Influence of the Extracting Solvent upon the Structural Properties of Amaranth (*Amaranthus hypochondriacus*) Glutelin

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Two amaranth glutelin preparations, Gt-bo extracted with borate buffer at pH 10 and Gt-na extracted with 0.1 N NaOH, were characterized and compared with the amaranth polymerized 11S globulin (Gp, globulin-P). Gt-bo and Gt-na presented very similar polypeptidic composition and a similar reactivity against an anti-Gp polyclonal antibody, although lower than that of Gp. It is demonstrated that Gt-na is composed of denatured and dissociated molecules, whereas Gt-bo consists of folded molecules. The size, polypeptidic composition, thermal stability, and denaturation enthalpy of Gt-bo molecules were similar to those of Gp subjected to a borate treatment at pH 10. The Gp immunoreactivity decreased to the level of Gt reactivity when subjected to alkaline treatment; this could be due to conformational changes. Results suggest that, like Gp, amaranth Gt molecules may be hexameric oligomers of approximately 300 kDa. They would be partially unfolded during the alkaline extraction.

KEYWORDS: Amaranth; glutelin; protein structure; storage protein

INTRODUCTION

Amaranth is a dicotyledoneus that produces large quantities of edible grains and presents many agronomic advantages. Amaranth seeds contain proteins of higher nutritional quality than that of many cereal proteins, roughly the same as some legume proteins (*1*−*3*). According to Osborne's classification (*4*) the amaranth proteins are mainly composed of albumins, globulins, and glutelins in similar proportions (*5*). Their composition is different from that of legume seeds, which have globulins as their major proteins, and it is also different from cereals with seed proteins, where glutelins and prolamins are found in abundance (*4*, *6*). Nevertheless, according to the classification based on gene structure and mechanism of accumulation (*4*), the majority of amaranth seed proteins are globulins, like most of the dicotyledoneous storage proteins. This can be attributed to the fact that amaranth glutelin, although having the same solubility properties as other glutelins, presents similar molecular characteristics to those of amaranth 11S-globulin. In fact, amaranth glutelin main polypeptides are homologous in size to legumine polypeptides, and some of them (those near 30 kDa) present a pI similar to that of the corresponding legumine polypeptides (*5*, *7*, *8*). It was also reported that amaranth glutelin shows some degree of immunochemichal homology with amaranth 11S-globulin as well as with rice glutelin and oat globulin (*8*). Because of the characteristics mentioned above, the amaranth glutelin may be compared to rice glutelin. Rice glutelin is also a legumine-like protein belonging to a globulin fraction, as far as its gene structure and mechanism of accumulation are concerned. However, like other cereal glutelins, it is insoluble in neutral saline solvents (*9*−*11*).

In addition to glutelin and the 11S globulin, the amaranth seed contains another 11S-like globulin, globulin-P, whose solubility properties are between globulins and glutelins (*12*, *13*). That fraction stands as a distinctive characteristic of the amaranth seed, and it has a state of aggregation intermediate between globulins and glutelins. Considering these characteristics, the comparison of glutelin and globulin-P properties may help in understanding the basis of the highest aggregation state of amaranth glutelins.

The study of the amaranth glutelin fraction is also interesting in understanding the glutelin physicochemical properties as well as in evaluating its participation in the physicochemical and functional properties of the amaranth protein isolates. Amaranth isolates containing glutelin should have not only high nutritional properties but also suitable functional properties for using them as food ingredients.

The aim of this work is to evaluate both the similarities and differences between amaranth glutelin and globulin-P and to characterize the amaranth glutelin physicochemical behavior. For this purpose, glutelin was extracted with different solvents, borate buffer and NaOH; these solvents were used to preserve this protein conformation and to obtain a good yield, respec-

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tively. Both preparations were analyzed using different methodologies and compared with globulin-P. The immunochemical reactivity against an anti-Gp polyclonal antibody was also tested.

MATERIALS AND METHODS

Materials. The seeds of *Amaranthus hypochondriacus* (commercial cultivar) 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), 1-mm mesh and screened by 10-mm mesh. Flour was defatted for 24 h, using hexane in a 10% (w/v) suspension 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 Nkong and Ballance's colorimetric method (*14*). The protein/nitrogen coefficient used was 5.85 (*5, 15*).

Protein Isolation. *Glutelin.* Glutelin was extracted according to the Osborne method, with a modification based on the method described by Konishi et al. (*12*) for albumin and globulin extraction. The extraction procedure was conducted at room temperature with a meal-extraction solution ratio (p/v) of 1:10. Flour was treated twice with water to extract albumin and then twice with 32.5 mM K_2HPO_4−2.6 mM KH_2PO_4, 0.4 M NaCl, pH 7.5 (buffer A), to extract globulin. Globulin-P was extracted by treating the residue twice with water, then glutelin was extracted from the last residue with 0.1 M borate buffer, pH 10 (**Gt-bo**), and with 0.1 N NaOH (**Gt-na**) separately. After each treatment, the extracted residue was separated by centrifugation at 9000*g* for 20 min at room temperature. The supernatants containing glutelin (either as soluble or dispersed protein) were adjusted to pH 6 using 2 N HCl. The resulting precipitate was suspended in water, neutralized with 0.1 N NaOH, and freeze-dried.

The protein content in the extraction solvents was determined by the Lowry method (*16*).

Treated Globulin-P. Treated globulin-P was prepared by suspending lyophilized globulin-P (Gp) in 0.1 M borate buffer, pH 10 (**Gp-bo**), and in 0.1 N NaOH (**Gp-na**) separately, in a ratio of 1 mg of protein to 10 μL of solvent. Each suspension was stirred for 1h at room temperature, and then the pH was adjusted to 6 with 1 N HCl. The precipitates were collected at 10 000*g* for 20 min at room temperature, suspended in water, neutralized at pH 7, and freeze-dried.

Chromatography. Proteins were analyzed by size exclusion chromatography at room temperature in a Superose 6B HR. 10/30 column using a Pharmacia LKB, FPLC System. Samples containing from 1 to 3 mg of protein in 200 μL of 0.1 M borate buffer, pH 10, were injected. Elution was performed using the same buffer at a flow rate of 0.2 mL/min. Fractions of 0.3 mL were collected, and the elution profile (absorbance at 280 nm) was obtained.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Linear gradient (6−12% in polyacrylamide) gels or 12% (w/v) of polyacrylamide separating gels with a stacking-gel of 4% (w/v) polyacrylamide were run in minislabs (BioRad Mini Protean II Model). Runs were carried out according to the Laemmli method (*17*), as modified by Petruccelli and Añón (*18*). Molecular masses of the polypeptides were calculated using the following protein standards (Pharmacia): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). 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.

Bidimensional SDS → SDS + 2-ME Gel Electrophoresis. The first dimension was performed under nonreducing denaturing conditions with 6−12% (w/v) polyacrylamide linear gradient separating gels. Samples were prepared in the same way as in one-dimensional electrophoresis. After the run, the first dimension gel portion was treated with 10 volumes of 62.5 mM Tris-HCl, pH 6.8, 1% SDS, 20% sucrose, 0.2 M 2-ME at 55 °C for 30 min, changing the solution at least twice. The second dimension gel was performed in a 12% polyacrylamide gel with a stacking gel of 4% (w/v) polyacrylamide.

Bidimensional Native → SDS Gel Electrophoresis. The first dimension was carried out in 4−7.5% (w/v) acrylamide linear gradient gels at pH 8.3 with the same buffer system used for SDS-PAGE but without SDS. The second dimension was performed in the same way as **SDS → SDS + 2-ME** but using the treatment buffer in the absence of 2-ME.

All gels were fixed and stained with Coomassie Brilliant Blue.

Differential Scanning Calorimetry (DSC). Runs were performed in a Polymer Laboratories (Rheometric Scientific Ltd., U.K.) calorimeter using Plus V 5.41 software. Calibration was carried out at a heating rate of 10 °C/min using the analytical grade standards (Rheometric Scientific Ltd., Surrey, U.K.) indium, lauric acid, and stearic acid.

For runs, 20% w/w suspensions of protein were prepared in distilled water. After preparation, all suspensions were allowed to rest for 20 min at room temperature. DSC samples consisted of hermetically sealed aluminum pans filled with 12−14 mg suspensions**.** They were run at a rate of 10 °C/min from 300 K (27 °C) to 393 K (120 °C), and a double, empty pan was used as a reference. After each run, the dry matter content was determined by puncturing the pans and by exposing them overnight to 107 °C. The dry protein content was calculated taking into account the percentage of protein (w/w) in the fraction, previously determined by the micro-Kjeldhal method. The denaturation parameters were calculated with the software equipment, with the denaturation temperature (T_d) being considered as the value corresponding to the maximum transition peak; the value of $\Delta T_{1/2}$ was obtained by determining the peak width at half the peak height, whereas the transition enthalpy (ΔH) values were calculated from the area below the transition peaks. At least three analyses were performed for each sample.

Immunochemical Analyses. Anti-globulin-P polyclonal antibodies were prepared as described by Castellani (*19*). New Zealand White rabbits were intra-dermally immunized every three weeks with 0.4 mL (1 mg/mL) of globulin-P prepared in our laboratory.

Direct ELISA. Polystyrene strips (Maxisorp; Nunc, Roskilde, Denmark) were sensitized overnight at 4 °C, with a 0.1-μg protein/well of Gt-na, Gt-bo, Gp, or Gp-na solution diluted in 0.1 M borate buffer, pH 10.0. After three washings with PBS-Tween 20 0.05% (PBS-T), the plates were blocked with 200 μL/well of a solution of PBS containing 3% skim milk. After 2 h of incubation at 37 °C, the plates were washed with PBS-T and serial dilutions of the anti-Gp serum in PBS-T containing 1% skim milk (diluent solution) were dispensed. After 1 h of incubation 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, the contents of each well were developed with a solution containing *o*-phenylenediamine (1 mg/mL, Merk, Darmstadt, Germany) and 30% H_2O_2 (1 μL/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 SO_4H_2. Absorbance was determined at 490 nm.

Competitive ELISA. Polystyrene strips were sensitized, washed, and blocked with Gt-na and Gt-bo separately, as previously described. A serial dilution of antigen (Gt-na or Gt-bo) was prepared in borate buffer, pH 10.0, 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 (1:4000 dilution) were mixed and incubated overnight at 4 °C in plastic tubes (preincubation). Then, 100 μL/well of each sample were incubated for 30 min at 37 °C in the coated wells. After washing, 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, the color reaction was developed and stopped, as previously outlined. Absorbance values were transformed by logit function (*20*). Logit-$p = \ln p/(1 - p)$, where $p = (A - A_o)/(A_M - A_o)$, where A is the absorbance from wells of samples and standard, A_M is the absorbance from well without antigen competitor, and A_o is the absorbance from well without antibody solution.

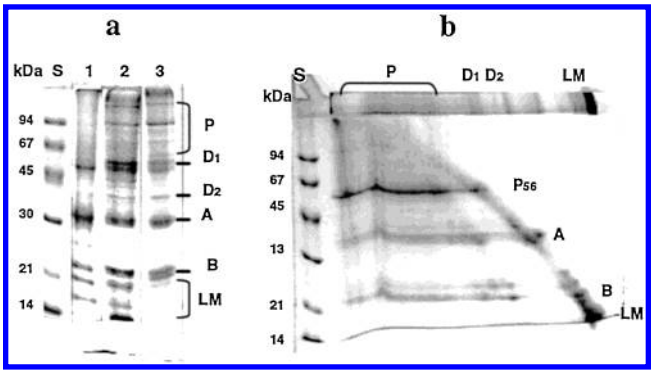

Figure 1. (a) SDS-PAGE in nonreducing conditions. Gt-na, lane 1; Gt-bo, lane 2; Gp, lane 3. S = standard proteins (molecular weights are indicated on the left side). Main polypeptides are shown on the right side: aggregated polypeptides, P; dimeric subunits, D1 and D2; approximately 30 kDa polypeptides, A; approximately 20 kDa polypeptides, B; low molecular mass polypeptides, LM. The acrylamide concentration was 12% (w/v). (b) Bidimensional (SDS → SDS + 2-ME) electrophoretic pattern of Gt-bo. The main polypeptides are shown in the figure. In the second dimension, P_{56} is the 56 kDa polypeptide, and the others are as those in (a). S = standard molecular mass proteins of second dimension. The first dimension gel was 6–12% (w/v) polyacrylamide, and the second dimension gel was 12% (w/v) polyacrylamide.

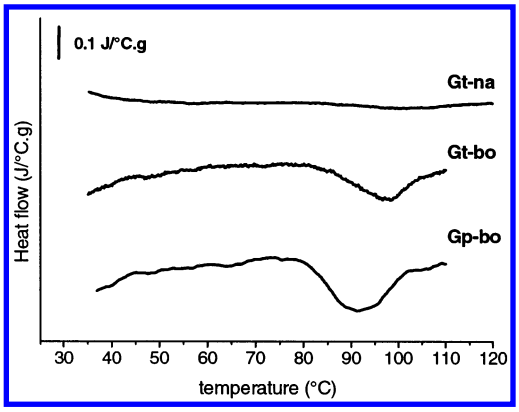

Figure 2. DSC thermograms of Gt-na, Gt-bo, and Gp-bo.

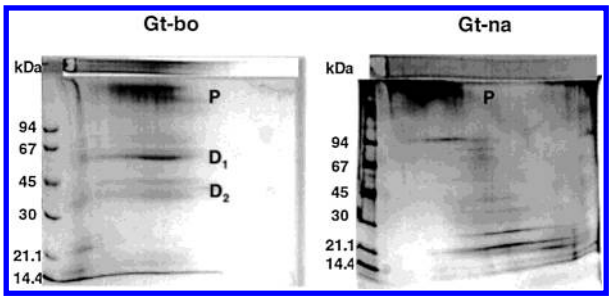

Figure 3. Bidimensional (native → SDS-PAGE) patterns of Gt-bo and Gt-na. Main polypeptides are indicated in the patterns. Standard molecular mass proteins of second dimension are on the left side. The first dimension gel was 4–7.5% (w/v) polyacrylamide, and the second dimension gel was 12% (w/v) polyacrylamide.

RESULTS AND DISCUSSION

The yield of amaranth glutelin extracted with 0.1 N NaOH (Gt-na) was 32.7 ± 1.3 mg/g flour, whereas glutelins extracted with 0.1 M borate buffer, pH 10 (Gt-bo), gave a lower yield (16.3 ± 0.5 mg/g flour). The latter treatment was not strong enough to extract all the glutelins, the use of 0.1% SDS being necessary to obtain the remaining glutelins. The addition of 2-ME did not improve the yield.

These results contradict those of Barba de la Rosa et al. (*21*), who reported similar yields for glutelins extracted with either borate buffer or 0.1 N NaOH. The difference may be partially attributed to differences in the sequence of the extracting solvents used, because we obtained globulin-P and glutelins as separated fractions, while the glutelin fraction used by the above authors is likely to contain Gp.

Both Gt-na and Gt-bo showed similar SDS-PAGE (**Figure 1a**, lanes 1 and 2) and 2D (**Figure 1b**, only one is shown) patterns. These patterns were similar to those of 11S-globulins; they showed that glutelin major subunits (D_1 in **Figure 1**, parts **a** and **b**) are composed of polypeptides of approximately 30 and 20 kDa (A and B) and that minor dimeric subunits (D_2 in **Figure 1**, parts **a** and **b**) are composed of polypeptides of molecular masses between 20 and 25 kDa. The Gt-na profile showed a lesser proportion of one of the D_1 polypeptides, which may be ascribed to the disulfide exchange of unfolded molecules in an alkaline medium. Such glutelin patterns are in line with the results obtained by Barba de la Rosa et al. (*21*) and Vasco-Méndez and Paredes-López (*8*). Amaranth glutelin also contained an important amount of a monomeric, 56 kDa polypeptide (P_{56}, **Figure 1b**) and aggregated polypeptides of molecular masses higher than 60 kDa (P in **Figure 1**), which were composed of P_{56}, A, and B polypeptides. P_{56} and P aggregates were characteristic of amaranth globulin-P (*22*). On the other hand, amaranth glutelin contained some polypeptides of a low molecular weight (LM in **Figure 1a**) that were absent in the Gp SDS-PAGE profiles (**Figure 1a** and in ref *22*).

Gt-bo and Gt-na were analyzed by DSC; their thermograms are shown in **Figure 2**. Gt-na showed no endotherm, indicating that this protein was denatured. On the other hand, Gt-bo presented an endotherm with a high denaturation temperature (T_d = 96.4 ± 1.3 °C) and a small denaturation enthalpy (ΔH = 5.0 ± 0.6 J/g). The high T_d indicates that Gt-bo had a high thermal stability, just like Gp (*13*). However, because the value of its ΔH was lower than that of Gp, it is feasible that Gt-bo presents a different conformation. Alternatively, this might indicate that Gt-bo molecules were only partially folded, with an open conformation evidenced by a low cooperativity, as shown by the large $\Delta T_{1/2}$ (11.6 ± 0.6). Inasmuch as glutelins and globulin-P have a very similar polypeptidic composition and thermal stability, it can be proposed that both proteins have very similar conformations, the alkaline treatment during extraction being the reason a partial unfolding of the glutelin took place. To explore this possibility, we also analyzed Gp subjected to alkaline (pH 10 borate buffer) treatment (Gp-bo, Materials and Methods). The thermogram of the treated globulin-P (Gp-bo) (**Figure 2**) showed a broad endotherm with a lower denaturation enthalpy (ΔH = 6.0 ± 0.4 J/g) than that for untreated Gp (ΔH = 14.0 ± 0.2 J/g, thermogram not shown), indicating that alkaline treatment modified the Gp structure, which became partially unfolded. This matches the results obtained by Castellani et al., who reported the low ΔH for alkaline treated Gp (*13*). The Gp-bo enthalpy value, similar to the Gt-bo enthalpy, may indicate that both proteins have similar conformations.

Glutelin molecules were also analyzed by bidimensional electrophoresis (native-SDS) and by FPLC molecular sieving eluting with borate buffer. According to the electrophoretic results (**Figure 3**) Gt-bo native-PAGE (first dimension) profile showed one wide band and some less intense bands of lower mobility. According to the second dimension profile, Gt-bo

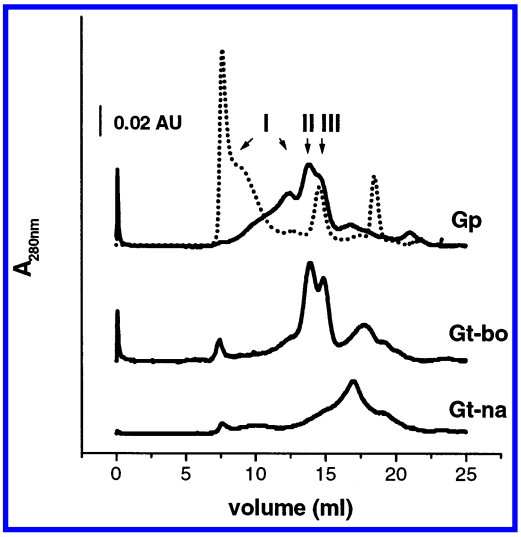

Figure 4. FPLC gel filtration. Full line profiles: Gp, Gt-bo, and Gt-na, eluted with 0.1 M borate, pH 10. Dotted line profile: Gp eluted with pH 8.5 phosphate. Peak I, high molecular mass aggregates (from 600 to 30000 kDa); peak II, low molecular mass aggregates (≈600 kDa); peak III, unitary molecules (≈300 kDa).

molecules were mainly built up by the nonreduced polypeptides D_1 and D_2. On the other hand, the native profile of the Gt-na was spread along the lane without defined bands (**Figure 3**). The second dimension showed that Gt-na protein mainly consisted of dissociated polypeptides of molecular masses ranging from 14 to 20 kDa (running faster in the first dimension) to above 80 kDa (P in **Figure 3**) with the lowest mobility in the first dimension.

In the FPLC analyses Gt-bo and Gt-na were compared with Gp-bo. The alkaline treated globulin-P (Gp-bo, **Figure 4**) eluted with pH 10 borate showed a profile different from that for untreated Gp in pH 8.5 phosphate (**Figure 4**, dotted profile). Compared with this later profile, Gp-bo profile contained a smaller amount of high molecular mass aggregates (component I in **Figure 4**) and a larger amount of low molecular mass aggregates (peak II) and unitary molecules (peak III). These results suggest that treatment at pH 10 caused the disruption of some aggregates into smaller molecules, as noted previously (*13*). Alternatively, the protein of peak II could correspond to the unitary molecules that showed a molecular mass of 280 kDa in buffer, pH 8.5 (peak III of the dotted profile). Due to a more open structure, they may have changed their hydrodynamic behavior, eluting at a lower volume in pH 10 borate. Some of the unitary molecules might be partially dissociated, giving subunits corresponding to peak III, which elutes at a lower volume.

Gt-bo profile (**Figure 4**) showed two major peaks, similar to Gp-bo peaks II and III, although Gt-bo profile presented a reduced amount of small-sized aggregates (component I). As regards Gt-na profile, it was observed that this fraction was composed of molecules of different sizes, most of them eluting at a lower volume than component III. According to native-PAGE results, they would correspond to dissociated polypeptides.

Gp was found to contain more antigenic determinants than Gt-na, as assessed by direct and competitive ELISA, using a polyclonal anti-Gp serum (*23*). Those immunoassays suggested that these two proteins exposed different surfaces to the antibody. Considering this and the above information, we tested the reactivity of Gt-bo and Gt-na against the same anti-Gp

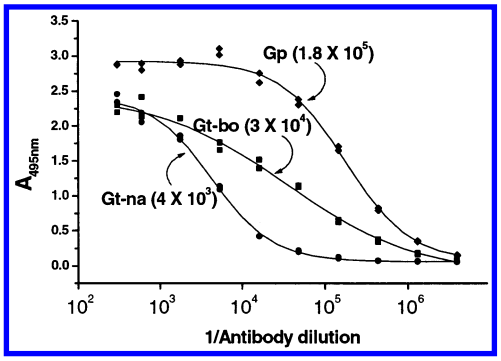

Figure 5. Titration curves for anti-Gp with Gp, Gt-bo, and Gt-na in the direct ELISA. Titers are indicated in the figure.

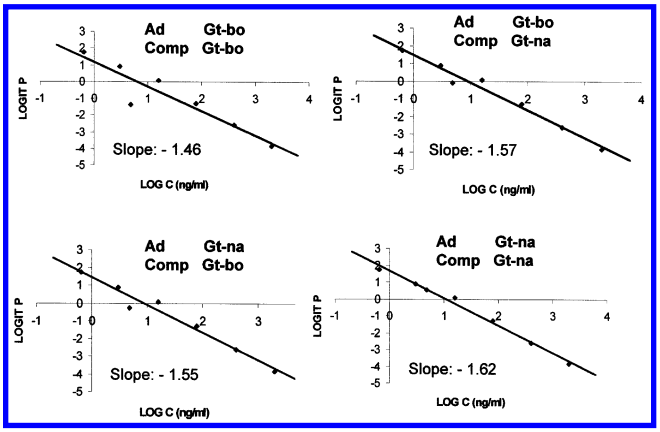

Figure 6. Logit-P curves for anti-Gp in the competitive ELISA with Gt-na and Gt-bo. In each curve, **Ad** = protein adsorbed on the microplate, and **Comp** = protein incubated with the serum as a competitor. Anti Gp serum was diluted 1:10^4.

serum. **Figure 5** shows the titration curves for anti-Gp binding to Gt-bo, Gt-na and Gp as control. The titers for the anti-Gp against the proteins are shown in **Figure 5**.

Gt-na presents a titer 100-fold lower than that of Gp, as already shown. Nevertheless, Gt-bo presents higher reactivity than Gt-na, much closer to Gp-reactivity, suggesting that both glutelin preparations exposed different surfaces when adsorbed on the microtitration plates. To assess whether this was still the case when Gt-na and Gt-bo were in solution, experiments by competitive ELISA were carried out. All the logit curves (**Figure 6**) obtained with Gt-na or Gt-bo as competitors, using either Gt-na or Gt-bo adsorbed on the microplates showed similar slope values, indicating that they have similar affinity to the anti-Gp serum.

Taking into account our previous results, which showed that Gt-bo presented similar structural characteristics to Gp subjected to the same alkaline treatment (Gp-bo), we analyzed whether the differences in immunochemical reactivity between Gt and Gp might be due to a different conformation. Thus, we compared the reactivity of Gp subjected to 0.1 N NaOH treatment (Gp-na, Materials and Methods) with the reactivity of Gt and Gp by direct ELISA. **Table 1** shows that Gp-na presented a low reactivity against the anti-Gp serum diluted 1:10^5, much lower than Gp reactivity, and also lower than that of Gt-bo.

These results indicate that the structural changes induced by alkaline treatment brought about a decrease in Gp immunoreactivity. To put it another way, results suggest that the Gt immunoreactivity is lower than that of Gp as a consequence of structural changes arising during the alkaline extraction.

Table 1. Percentage Cross-Reaction of Polyclonal Anti-Gp with Amaranth Proteins (antibody dilution 1×10^{-5})

protein fraction	percentage reactivity
Gp	100
Gt-bo	44
Gt-na	7
Gp-na	24

CONCLUSIONS

Amaranth glutelin, like rice glutelin, is a legumine-like protein that, due to its solubility properties, has to be extracted from the flour, using extreme pH or denaturing conditions. Thus, it is difficult to obtain glutelins with the same structural characteristics as those we find in the seed (*22*, *24*, *25*). In the present study, we extracted amaranth glutelins using mild conditions (Gt-bo) in order to preserve certain structural properties**.** Nonetheless, the yield obtained was lower than one corresponding to glutelin extracted with 0.1 N NaOH (Gt-na).

Gt-na was composed of denatured and dissociated molecules. On the other hand, Gt-bo contained folded molecules that, by their size, polypeptidic composition, thermal stability, and denaturation enthalpy were similar to those of Gp subjected to pH 10 borate treatment. These findings suggest that, like legumins, amaranth Gt molecules might be hexameric oligomers of approximately 300 kDa. They might remain in a more aggregated state when they are in the seed; such a state could be disrupted by alkaline treatment during extraction, with a concurrent loss of conformational epitopes, leading to a lesser reactivity of glutelins against an anti-Gp antibody. This may also be true for rice glutelins, which were composed of dissociated polypeptides when extracted under hard conditions (*24*, *26*) and which showed some degree of folding when extracted under mild conditions (*27*, *28*).

In showing the influence of the extracting solvent on the yield and physichochemical properties of the amaranth glutelin, these results make up the basis for the preparation of amaranth isolates containing glutelin in different proportions and with a different structure. These isolates may show different functional properties behaving as ingredients suitable for different foods. The functional properties of these kinds of amaranth isolates are under study in our laboratory.

ACKNOWLEDGMENT

We thank the Facultad de Ciencias Químicas y Farmacéuticas y Departamento Ciencia de los Alimentos y Tecnología Química Universidad de Chile.

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Received for review September 10, 2002. Revised manuscript received April 10, 2003. Accepted April 24, 2003. Financial support provided by the Agencia Nacional de Promoción Científica y Tecnológica (FOCCyT, PICT 98-09-04265).

JF025949E