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Surface Physicochemical Properties of Globulin-P Amaranth Protein

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Globulin-P, the polymerized 11S amaranth globulin, is composed of 280 kDa unitary molecules (UM, 23%) and aggregates larger than 500 kDa (A, 70%). Antibodies against these proteins were prepared to study their surface characteristics and to assess their homology with other storage proteins. Results showed that globulin-P unitary molecules and aggregates had similar reactive surfaces. A polypeptide of 56 kDa was found to be the most reactive to the antibodies assayed, followed by the acidic polypeptides. Such results support previous information, according to which these polypeptides appeared to be the most exposed on the molecule surface. Globulin-P fraction presented cross-reactivity with the remaining amaranth protein fractions: 11S-globulin, glutelins, and albumins. Globulin-P and 11S-globulin showed similar reactive surfaces whereas glutelin and albumins presented a lower cross-reactivity. The reactivity of the glutelin fraction depended on its sequence. Globulin-P fraction presented cross-reactivity with quinoa globulins, and to a lesser extent with globulins of sunflower and rice. Moreover, the anti-Gp serum was unable to detect either conformational or sequence epitopes in globulins of soybean, wheat, buckwheat, rice, and rye.

KEYWORDS: amaranth proteins; globulin-P; surface properties; cross-reactivity

INTRODUCTION

Amaranth is a crop that can be grown in different soils and climates, being resistant to high temperatures, drought, and some pests (*1*). Interestingly, its seeds as well as its leaves may be used as food. Amaranth seeds contain a large proportion of proteins (17−19% w/w) of high nutritional value. While amaranth is dicotyledonous, it shares some characteristics with cereals and for this reason is classified as a pseudocereal (*2*).

Globulin-P is one of the main amaranth proteins; it shows some peculiarities that make it a hallmark of amaranth. Globulin-P is extracted with water after globulin extraction (*3*, *4*). The proteins of this fraction have high concentrations of the essential amino acids Val, Ile, and Leu. (*5*). Globulin-P revealed a strong trend toward polymerizing; it is composed of 75% of high molecular mass aggregates and 23% of unitary molecules of 280 kDa (*6*). Its polypeptidic composition is similar to that of 11S globulins; it contains A and B polypeptides linked by a disulfide bond, but it also contains higher amounts of a distinctive polypeptide of 56 kDa (P56), which is involved in the polymer stabilization (*6*). According to Gorinstein et al. (*7*), the proteins of this fraction have a less compact and more hydrophobic structure than that of 11S-globulins. Globulin-P shares some structural characteristics with 11S-globulins and other structural and solubility properties with glutelins and prolamins (*4*, *8*, *9*).

Different studies have been performed to determine the surface homology level and conformation in different vegetable storage proteins (*10*−*13*). In most cases, an immunochemical approach has been used on the basis of the specificity of reaction between the antibodies used and the proteins to be analyzed. Results obtained have shown similarities in the primary amino acid sequences as well as in the conformation of different 11S seed globulins from monocotyledonous and dicotyledonous plants (*13*). Carter et al. (*14*) used a polyclonal antibody against the soybean 11S storage protein; they found cross-reactivity between pea 11S-globulin and the 7S-globulins of soybean and pea, suggesting homologies between 11S and 7S storage proteins. Barroto and Dure (*15*) have also shown homology between soybean 11S and oat 12S proteins by dot matrix analysis of basic subunit amino acid sequences. On the other hand, Vasco-Méndez and Paredes-Lopez (*12*), have suggested a structural homology between amaranth glutelin and globulin fractions.

The aim of this work was to obtain information about surface characteristics of globulin-P using an immunochemical approach and to assess the homology (sequential and/or conformational similarities) that this protein may have with other amaranth protein fractions and seed storage proteins.

MATERIALS AND METHODS

Plant Materials. *Amaranth hypochondriacus* (Mercado cultivar) was grown at the Experimental Station of the Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Chapingo,

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Mexico. The harvested seeds were kindly donated to our laboratory. Mature seeds were ground in an Udy mill (Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina), 1-mm mesh and screened at 10-mm mesh. Flour was defatted using hexane in a 10% (w/v) suspension under continuous stirring for 24 h, 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 Kjeldhal method, (*16*) using the coefficient 5.85 (*8*).

Protein Isolation. Amaranth protein fractions were prepared according to the procedure reported elsewhere (*4*). The defatted amaranth flour was successively treated three times with water to extract albumin, three times with buffer A (32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4, pH 7.5, 0.4 M NaCl) for 11S-globulin extraction, three times with water for globulin-P extraction, and three times with 0.1N NaOH to extract glutelins. Each extraction step, at a ratio of 10 mL solvent/g flour, was performed at room temperature, and after each treatment, the extraction residue was separated by centrifugation at 9000*g* for 20 min at room temperature. Globulin-P, 11S-globulin and glutelins were isolated from the corresponding supernatants by precipitation at pH 6 using 2 N HCl. The precipitates were suspended in water, neutralized with 0.1 N NaOH, and freeze-dried.

Purification of Globulin-P. The components of globulin-P, unitary molecules of 280 kDa (UM) and aggregates of molecular mass > 500 kDa (A) 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. The column was packed and equilibrated with three bed volumes of buffer B (33.3 mM K_2HPO_4, 1.7 mM KH_2PO_4, pH 8.5) containing 0.02% (w/v) NaN_3.

A 4-mL sample containing 50 mg of globulin-P was put 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 pressed using a Gilson Minipuls 2 peristaltic pump. Fractions (3 mL) were collected with a Buchler (Fractomet e Alpha 400) fraction collector. Absorbance at 280 nm was measured with a UV Bio-Rad (Econo UV) detector. Protein elution profile was plotted using a Gilson N2 recorder. Fractions corresponding both to unitary molecules (UM) or aggregates (A) were pooled and concentrated with an Amicon 8020 (Amicon Inc.) ultrafiltration cell using a 10 000 Da pore membrane (Amicon, YM10). The resulting fractions were lyophilized and stored at −20 °C.

Production of Rabbit Polyclonal Antibodies. Two polyclonal antibodies were prepared by immunizing rabbits either with globulin-P UM or A. New Zealand White rabbits were intradermally immunized with 0.4 mL (1 mg/mL) of globulin-P components in phosphate buffered saline (PBS) with Freunds complete adjuvant. They were later inoculated every 3 weeks with the same immunogen prepared in Freunds incomplete adjuvant. Serum samples were analyzed 7−10 days after each booster.

Enzymatic Treatments. Globulin-P was hydrolyzed with two enzymes, papain and trypsin. Two sets of globulin-P suspensions in buffer B (30 mg/mL) were prepared for hydrolysis. One set was hydrolyzed with trypsin (Type III from bovine pancreas, Sigma Chem. Co., St. Louis, MO) 14 IU/mg protein, while the other was hydrolyzed with papain (Sigma Chem. Co., St. Louis, MO) 0.018 IU/mg protein. Each suspension corresponded to one time of reaction. After incubation at 25 °C at the time points indicated in **Figure 3**, the enzymatic reactions were stopped by adding cold 0.1 N NaOH. An equal volume of SDS-PAGE sample buffer (reducing conditions) was added to each sample, then they were analyzed by SDS-PAGE and western blotting.

Sodium-Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS−PAGE). Gels were prepared in minislabs (Bio Rad Protean II Model). Runs were carried out as described elsewhere (*4*), using linear gradient (6−12% in polyacrylamide) gels. Molecular masses of the polypeptides were calculated using the following protein standards (Pharmacia): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovoalbumin (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% w/v sodium dodecyl sulfate and 0.05% w/v bromophenol blue). Under reducing conditions, 5% v/v 2-mercaptoethanol was added, and samples were heated at 100 °C for 3 min. Gels were fixed and stained with Coomasie Brillant Blue.

Western-Blot Analysis. Following separation by SDS-PAGE, the protein fractions were wet-blotted onto nitrocellulose supporter at 250 mA for 1 h using buffer containing 20 mM Tris, 0.193 M glycine, 20% methanol, pH 8.35 in Transblot System equipment (BIO-RAD, Richmond, Calif., USA).

Western analyses were performed using the method described by Towbin et al. (*17*). The nitrocellulose membranes were blocked with a solution of TBS (50 mM Tris-HCl, 150 mM NaCl pH 7.4) containing 2% skimmed milk. After incubation overnight at 4 °C, a dilution of the polyclonal antiserum was added. After washing with 0.05% Tween/TBS, immunoreactive proteins were detected by incubating the membrane in the presence of peroxidase−conjugate goat−anti-rabbit immunoglobulin G. A solution (3 mg/mL) containing 4-chloronaphthol in methanol, TBS and 30% H_2O_2 was used as a substrate for the peroxidase. Immunoreactive peptides were visualized as black bands following the enzymatic reaction.

ELISA. Polystyrene strips (Maxisorp; Nunc, Roskilde, Denmark) were sensitized overnight at 4 °C with antigen solutions diluted to 0.1 μg protein/well with buffer B, pH 10, 0.1 M borate buffer, 9 M urea, or pH 10, 0.1 M borate buffer + 0.1% SDS, as indicated in the text. After washing three times with PBS−Tween 20 0.05% (PBS−T), the plates were blocked with 200 μL/well of a solution of PBS containing 3% skimmed milk. After incubating for 2 h at 37 °C, the plates were washed with PBS-T, and serial dilutions in PBS-T containing 1% skimmed milk (diluent solution) of the anti-Gp serum were dispensed. After incubating for 1h at 37 °C, they were washed with PBS-T and 100 μL/well of a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (BioRad, diluted 1:8000 in diluent solution) were added. The plates were incubated for 1 h at 37 °C. After washing with PBS-T, the content of each well was developed with a solution containing *o*-phenylenediamine (1 mg/mL, Merk, Darmstadt, Germany) and 30% H_2O_2 (1μL/mL) in 0.1 M citrate−phosphate buffer pH 5.0; the reaction was stopped after 20 min with 40 μL/well of 4 N H_2SO_4. Optical density (OD) was determined at 492 nm with a Tecan detector (Spectra Rainbow SLT).

Experiments were performed at least in duplicate. Each experiment consisted of three titration curves per protein fraction in each condition. The data so obtained were statistically evaluated by variance analysis (ANOVA). The comparison of means was done by the least significant difference (LSD) test at a significance level (α) of 0.05.

Competitive ELISA. Polystyrene strips were sensitized with protein fractions diluted in buffer B, washed, and blocked as previously described. Serial dilutions of albumin, 11S-globulin or globulin-P were prepared in buffer B or borate buffer pH 10.0 where indicated, providing solutions ranging from 0.8 ng/mL to 4×10^4 ng/mL of protein. Equal volumes of these antigen solutions and antibody solutions (1:100 000 dilution) were mixed and incubated overnight at 4 °C in plastic tubes (preincubation). Then, 100 μL/well of each sample was incubated for 30 min at 37 °C in the coated wells. After washing, the plates were incubated with goat anti-rabbit IgG HRP conjugate (BioRad, diluted 1:8000 in diluent solution), 1 h at 37 °C. After three cycles of washing with PBS-T, color reaction was developed, stopped, and then absorbance at 492 nm was determined as described previously.

RESULTS AND DISCUSSION

Characterization of Antisera. Both anti-UM and anti-A antibodies evidenced a similar reactivity when analyzed by ELISA using unitary molecules (UM, 280 kDa) or globulin-P aggregates (A > 500 kDa) as antigen (**Figure 1**). Hence, quite similar reactive surfaces would exist in both types of molecules.

The difference of titer detected between unitary molecules ($3.0 \pm 0.3 \times 10^5$) and aggregates ($1.5 \pm 0.2 \times l0^6$) could be due to an improved microplate coating with the aggregates, which would present a greater number of reactive sites and/or a spatial arrangement of epitopes, thereby favoring multiple interactions of the aggregates with the antibodies. Consequently, the antibodies would interact with aggregates rather than with the UM.

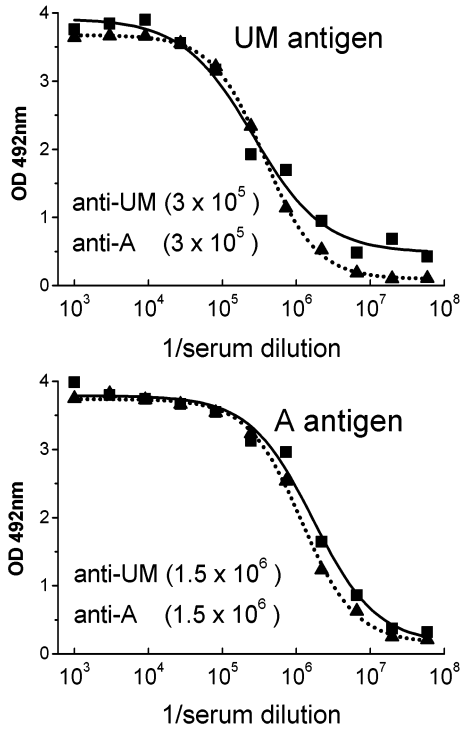

Figure 1. Direct ELISA. Titration curves for anti-unitary molecules (■ anti-UM) and anti-globulin-P-aggregates (● anti-A) sera, with UM (Gp-unitary molecules) and A (Gp-aggregates) antigens. Titers are indicated in the figure.

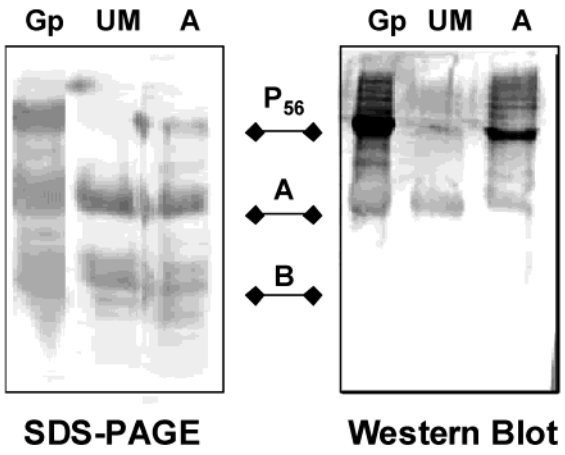

Figure 2. SDS-PAGE and western blot of globulin-P and its purified components, unitary molecules (UM) and aggregates (A). Western blot of the gel was performed using anti-UM serum diluted 1:3000 (v/v). Main polypeptides, P56, A and B, are indicated in the figure.

Characterization of antigenic compounds in the globulin-P fraction. Total globulin-P fraction and purified unitary molecules and aggregates (Materials and Methods) were subjected to western blot analysis for identification of the antigenic compounds. Both types of antibodies assayed (anti-UM and anti-A) yielded the same reactivity; hence, only those results obtained with anti-UM serum are depicted in **Figure 2**. The SDS-PAGE profiles of globulin-P and aggregates (**Figure 2**, SDS-PAGE, lanes Gp and A) evidenced equivalent protein species. There, the P56, acidic, A (30−32 kDa) and basic, B (20−22 kDa) polypeptides were clearly observed.

Unlike these fractions, globulin-P unitary molecules presented a slightly lower content of the polypeptide P56 (**Figure 2**, SDS-PAGE, lane UM). In all the western blot profiles (**Figure 2**, western blot), the 56 kDa polypeptide was found to be the most reactive, followed by the A polypeptides in a minor proportion. Western blots of globulin-P and its aggregates showed series of high molecular weight reactive protein species (MM>80 kDa), though they could not be detected in the SDS-PAGE profiles. These high molecular weight polypeptides have been studied in globulin-P in the absence of 2-mercaptoethanol, evidencing high content of P56 (*18*).

Considering the high reactivity of P56 to the anti-Gp and its sensitivity to a protease action (*18*), the time course of globulin-P hydrolysis by trypsin and papain under mild conditions was analyzed by western blot utilizing the anti-Gp serum. Results are shown in **Figure 3**. SDS profiles of trypsin-treated globulin-P showed that the polypeptides produced by hydrolysis were four bands in the molecular weight range 40−50 kDa, which were weakly stained on the western blot and are presumably products of P56 degradation. There was considerable residual P56. The polypeptides A, at 30 kDa, were almost completely hydrolyzed by 120 min (i.e., 30 000 polypeptides were not produced with this enzyme). With papain, there were also considerable undigested P56 and western positive hydrolysis products between 56 and 30 kDa. It is difficult to say whether the 30 kDa band is unhydrolyzed polypeptide A or a hydrolysis product of P56.

SDS-PAGE profiles indicated that the polypeptides A are more sensitive to trypsin and papain than P56 in these conditions. Western-blot results suggested that some P56 epitopes remained after the two proteases action.

Cross-Reactivity between Different Amaranth Protein Fractions. *Western Blots.* Reactivity of the different protein fractions of amaranth (albumins, 11S-globulin, globulin-P, and glutelin) against anti-UM serum was compared. The SDS-PAGE profiles of globulin-P, 11S-globulin, and glutelin fractions were found to be quite similar (**Figure 4** SDS-PAGE, lanes Gp, G, and Gt). Their differences lay only in the P56 polypeptide content (which is low in 11S-globulin fractions), and in the quality and quantity of those polypeptides with a molecular mass lower than 25 kDa. The albumin fraction showed a different profile with a greater proportion of protein species ranging from 20 to around 35 kDa. The corresponding western blots (**Figure 4**) documented that the albumin fraction was not responsive to anti-UM serum, while the other fractions, globulin-P, 11S-globulin, and glutelins, evidenced cross reactivity, suggesting certain homology among their polypeptides. Again, P56 was the most reactive polypeptide, with a much lower content in the 11S-globulin fraction. Finally, minor reactivity could be

Table 1. Anti-Gp Titers against the Amaranth Protein Fractions Dissolved in Different Media (Means ± SD)

	buffer B	borate pH 10	9 M urea	borate–SDS
globulin-P	$1.1 \pm 0.5 \times 10^{5(e)}$	$4.3 \pm 0{,}1 \times 10^{5(g)}$	$1.2 \pm 0{,}2 \times 10^{4(a,b,c)}$	$1.3 \pm 0{,}2 \times 10^{4(b,c)}$
11S-globulin	$3.3 \pm 0.1 \times 10^{4(d)}$	$3.2 \pm 0{,}1 \times 10^{5(f)}$	$6 \pm 3 \times 10^{3(a,b,c)}$	$1.4 \pm 0{,}1 \times 10^{4(c)}$
glutelin	$3.2 \pm 0.3 \times 10^{3(a)}$	$8.9 \pm 0{,}5 \times 10^{3(a,b,c)}$	$5 \pm 2 \times 10^{3(a,b)}$	$7.1 \pm 0{,}2 \times 10^{3(a,b,c)}$
albumin	$3.8 \pm 0.1 \times 10^{4(d)}$	$9.3 \pm 0{,}1 \times 10^{3(a,b,c)}$	$3 \pm 2 \times 10^{3(a)}$	$3 \pm 2 \times 10^{3(a,b,c)}$

[a−f] Different letters denote significant differences at $P < 0.05$.

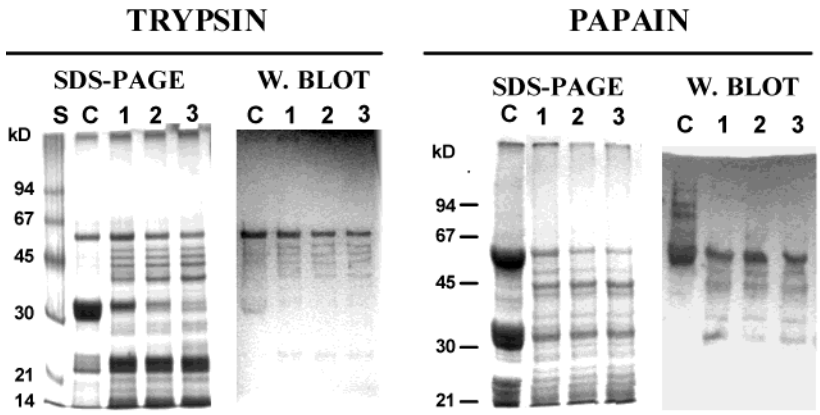

Figure 3. SDS-PAGE and western blot of globulin-p (Gp) hydrolyzed with trypsin and with papain. Western blot of the gels was performed using anti-UM serum diluted 1:3000 (v/v). Trypsin: standard proteins, lane S; unhydrolyzed Gp, lane C; Gp hydrolyzed for 5 min, lane 1; 30 min, lane 2; 120 min, lane 3. Molecular weights are indicated on the left side. Papain: unhydrolyzed Gp, lane C; Gp hydrolyzed for 1 min, lane 1; 30 min, lane 2; 120 min, lane 3. Molecular weights are indicated on the left side.

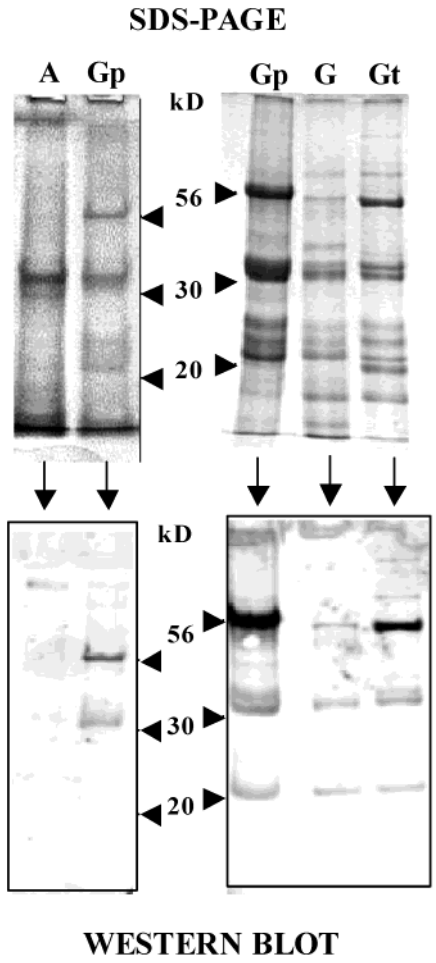

Figure 4. SDS-PAGE and western blot of amaranth protein fractions. Albumin, lane A; globulin-P, lane Gp; globulin, lane G; glutelin, lane Gt. Western blot of the gels was performed using anti-UM serum diluted 1:3000 (v/v). Molecular masses are indicated in the middle.

detected in two molecular species around 30 (polypeptides A) and 20 kDa (polypeptides B).

ELISA. Globulin-P, 11S-globulin, albumins, and glutelin partially purified fractions were analyzed via ELISA. The corresponding curves and titers are described in **Figure 5** and **Table 1**. In buffer B, globulin-P showed an enhanced reactivity when compared with that of the other fractions. It must be noted that glutelin is rather insoluble in this buffer (*19*, *20*), and that 11S-globulin also presents low solubility in the same buffer, though in both cases there is enough soluble protein for the plates to be sensitized. Albumin showed certain reactivity, though lower than that of globulin-P, which is not in agreement with the results obtained by western blot.

The solubility of 11S-globulin and glutelin increased in pH 10 borate buffer, and there is a partial unfolding of protein (*8*, *20*, *21*). In these conditions (**Figure 5**), similar titers were observed for both globulin-P and 11S-globulin, whereas they were 10-fold lower for glutelins, and even lesser for albumins. Such differences could be primarily ascribed to fewer reactive epitopes in glutelins and albumins. It is interesting to remark that globulin-P maintained almost similar titers either in buffer B or in borate buffer; the titer of glutelin and 11S-globulin is 10-fold higher in borate buffer than in buffer B; whereas albumins show a lower titer in borate than in buffer B. In view of the fact that glutelins are less aggregated in borate buffer than in buffer B (*20*), it was reasonable to assume that the same occurred with 11S-globulin. It seems that these fractions showed lower reactivity in buffer B than in borate buffer due to the large amount of aggregates. Such aggregates could present less reactive sites when 11S-globulins and glutelin are embedded in the microplate. On the other hand, the behavior of albumins could be related to the loss of conformational epitopes.

To corroborate the above assumptions, the assay was performed again in the presence of sodium dodecyl sulfate and 9 M urea, which promote the unfolding of protein molecules (**Figure 5**). These reagents may also affect the binding of the proteins to the wells. Under both conditions, the titers of globulin-P and 11S-globulin diminished while glutelins showed a titer similar to that found in the other conditions, and albumins presented low reactivity.

The rationale for ELISA results is that both globulin-P and 11S-globulin have a greater number of antigenic determinants detected by the polyclonal antiserum and that glutelins and globulins would have a different tridimensional structure. Moreover, considering only the unfolding effect of SDS and urea, the reactivity of the two globulins would depend on their structure, whereas that of glutelins would be related to its sequence. The behavior of albumins, opposite to that of glutelins, could be explained by assuming that albumin reactivity with anti-UM serum would be due to the presence of conformational epitopes in this fraction.

Competition Assays. Different competition assays were carried out to determine relative binding strengths. Proteins were

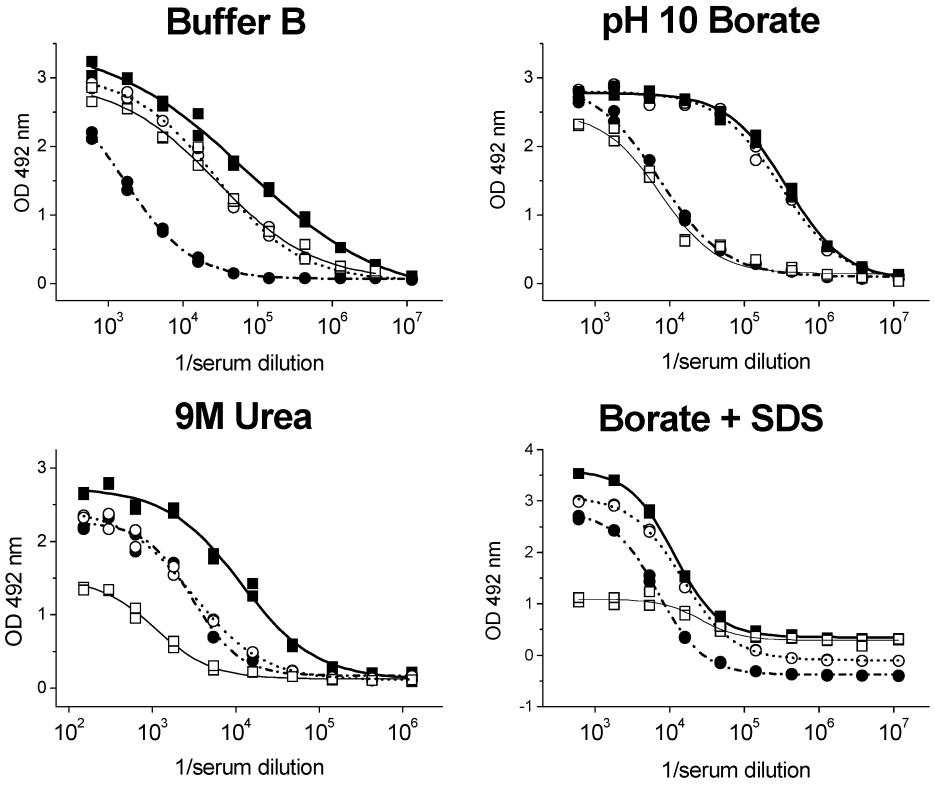

Figure 5. Direct ELISA. Titration curves for anti-Gp-unitary molecules (anti-UM) serum using amaranth protein fractions as antigens. ■, globulin-P; ○, 11S-globulin; ●, glutelin; □, albumin. Buffer B: antigens in buffer B, pH 10 Borate; antigens in 0.1 M pH 10 borate buffer, 9 M Urea; antigens in 9 M urea, borate + SDS; antigens in 0.1 M pH 10 borate buffer, 1% SDS.

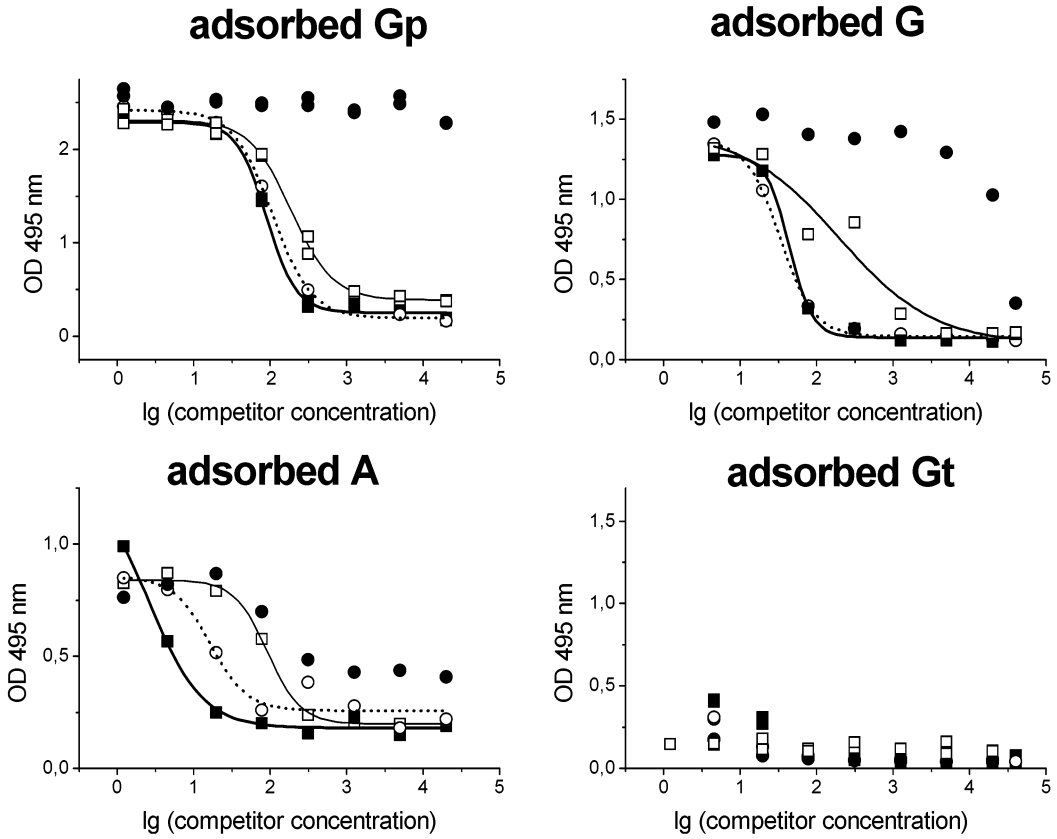

Figure 6. Titration curves for anti-UM serum in the competitive ELISA. Competitors: ■, Globulin-P; ○, 11S-globulin; ●, Glutelin; □, Albumin. Protein adsorbed, globulin-P, Gp; 11S-globulin, G; glutelin, Gt; albumin, A. Proteins were in buffer B. Assays were performed with the serum diluted 1:100 000 (v/v).

dissolved using either buffer B (**Figure 6**) or pH 10, 0.1 M borate buffer (**Figure 7**).

Results showed that globulin-P and 11S-globulin were competitors, whereas the albumin fraction, although also

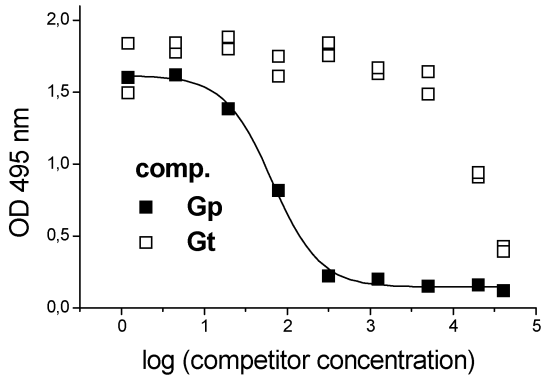

Figure 7. Titration curves for anti-UM serum in the competitive ELISA. Protein adsorbed, globulin-P diluted in buffer B. Competitors: ■, Globulin-P; □, Glutelin. The competitors were diluted in 0.1 M, pH 10 borate buffer. Assays were performed with the serum diluted 1:100 000 (v/v).

Table 2. Percentage Cross-Reaction of Anti-Gp Serum with Globulins from Different Special

globulin	percentage reactivity
amaranth	100
wheat	3.2 ± 0.6
buckwheat	3.4 ± 0.8
soybean	1 ± 1
quinoa	100 ± 3
rye	2 ± 1
sunflower	20 ± 4
rice	3 ± 1
oat	16 ± 4

competing with the above-mentioned fractions, would not easily displace the antibody. The soluble glutelin fraction was unable to compete with globulin-P, 11S-globulin as well as albumins in buffer B. In addition, glutelins can be displaced by these fractions. This behavior could be attributed to the fact that glutelins, which are quite insoluble in buffer B, could appear as aggregates with no reactive epitope in solution and/or to the fact that antibodies have very low affinity for glutelin epitopes.

In 0.1 M borate buffer (**Figure 7**) glutelins are more soluble and evidenced an equivalent behavior to the one previously shown. Hence, the differences arising from these assays between globulins and glutelins could not be ascribed to their aggregation state.

The competition assays demonstrated that the same reactive surfaces in buffer B could be found in globulin-P and 11S-globulin, while only some of their epitopes could be shared with albumins. On the other hand, glutelins are unable to compete with globulin-P in the conditions assayed (buffer B or borate buffer), indicating the existence of conformational differences between these fractions.

Homology Between Amaranth and Other Seed Proteins. Total proteins from different mono and dicotyledonous plants, namely wheat, rye, oat, rice, buckwheat, soy, sunflower, and quinoa, were analyzed by SDS-PAGE and western blot to study the existence of a possible homology between amaranth and other seed proteins. Results are depicted in **Figure 8**.

The SDS-PAGE profiles show a large number of polypeptides in different flours, some of them with molecular weights similar to those of amaranth. However, as shown by western blot, only amaranth proteins were found to be reactive to anti-UM serum. At higher serum concentration, quinoa protein evidenced little reactivity (results not shown). The same type of assay was performed with the globulin fractions of soy, sunflower, rye,

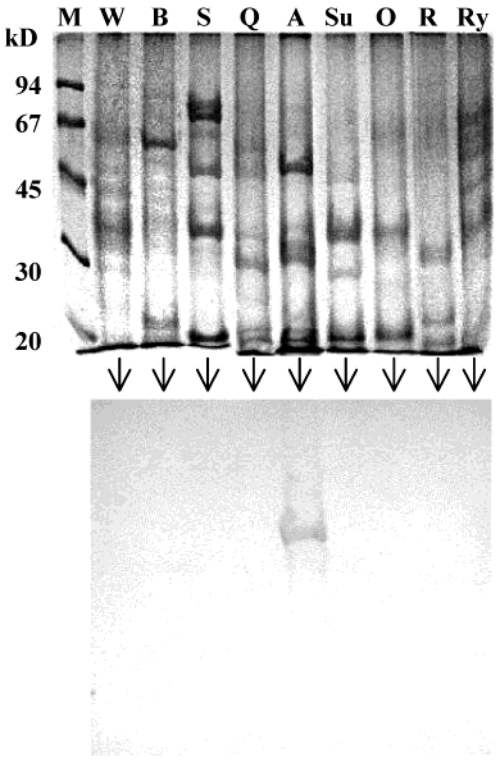

Figure 8. SDS-PAGE and western blot of proteins from seeds. Wheat, lane W; buckwheat, lane B; soybean, lane S; quinoa, lane Q, amaranth, lane A; sunflower, lane Su; oat, lane O; rice, lane R, rye, lane Ry; molecular weight standard proteins, lane M. Molecular masses (kDa) are indicated on the left. Western blot of the gel was performed using anti-UM serum diluted 1:3000 (v/v)

and quinoa; results showed that, in addition to globulin-P being used as control, only the globulin fraction of quinoa was reactive against the antiserum utilized (data not shown). The likely surface homology between globulins from mono and dicotyledonous plants and globulin-P was analyzed by ELISA, using anti-UM (10^5 fold dilution).

As seen in **Table 2**, only the globulin of quinoa evidenced the same reactivity as globulin-P against the antiserum, whereas globulins from sunflower and oat showed a slightly lower homology values (20 ± 4% and 16 ± 4%, respectively).

This study showed that unitary molecules (280 kDa) and aggregates (A > 500 kDa) of globulin-P had similar reactive surfaces. The polypeptide of 56 kDa was found to be the most reactive to the antibodies assayed, followed by the acidic ones. This result supports studies performed with globulin-P proteolytic treatments (*6*) proposing that the P56 and A polypeptides are more exposed on the molecule surface.

Globulin-P fraction presented cross-reactivity with the remaining amaranth protein fractions 11S-globulin, glutelins, and albumins. Western blot, direct, and competitive ELISA assays showed globulin fractions (11S-globulin and globulin-P) with similar reactive surfaces. Conversely, reactivity of the glutelin fraction depended on its sequence, and results corroborated that glutelins and globulins show different conformations. The albumin fraction, which did not show structural similarities compared with globulins or glutelins (*4*, *22*, *23*), would have some surface characteristics similar to those of globulins (11S-globulin and globulin-P).

From the evidence that amaranth globulin-P fraction has cross-reactivity with quinoa globulins, and to a lesser extent, with globulins of sunflower and oat, it can be concluded that

globulin-P shares some epitopes with these proteins, but not with the globulins of soybean, wheat, buckwheat, rice, and rye. Moreover, the anti-Gp serum was unable to detect either conformational or sequence epitopes in globulins of soybean, wheat, buckwheat, rice, and rye. In differentiating clearly the amaranth globulin-P from the other storage globulins, excepting quinoa, our results do not agree with those reported by Marcone et al. (*13*), who found common epitopes to both amaranth 11S-globulin and globulins from some monocotyledonous.

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