The positive correlation between the disappearance of polymers and M polypeptide was also observed in amaranth protein isolates hydrolyzed with papain or cucurbita (Scilingo, personal communication). These results suggest that the M subunit might participate in the stabilization of glb-P polymers.

Polypeptides integrating hydrolyzed glb-P unitary molecules (S2) were analyzed by bi-dimensional electrophoresis. Figure 4a shows the nonreducing patterns of nonhydrolyzed glb-P corresponding to a control. The patterns of S1 and S2 show as main components the high-molecular-mass polypeptides (P) and the dimeric subunits (D). Also present are the A and B polypeptides and B_2 band. As reported by Martínez et al. (1997), the P polypeptides were composed of the M subunit and the A and B polypeptides, D subunits were integrated by A and B polypeptides (model in Figure 5). B_2 was also present, and it was described as a dimer composed of two polypeptides of the same size as B polypeptides (Martínez et al., 1997).

Figure 4b shows the first- and second-dimension patterns of glb-P under intermediate hydrolytic conditions (C6). In the first dimension, the bands in the region of molecular masses lower than 30 kDa represent monomeric chains which run in the diagonal of the second dimension where they are labeled as B, H1, and H2. B identifies free B polypeptides, and H1 and H2 correspond to hydrolytic products, as shown in the model of Figure 5. Above 30 kDa the first dimension shows bands A, B_2, 1, 2, D, and P. In the second

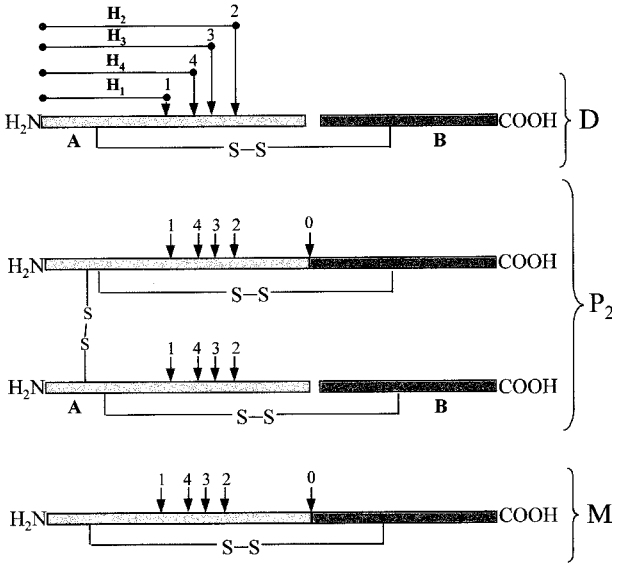

Figure 5. Model of glb-P polypeptides and hypothetical points of cleavage. M, monomeric subunit; D, dimeric subunit; P_2, minor high molecular mass polypeptide; A and B, monomeric polypeptides; H_1, H_2, H_3, and H_4, hydrolyzed peptides. Arrows indicate the points of cleavage numbered from 0 to 4.

dimension A runs on the diagonal corresponding to monomeric chains, B_2 is formed by two polypeptides of the same size as B polypeptides, 1 is composed of H2 and B chains, and 2 may be integrated by two H2 polypeptides. The second dimension diagram shows that D bands are formed by chains A, H2, and B. This suggests the presence of hydrolysis products coming from P polypeptides in addition to the D subunits in the D region of the first dimension. H2 polypeptides may also come from an hydrolyzed M subunit having intrachain disulfide bridges (Figure 5). P-aggregated polypeptides (first dimension) were integrated by A, B, and H2

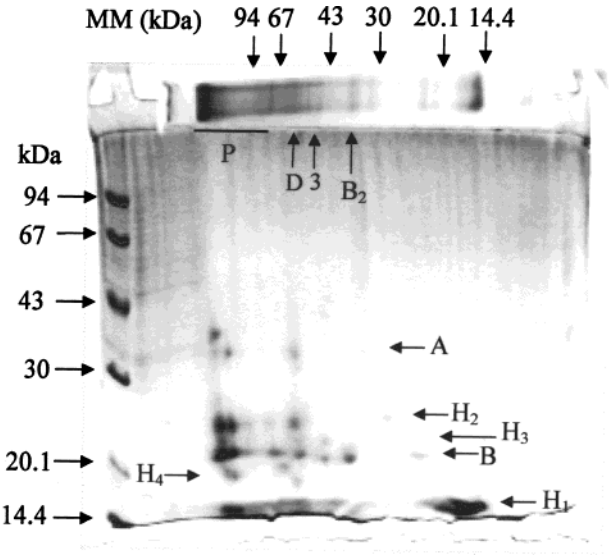

Figure 6. Bidimensional (SDS → SDS + 2-ME) electrophoretic pattern of globulin-P treated under condition C7 (see Table 1). In the first dimension standard molecular masses (kDa) are indicated on the top. St, standard molecular mass proteins of second dimension. First- and second-dimension gels were 12% polyacrylamide.

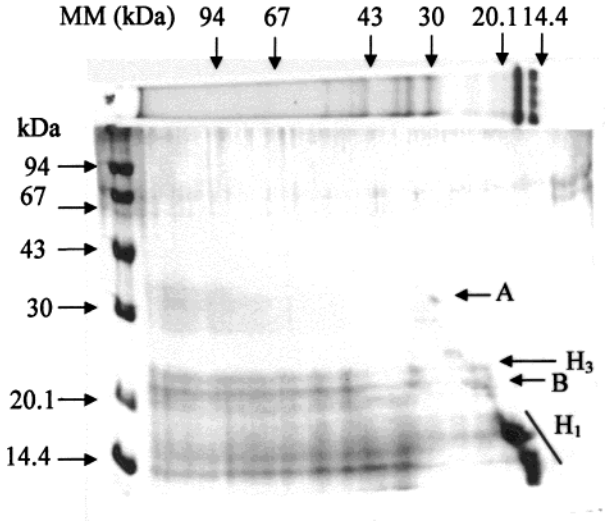

Figure 7. Bidimensional (SDS → SDS + 2-ME) electrophoretic pattern of the globulin P treated under condition C8 (see Table 1). In the first dimension standard molecular masses (kDa) are indicated on the top. St, standard molecular mass proteins of second dimension. First-dimension gel was 6−12% of polyacrylamide and second-dimension gel was 12% of polyacrylamide.

chains (second dimension) suggesting the presence of hydrolyzed aggregates coming from higher-molecular-mass polypeptides.

As shown in Figure 6 stronger hydrolytic treatment caused the decrease of A polypeptides and an increase in the intensity of the hydrolysis products H1. These chains were present as free polypeptides in the second dimension diagonal and composing dimeric and polymeric hydrolyzed subunits. A new species (band 3 in the first dimension) formed by the polypeptide B and the H3 polypeptide was also present. This polypeptide, smaller than H2, may come from a further processing of A chain (Figure 5).

In the pattern displayed in Figure 6, B_2 band (first dimension) was more intense than in the profile of Figure 4b indicating that new B_2 polypeptides have been formed. This may be explained considering that the new B_2 are hydrolytic products of D subunits in which A polypeptides were excised to the same size as B chains. Concerning the first dimension, bands in the D and P regions were mainly formed by B chains and H1 and H2 hydrolysis products, according to the second dimension pattern. They also contained a low amount of A chains and H4 hydrolysis products.

At even stronger hydrolytic conditions (Figure 7, first dimension) the major species were hydrolysis products of low (H1), intermediate (between 30 and 67 kDa), and high (higher than 67 kDa) molecular masses. H1 chains were found as spots on the second dimension diagonal corresponding to free polypeptides. They were also bound to B chains composing hydrolysis products of intermediate and high molecular masses as shown by their position in the second dimension. The profile obtained indicated that nonhydrolyzed D subunits and P polypeptides were absent in this hydrolytic condition because there was no A chain on the second dimension under D and P regions. Nonhydrolyzed A chains were found in low amount only as free polypeptides on the diagonal of the second dimension profile.

DISCUSSION

The different hydrolytic conditions studied in this work may correspond to different stages of the papain hydrolytic process. From our results, it can be argued that the first structural modification under mild papain hydrolytic conditions was the disruption of polymers which turned into their unitary constituents, the 280 kDa molecules. In the first stages, these molecules, which can be considered high-molecular-weight hydrolysis products, showed a molecular mass not different ($p \leq 0.05$) from that of the nonhydrolyzed unitary molecules. Under stronger hydrolytic conditions, the hydrolyzed glb-P (glb-PH) unitary molecules were 13% smaller than those of the nonhydrolyzed globulin. This result was similar to those reported for 11S-globulins from soybean, faba bean, and pea hydrolyzed with trypsin. The results also showed a high-molecular-weight intermediary smaller than the nonhydrolyzed molecule (Shutov et al., 1991; Schwenke et al., 1995; Plumb et al., 1989). The amaranth glb-PH might have released hydrolyzed peptides as other hydrolyzed 11S-globulins have done (Plumb et al., 1994; Henning et al., 1998).

The glb-PH unitary molecules remained assembled, even though they contained degraded polypeptides. These chains may be joined by secondary forces and disulfide bridges. Only under the described hydrolytic conditions where almost every polypeptide was cut (C7 condition) glb-PH unitary molecules began to disassemble.

Regarding the results obtained for polypeptide analyses, early hydrolysis of the M subunit suggested that these subunits might be located in an exposed site of the molecules. M subunits might stabilize glb-P polymers by disulfide bridges with subunits of other unitary molecules. They may also have intramolecular disulfide linkages, as well as disulfide linkages with subunits of the same unitary molecule. These interactions would originate high-molecular-mass polypeptides P after glb-P SDS dissociation as shown in the SDS−PAGE patterns (described in Figure 5 model). For this reason, the hydrolytic cleavage of M subunits may lead to products of many different sizes.

In patterns of glb-PH under conditions where the M subunit was absent, monomeric polypeptides of sizes higher than A chains were not detected. This result suggested that the site of cleavage may be located to give products of the same size as A and B chains (site of cleavage 0 in the model of Figure 5). Considering the proposal that M is an unprocessed A S−S B subunit (Martínez et al., 1997; Barba de la Rosa et al., 1996) and the unitary molecules are hexamers as other 11S globulins as reported by Marcone et al. (1994), the region between the domains A and B may be more flexible and exposed for the enzymatic cleavage. Another possibility would be that, after hydrolysis, M turned into two peptides of the same size as H2 peptides (site of cleavage 2 instead of 0 in the model of Figure 5).

Other possibilities cannot be disregarded, such as that the M subunit had been completely excised giving products smaller than the B polypeptide, or that the hydrolyzed M subunit had been released from the unitary molecule. However, these two proposals are improbable because (1) the bidimensional pattern glb-PH molecules with intermediate treatment (Figure 4b) do not show an amount of low-molecular-mass polypeptides equivalent to the amount of M subunit in the nonhydrolyzed sample; and (2) the glb-PH molecules have decreased their molecular masses by 13% but not by 23% which is the mass proportion corresponding to M polypeptides (results not shown).

Electrophoretic results also showed that following M subunit, A chains are preferentially attacked by the protease, whereas B chains remained with a size similar to the original one. This behavior was similar to that observed with 11S globulins (Kamata and Shibasaki, 1978), which structure was proposed to consist of the basic chains buried within the interior of the protein molecule and the A chains more exposed (Lambert and Yarwood, 1992). This similarity led us to suppose that glb-P molecules, and also amaranth 11S-globulin, have structures similar to those of legumins: with A chains exposed to the outside. At the same time, these results agreed the model proposed by Marcone et al. (1994).

Considering that the first proteases acting in protein mobilization during germination are cysteine proteases such as papain (Müntz, 1996), it is legitimate to think that events similar to those described in this paper might carry out globulin-P during amaranth grain germination.

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