Calcium- and Magnesium-Dependent Aggregation of Legume Seed Storage Proteins

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The solubility characteristics and sedimentation behavior of total or individual globulins from legume seeds [*Lupinus albus* L., *Pisum sativum* L., and *Glycine max* (L.) Merr.] were investigated. The typical insolubility of globulins detected during their extraction seems to be due to the presence of a low molecular weight factor(s) in the seed extract. The solubility of the purified globulins decreases with increasing concentrations of calcium and/or magnesium, but not of other cations, showing minimum values at concentrations that vary with the particular globulin considered. Ultracentrifugation analyses revealed that the Ca^{2+}- and/or Mg^{2+}-induced insolubilization of the globulins involves the formation of high-order aggregates of molecules of the same or of different globulins. These macromolecular structures are dissociated under conditions of high ionic strength, suggesting the involvement of electrostatic interactions in the aggregation process. The degree of association relies heavily on the amount of Ca^{2+} and/or Mg^{2+} available, on the presence of chelating agents for these divalent cations, and on the ionic strength of the surrounding medium. The possible physiological significance of the findings is discussed.

Keywords: *Calcium; legume seeds; Lupinus albus; sedimentation coefficient; solubility; storage proteins*

INTRODUCTION

Legume seed storage proteins are typically represented by a relatively small number of molecular species but account for ~70% of the seed nitrogen (Mossé and Pernollet, 1983). They are located in the mature seed within specialized organelles, named protein bodies (Shewry et al., 1995). A plausibly important characteristic of storage proteins inside the protein bodies is their capacity to undergo a highly compact packing.

Osborne and Campbell (1898) initially separated the globulin fraction from *Pisum sativum* seeds into two major components, which were named vicilin and legumin. The presence of similar fractions was subsequently demonstrated in a variety of legume seeds (Derbyshire et al., 1976). Danielsson (1949) studied the seeds from 34 species of legumes and found, with a few exceptions, that they all contain two globulin components with sedimentation coefficients of approximately 7 and 11 S. These protein components were designated vicilin and legumin, respectively, on the sole basis of their sedimentation values. This nomenclature has been adopted and extensively used since the pioneer work of Danielsson (1949).

One of the characteristics of legume seed globulins is their capacity to show an association/dissociation behavior, particularly under conditions of varying ionic strength or pH (Derbyshire et al., 1976; Duranti et al., 1988). This phenomenon is well documented in the literature. For example, legumin hexamers can not only stepwise dissociate into subunits, with often stable intermediary trimers, but also associate into dodecamers or possibly superoligomers with larger degrees of polymerization (Mossé and Pernollet, 1983). This association capacity of legume storage proteins, leading to an increased fitness to compacting, is likely to fulfill a physiologically relevant role in vivo. The vicilin fraction of *P. sativum* associates into an 11 S form at low ionic strength at pH values of 6.2 and 7.0 (Derbyshire et al., 1976). γ-Conglutin from *Lupinus* seeds, a globulin distinct from vicilin and legumin, occurs in vitro in several oligomeric forms, with sedimentation coefficients up to 10.6 S (Blagrove and Gillespie, 1975; Blagrove et al., 1980). Even globulin subunits from distant plant species, such as *Glycine max* and *Sesamum indicum*, can associate with each other and undergo in vitro molecular hybridization (Mori et al., 1979). On the other hand, the reported sedimentation coefficients of supposedly identical globulins sometimes differ considerably—this is the case, for instance, of arachin, a peanut globulin, for which sedimentation coefficients of 10.8 to 14.6 S have been reported (Derbyshire et al., 1976). These variations may result from the use of distinct methodologies by different authors. They may also be partly due to post-translational modifications of the globulins. The variations reported for the sedimentation coefficients of legume seed globulins cast some doubt on the apparently well-established classification of these proteins into 7 and 11 S species.

In this work we have studied the solubility characteristics and the sedimentation behavior of total or individual globulins from *Lupinus albus* L., *Pisum sativum* L., and *Glycine max* (L.) Merr. The effects of

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cations, ionic strength, and chelating agents were investigated.

MATERIALS AND METHODS

Plant Material. Dry seeds of white lupin (*L. albus* L.), cultivar Multolupa, kindly supplied by Dr. J. N. Martins, were hand-sorted, under ultraviolet (UV) light, to certify their sweet character (Ferreira et al., 1995). Dry seeds of pea (*P. sativum* L.) and soybean [*Glycine max* (L.) Merr.] were obtained in a local market. Duckweed (*Lemna minor* L.) was grown as described before (Ferreira and Teixeira, 1992).

Isolation of Total Globulins. Total globulins from legume seeds were extracted and isolated according to a modification of the procedure described by Melo et al. (1994), which is a modified version of the method of Blagrove and Gillespie (1978). The dry cotyledons were milled (0.2 mm sieve), and the resulting meal was defatted with *n*-hexane (34 mL per gram of flour) for 4 h with agitation and air-dried after decantation of the hexane. The albumin fraction of the proteins was extracted by mixing 1 g of flour per 34 mL of water (pH adjusted to 8.0) containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 mM phenylmethanesulfonyl fluoride (PMSF) for 4 h. The suspension was centrifuged for 1 h at 30000*g*, and the supernatant, containing the albumins, was discarded. The total insoluble fraction of the proteins was subsequently extracted by stirring the pellet with 0.1 M Tris-HCl buffer, pH 8.0, containing 10% (w/v) NaCl, 0.05% (w/v) NaN_3, 1 mM PMSF, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (34 mL per gram of original flour), for 12 h. The globulin-containing solution was centrifuged for 1 h at 30000*g* and the supernatant desalted on a PD-10 prepacked Sephadex G-25 column (Pharmacia/LKB), previously equilibrated in 50 mM Tris-HCl buffer, pH 7.5, and used as the source of total globulins. All operations were performed at 4 °C. This procedure completely eliminates the albumin fraction, free of contaminating globulins, allowing an efficient extraction of the globulin fraction.

Purification of Proteins. α-Conglutin, β-conglutin, and γ-conglutin were purified from *L. albus* seeds by anion-exchange chromatography of the total globulin fraction (~5 mg) on a Mono Q HR5/5 column (Pharmacia/LKB) as described by Melo et al. (1994). The column was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, and the bound proteins were eluted with a continuous, linear gradient of NaCl (0−1 M). γ-Conglutin, a minor globulin component, did not bind to the anion exchanger at pH 7.5. SDS−PAGE analyses have revealed that this protein is composed of one main type of subunit (40 kDa) containing two polypeptide chains (18 and 26 kDa) linked by disulfide bonds (Blagrove and Gillespie, 1975; Melo et al., 1994). It occurs in vitro in oligomeric form, with a sedimentation coefficient of 10 S (Blagrove et al., 1980). β-Conglutin, the main globulin component, eluted from the Mono Q column with ~0.3 M NaCl. It is composed of 10−12 major types of subunits (15−72 kDa) and a considerable number of minor subunits, with no disulfide bonds (Melo et al., 1994). Finally, α-conglutin, a major globulin component, eluted from the Mono Q column with 0.40−0.45 M NaCl. It contains four main types of subunits (53, 60, 66, and 70 kDa) as well as a number of minor subunits. Upon reduction, each of these main subunits is split into a heavier polypeptide chain (31, 36, 42, and 46 kDa, respectively) and a lighter polypeptide chain (19 kDa), which is common to all four subunits (Blagrove and Gillespie, 1975; Melo et al., 1994).

Vicilin and legumin were purified from *P. sativum* seeds by anion-exchange chromatography of the total globulin fraction on the Mono Q column of the FPLC, as described before. Vicilin elutes from the Mono Q column with ~0.3 M NaCl, whereas legumin is eluted with ~0.4 M NaCl (data not shown).

Ribulose bisphosphate carboxylase was purified from *L. minor* by FPLC anion-exchange chromatography on a Mono Q column followed by gel filtration on a Superose 12 column as described by Ferreira and Teixeira (1992).

Immunoglobulins G were purified from goat blood by FPLC affinity chromatography on a protein G−Superose column. Goat blood was obtained from a local farm and allowed to clot. The serum was desalted on a PD-10 column previously equilibrated in 20 mM sodium phosphate buffer, pH 7.0, and injected onto the protein G column equilibrated in the same buffer. Immunoglobulins G were eluted with 0.1 M glycine buffer, pH 2.7. Fractions (1 mL) were collected into test tubes containing enough (44 μL) 1 M Tris-HCl buffer, pH 9.0, to neutralize the protein solutions. Purified immunoglobulins G were finally desalted into 50 mM Tris-HCl buffer, pH 7.5.

Isopycnic Glycerol Density Gradient Ultracentrifugation. The glycerol gradients were prepared in ultraclear 38 mL centrifuge tubes and were composed of 45% (4 mL), 40% (4 mL), 35% (4 mL), 30% (4 mL), 25% (4 mL), 20% (4 mL), 15% (4 mL), 10% (4 mL), and 5% (4 mL) glycerol (v/v) made up in 50 mM Tris-HCl buffer, pH 7.5. Starting with the highest concentrations, each solution was layered in the centrifuge tube and immediately frozen in liquid nitrogen. After preparation, the 36 mL gradients were stored at −70 °C until needed. These step gradients were converted into continuous gradients by thawing followed by incubation at 4 °C during 24 h (results not shown). A similar linearization procedure was reported before for sucrose gradients (Luthe, 1983). Subsequent ultracentrifugation (performed under the conditions described below) did not alter the shape of the continuous gradient (data not shown). Glycerol concentrations were determined spectrophotometrically at 230 nm. When appropriate, the gradients were prepared to contain several additives such as 1 mM $CaCl_2$ + 1 mM $MgCl_2$, 5 mM $CaCl_2$ + 5 mM $MgCl_2$, 10 mM $CaCl_2$ + 10 mM $MgCl_2$, 10% (w/v) NaCl, 10 mM EDTA + 10 mM EGTA, or 10 mM EDTA + 10 mM EGTA + several concentrations of $CaCl_2$ and $MgCl_2$.

A sample (2 mL; 1.5 mg of protein) containing total globulins from *L. albus*, *P. sativum*, or *G. max* or the individual globulins from *L. albus* or *P. sativum* was desalted into 50 mM Tris-HCl buffer, pH 7.5, containing the same additives as the glycerol gradient, layered on the top of the gradient and centrifuged at 110000*g* for 24 h at 4 °C in a Beckman XL-90 analytical ultracentrifuge. Immediately after ultracentrifugation, the gradients were collected (1.5 mL fractions) using a peristaltic pump and the A_{280} was continuously monitored. The precipitate was suspended in 200 μL of 50 mM Tris-HCl buffer, pH 7.5, and divided in two aliquots: one aliquot (100 μL) was added to sample buffer and analyzed by SDS−PAGE; the other aliquot (100 μL) was added to 900 μL of 50 mM Tris-HCl buffer, pH 7.5, containing 10% (w/v) NaCl to solubilize the suspended globulins and used to measure A_{280}.

Sedimentation coefficients of protein fractions obtained after ultracentrifugation were determined according to the method of Martin and Ames (1961) using horse heart cytochrome *c* (1.8 S; 12.4 kDa), bovine serum albumin (4.4 S; 66 kDa), goat immunoglobulin G (6.6 S; 150 kDa), bovine liver catalase (11.3 S; 232 kDa), *Lemna* ribulose bisphosphate carboxylase (15 S; 530 kDa), and rabbit muscle phosphorylase kinase (22 S; 1.28 MDa) as standards (data not shown; Martin and Ames, 1961; Tominatsu, 1980; Ferreira and Davies, 1987; Gueguen and Barbot, 1988).

Turbidity Measurements. Turbidity measurements of conglutin solutions were made spectrophotometrically at 600 nm in 1 mL silica cuvettes, following the procedure described by Okubo et al. (1976) to detect glycinin turbidity in the presence of increasing concentrations of each globulin and the period of incubation of each protein in the presence of various Ca + Mg concentrations. A globulin concentration of 0.5 mg/mL and an incubation period of 5 min were selected. Longer periods of incubation did not result in significant increases in absorbance.

General Assays. Protein concentration was measured by using a modification of the Lowry method (Bensadoun and Weinstein, 1976). Turbidimetric measurements were performed spectrophotometrically at 600 nm (Okubo et al., 1976). A_{600} values were measured in 50 mM Tris-HCl buffer, pH 7.5, after a 5 min incubation, in a 1 mL silica cuvette containing

0.5 mg of protein/mL, against an identical cuvette containing exactly the same components with the exception of the protein.

PD-10 prepacked Sephadex G-25 columns, previously equilibrated in 50 mM Tris-HCl buffer, pH 7.5, were utilized when required to exchange buffers or to desalt the globulin solutions.

Reducing SDS−PAGE was performed in 12.5% (w/v) acrylamide slab gels as previously described (Ferreira and Davies, 1987), except that *m*-cresol purple was used as the front dye marker and 0.1 M sodium acetate was included in the anode buffer. These modifications allow the resolution of polypeptides with molecular masses ranging from 2.5 to >200 kDa (Christy et al., 1989). Prior to SDS−PAGE, protein samples were boiled for 3 min in sample buffer (80 mM Tris-HCl buffer, pH 6.8, containing 0.1 M 2-mercaptoethanol, 2% w/v SDS, 15% v/v glycerol, and 0.006% w/v *m*-cresol purple). Total polypeptides in gels were stained with Coomassie brilliant blue R. Molecular mass standards for SDS−PAGE were myosin (205 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and α-lactalbumin (14 kDa).

RESULTS AND DISCUSSION

Effect of Cations and Chelating Agents on the Solubility of Legume Seed Globulins. Seed storage proteins have been extensively studied from the turn of the century, when Osborne (1924) classified them into four groups on the basis of their extraction and solubility characteristics: albumins, soluble in water; globulins, the major storage proteins in legume seeds, insoluble in water but soluble in dilute salt solutions; prolamins, insoluble in the above solutions but soluble in alcohol/water mixtures; and glutelins, insoluble in the above solutions but soluble in weak acidic or basic solutions (Ashton, 1976; Shewry et al., 1995). Therefore, a suitable methodology to isolate total globulins from legume seeds must involve the following steps: (i) the seeds are usually milled and defatted prior to extraction, particularly when seeds of high oil content are being used; (ii) removal of the total albumin fraction is performed by treatment of the flour with water; (iii) the total globulin fraction is subsequently extracted with a buffered solution containing 10% (w/v) sodium chloride. Desalting the total globulins into 50 mM Tris-HCl buffer, pH 7.5, yields reasonably clear solutions. However, addition of calcium and magnesium ions to the desalted globulins insolubilizes the proteins, producing a large enhancement in the turbidity of the solution, as detected by the increment in A_{600}. This increase in A_{600} depends on the globulin concentration, on the calcium and magnesium concentrations, and on the period of incubation of the proteins in the presence of the cations. After performing a series of preliminary experiments (results not shown), we selected a globulin concentration of 0.5 mg/mL and an incubation period of 5 min.

Total globulins were isolated from *L. albus* seeds and utilized to purify α-, β-, and γ-conglutins by anion-exchange chromatography on the Mono Q column of the FPLC. The data presented in Figure 1A show the turbidity (detected by A_{600}) of the resulting protein solutions (total globulins, α-conglutin, β-conglutin, or γ-conglutin; 0.5 mg of protein/mL in 50 mM Tris-HCl buffer, pH 7.5) in the presence of different concentrations (0−100 mM) of calcium chloride and magnesium chloride. In all cases, the A_{600} values increase with increasing concentrations of the cations until reaching a maximum, decreasing thereafter. However, different protein fractions show A_{600} maxima at distinct Ca^{2+} + Mg^{2+} concentrations: 5 mM Ca^{2+} + 5 mM Mg^{2+} for total globulins, 10 mM Ca^{2+} + 10 mM Mg^{2+} for α-conglutin,

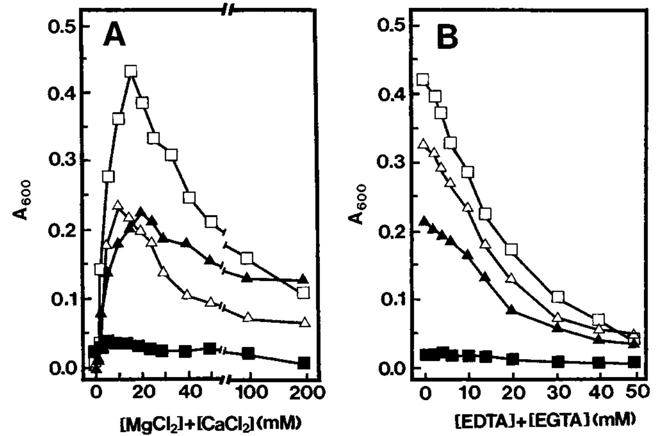

Figure 1. Effect of calcium, magnesium, and chelating agents on the solubility of *L. albus* globulins. Total globulins (△), α-conglutin (▲), β-conglutin (□), and γ-conglutin (■) were purified from *L. albus* as described under Materials and Methods. (A) The protein solutions (0.5 mg/mL) in 50 mM Tris-HCl buffer, pH 7.5, were incubated for 5 min in a 1 mL silica cuvette in the presence of increasing concentrations of $CaCl_2$ + $MgCl_2$. (B) The protein solutions (0.5 mg/mL) in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM Ca^{2+} + 5 mM Mg^{2+} for total globulins (△), 10 mM Ca^{2+} + 10 mM Mg^{2+} for α-conglutin (▲), 7.5 mM Ca^{2+} + 7.5 mM Mg^{2+} for β-conglutin (□), or 2.5 mM Ca^{2+} + 2.5 mM Mg^{2+} for γ-conglutin (■) were incubated for 5 min in a 1 mL silica cuvette in the presence of increasing concentrations of EDTA + EGTA.

and 7.5 mM Ca^{2+} + 7.5 mM Mg^{2+} for β-conglutin. The turbidity of γ-conglutin solutions is not affected in a significant way by the presence of Ca^{2+} + Mg^{2+}, reaching a maximum for 2.5 mM Ca^{2+} + 2.5 mM Mg^{2+}. High concentrations of Ca^{2+} and Mg^{2+} produce a gradual decrease in A_{600} for all proteins, probably reflecting the increase in ionic strength (Sakakibara and Noguchi, 1977; Peng et al., 1984).

The observation that divalent cations increase the A_{600} values of *L. albus* globulin solutions suggests that the simultaneous addition of the chelating agents EDTA and EGTA should reduce A_{600} due to a solubilization of the globulins caused by a reduction in available Ca^{2+} + Mg^{2+}. The experiment illustrated in Figure 1B shows the effect of increasing concentrations of EDTA + EGTA on the turbidity of globulin solutions incubated in the presence of Ca^{2+} + Mg^{2+} concentrations responsible for maximum insolubility; these are 5 mM Ca^{2+} + 5 mM Mg^{2+} for total globulins, 10 mM Ca^{2+} + 10 mM Mg^{2+} for α-conglutin, 7.5 mM Ca^{2+} + 7.5 mM Mg^{2+} for β-conglutin, and 2.5 mM Ca^{2+} + 2.5 mM Mg^{2+} for γ-conglutin. As expected, the A_{600} values decrease sharply, reaching values almost identical to those obtained in the absence of added cations for concentrations of 25 mM EDTA + 25 mM EGTA.

In a separate experiment, the effect of a variety of individual cations on the solubility of *L. albus* globulins was tested. Increasing concentrations (from 0 to 10 mM) of Cd, Cu, Zn, Mn, or K were added to a 1 mL silica cuvette containing *L. albus* globulins (total globulins, α-, β-, or γ-conglutins; 0.5 mg/mL) in 50 mM Tris-HCl buffer, pH 7.5. No significant alterations were detected in A_{600} (data not shown), indicating that these cations do not affect the solubility of globulins. With respect to Ca^{2+} and Mg^{2+}, the data illustrated in Figure 2 show the individual or combined effect of these cations on the solubility of *L. albus* β-conglutin. Calcium alone is the most effective in promoting β-conglutin insolubility (with an A_{600} maximum obtained at ~15 mM), followed

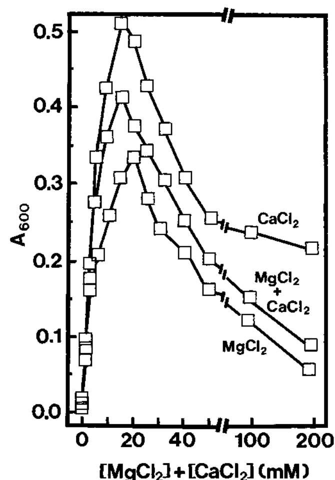

Figure 2. Individual or combined effects of calcium and magnesium on the solubility of *L. albus* β-conglutin. β-Conglutin was purified from *L. albus* as described under Materials and Methods. The protein solution (0.5 mg/mL) in 50 mM Tris-HCl buffer, pH 7.5, was incubated for 5 min in a 1 mL silica cuvette in the presence of increasing concentrations of $CaCl_2$, $MgCl_2$, or $CaCl_2$ + $MgCl_2$.

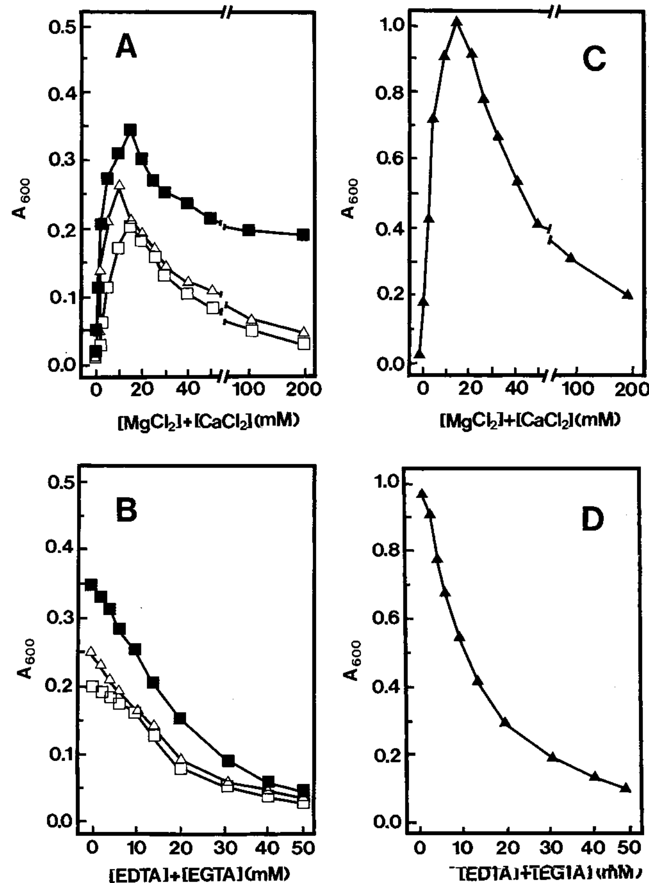

Figure 3. Effect of calcium, magnesium, and chelating agents on the solubility of *P. sativum* and *G. max* globulins. Total globulins from *P. sativum* (△; A, B) or *G. max* (▲; C, D) and vicilin (□) and legumin (■) from *P. sativum* (A, B) were purified as described under Materials and Methods. (A, C) The protein solutions (0.5 mg/mL) in 50 mM Tris-HCl buffer, pH 7.5, were incubated for 5 min in a 1 mL silica cuvette in the presence of increasing concentrations of $CaCl_2$ + $MgCl_2$. (B, D) The protein solutions (0.5 mg/mL) in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM Ca^{2+} + 5 mM Mg^{2+} for *P. sativum* total globulins (△) or 7.5 mM Ca^{2+} + 7.5 mM Mg^{2+} for vicilin and legumin from *P. sativum* or *G. max* total globulins (□, ■, ▲) were incubated for 5 min in a 1 mL silica cuvette in the presence of increasing concentrations of EDTA + EGTA.

by Ca^{2+} + Mg^{2+} (with A_{600} maximum at 7.5 mM Ca^{2+} + 7.5 mM Mg^{2+}) and finally magnesium alone (with A_{600} maximum at ~20 mM).

To analyze the effect of calcium and magnesium ions on the solubility of seed globulins from other legume species, an experiment identical to the one presented in Figure 1 was performed with the total globulins, vicilin, and legumin from *P. sativum* (Figure 3A,B) and with the total globulins from *G. max* (Figure 3C,D). The results obtained are very similar to those observed for *L. albus* globulins, suggesting that the solubility behavior of seed globulins in the presence of Ca^{2+} + Mg^{2+} alone or in combination with chelating agents is common to all legume species. As a control, the effect of Ca^{2+} and Mg^{2+} on the solubility of other proteins was investigated, namely on immunoglobulin G (a globulin of animal origin, purified from goat serum), ribulose bisphosphate carboxylase (purified from *L. minor*), bovine serum albumin (of commercial origin), and egg albumin (of commercial origin). No changes in A_{600} were detected when each of these proteins (0.5 mg/mL in 50 mM Tris-HCl buffer, pH 7.5) was incubated in a 1 mL silica cuvette for 5 min in the presence of increasing concentrations (0−100 mM) of $CaCl_2$ + $MgCl_2$ (results not shown).

Interaction of calcium and magnesium with seed storage proteins has already been reported in the case of soybean. Saio et al. (1968), Rao and Rao (1975), and Sakakibara and Noguchi (1977), for example, have utilized the bivalent cations in the extraction and purification of soybean globulins. Wolf and Briggs (1958) observed that soy globulins have a high affinity for bivalent cations at pH values above their isoelectric point. In particular, protein fractions 7 and 11 S exhibit distinct solubility behaviors in the presence of 10 mM calcium (Saio et al., 1968). The 11 S protein precipitates at lower calcium concentrations than the 7 S protein (Saio and Watanabe, 1973). This observation allowed the fractionation of soy 7 and 11 S proteins at pH 8.0−8.5 on the basis of the relative solubility of the 7 S component and of the insolubility of the 11 S component in 10 mM $CaCl_2$. The insolubility of the 11 S component at 10 mM calcium or magnesium concentrations does not seem to cause association or dissociation of the protein or any alteration in its sedimentation coefficient (Peng et al., 1984). Addition of EDTA or 0.5 M NaCl suppresses the precipitation, drastically reducing the interactions of calcium and magnesium with the globulins (Sakakibara and Noguchi, 1977; Peng et al., 1984). Furthermore, EDTA causes considerable dissociation of 11 S protein into 7 and 4 S, with the result being the formation of a solution of mixed molecular species of 11, 7, and 4 S (Wolf and Briggs, 1958). This dissociation can be reverted by the addition of the divalent cations (Peng et al., 1984).

The results presented in Figures 1 and 3 allow us to understand the improved methodology used to isolate the total globulins from legume seeds that greatly increases the efficiency and selectivity of their extraction (Franco et al., 1997). This improved methodology must include (i) removal of the fat from the milled flour, prior to extraction; (ii) selective removal of the albumin fraction by treatment of the fatfree flour with water (pH adjusted to 8.0) containing 10 mM Ca^{2+}, 10 mM Mg^{2+}, and 1 mM PMSF (this treatment insolubilizes the

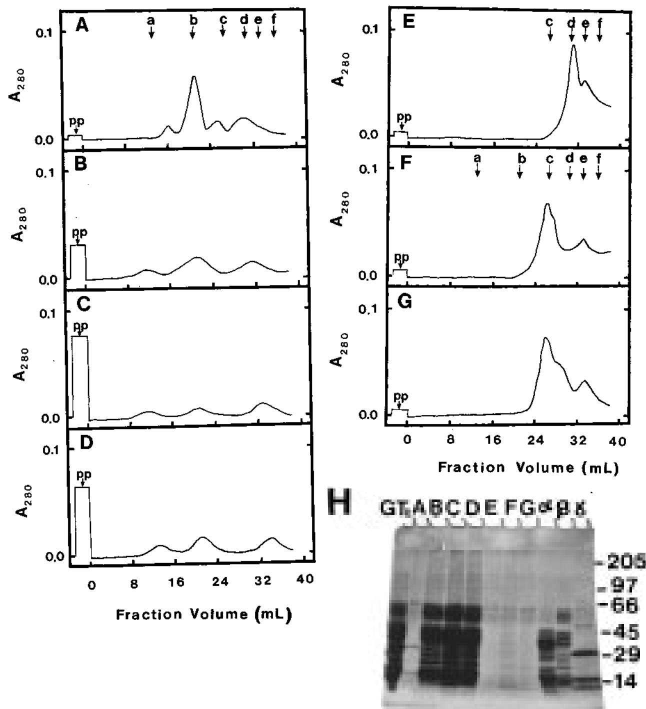

Figure 4. Effect of calcium, magnesium, and chelating agents on the sedimentation coefficients of *L. albus* total globulins. Total globulins (0.75 mg/mL; 2 mL) from *L. albus* were purified and subjected to ultracentrifugation in the presence of no additives (A), 1 mM $CaCl_2$ + 1 mM $MgCl_2$ (B), 5 mM $CaCl_2$ + 5 mM $MgCl_2$ (C), 10 mM $CaCl_2$ + 10 mM $MgCl_2$ (D), 10% (w/v) NaCl (E), 10 mM EDTA + 10 mM EGTA (F), or 10 mM EDTA + 10 mM EGTA + 5 mM $CaCl_2$ + 5 mM $MgCl_2$ (G), as described under Materials and Methods. The sediments in the bottom of the centrifuge tubes (pp) were dissolved and utilized to measure A_{280} or analyzed by SDS−PAGE (H). (A−G) Sedimentation coefficient markers: a, phosphorylase kinase (22 S); b, ribulose bisphosphate carboxylase (15 S); c, catalase (11.3 S); d, immunoglobulin G (6.6 S); e, serum albumin (4.4 S); f, cytochrome *c* (1.8 S). (H) Lanes A, B, C, D, E, F, and G, precipitates from A, B, C, D, E, F, and G, respectively; GT_L, α, *β*, *γ*, *L. albus* total globulins, α-conglutin, *β*-conglutin, and *γ*-conglutin, respectively; molecular masses of markers are indicated in kilodaltons.

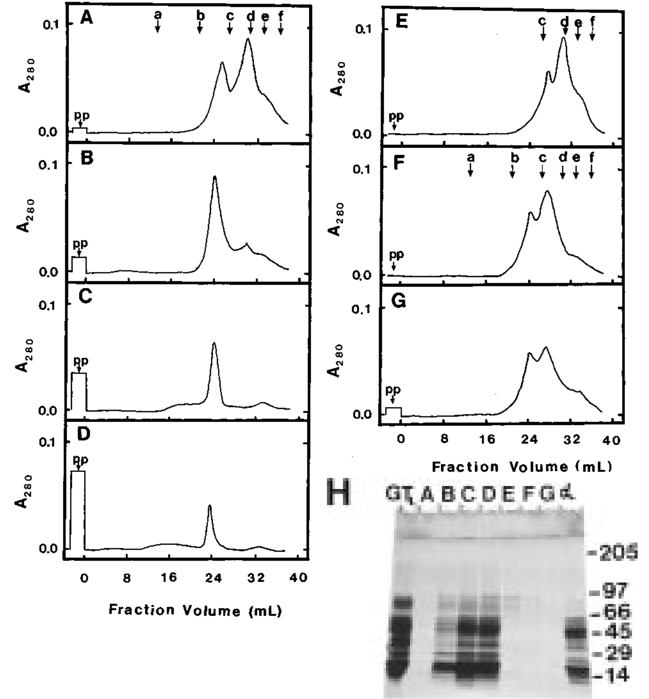

Figure 5. Effect of calcium, magnesium, and chelating agents on the sedimentation coefficient of *L. albus* α-conglutin. α-Conglutin (0.75 mg/mL; 2 mL) from *L. albus* was purified and subjected to ultracentrifugation in the presence of no additives (A), 1 mM $CaCl_2$ + 1 mM $MgCl_2$ (B), 5 mM $CaCl_2$ + 5 mM $MgCl_2$ (C), 10 mM $CaCl_2$ + 10 mM $MgCl_2$ (D), 10% (w/v) NaCl (E), 10 mM EDTA + 10 mM EGTA (F), or 10 mM EDTA + 10 mM EGTA + 5 mM $CaCl_2$ + 5 mM $MgCl_2$ (G), as described under Materials and Methods. The sediments in the bottoms of the centrifuge tubes (pp) were dissolved and utilized to measure A_{280} or analyzed by SDS−PAGE (H). Markers and symbols: same as in the caption to Figure 4.

globulins, ensuring that albumins are removed free of contaminating globulins); and (iii) extraction of the total globulin fraction with buffer, pH 8.0, containing 10% (w/v) NaCl, 10 mM EDTA, and 10 mM EGTA (this solution completely solubilizes the globulins, leading to an efficient extraction of these proteins). Although the presence of EDTA + EGTA produced a marked alteration in globulin structure (see, for example, Figures 4−7), their effect is totally reversible. Indeed, extraction of the total globulin fraction with or without the chelating agents produces identical results in what concerns the structure of the individual conglutins (data not shown). Desalting the total globulins obtained according to this procedure into 50 mM Tris-HCl buffer, pH 7.5, yields clear solutions (results not shown), indicating that globulins are soluble in this buffer and suggesting that some unknown, low molecular weight factor(s) (presumably Ca^{2+} and/or Mg^{2+}) present in the defatted meal, responsible for the typical insolubility of globulins in water (or low ionic strength buffer), was (were) totally or partially removed by the desalting step. This observation suggests that the characteristic water insolubility of legume seed storage proteins does not result from the protein molecules themselves but arises through their interaction with one or more seed components.

Effect of Calcium, Magnesium, and Chelating Agents on the Sedimentation Coefficients of Legume Seed Globulins. To determine whether the turbidity of globulin solutions in the presence of Ca^{2+} and Mg^{2+} arises from the cation-dependent formation of high-order aggregates of globulin molecules, a number of ultracentrifugations were performed. Using isopycnic density gradient centrifugation, we studied the variations in the sedimentation coefficients of the different protein components in the presence of various additives.

Total globulins were isolated from *L. albus* cotyledons and subjected to glycerol density gradient centrifugation as described under Materials and Methods. The results obtained, illustrated in Figure 4A, show that *L. albus* globulins comprise four main fractions with sedimentation coefficients of 19.6, 15, 13.5, and 4.5 S. SDS−PAGE analysis of each of these fractions (results not shown) has revealed that components 19.6 and 15 S are each composed of α- and *β*-conglutins; component 13.5 S is composed of α-conglutin with traces of *γ*-conglutin, and component 4.5 S by α- and *γ*-conglutins. This observation suggests that α-, *β*-, and *γ*-conglutins may associate in vitro with each other to form molecular aggregates of distinct sedimentation coefficients. However, when the ultracentrifugation was perfomed in the presence of increasing concentrations of Ca^{2+} and Mg^{2+} (1 mM Ca^{2+} + 1 mM Mg^{2+}, Figure 4B; 5 mM Ca^{2+} + 5 mM Mg^{2+}, Figure 4C; 10 mM Ca^{2+} + 10 mM Mg^{2+}, Figure

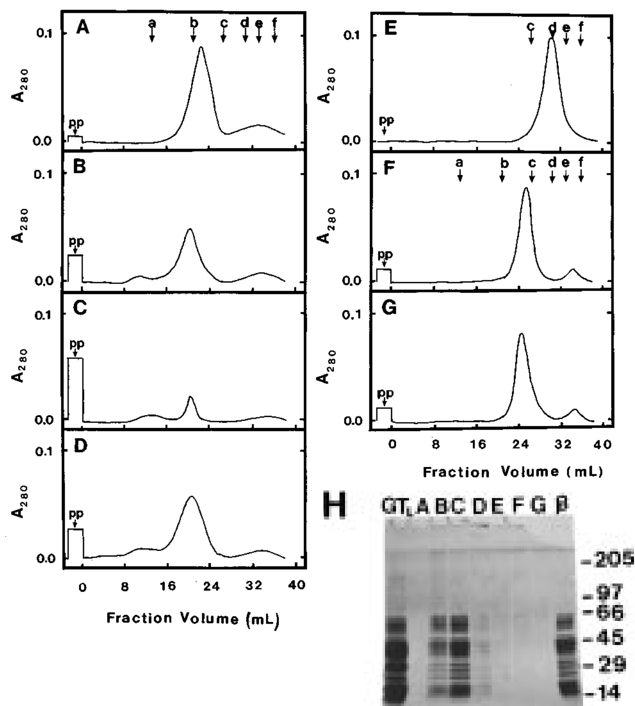

Figure 6. Effect of calcium, magnesium, and chelating agents on the sedimentation coefficient of *L. albus* β-conglutin. β-Conglutin (0.75 mg/mL; 2 mL) from *L. albus* was purified and subjected to ultracentrifugation in the presence of no additives (A), 1 mM $CaCl_2$ + 1 mM $MgCl_2$ (B), 5 mM $CaCl_2$ + 5 mM $MgCl_2$ (C), 10 mM $CaCl_2$ + 10 mM $MgCl_2$ (D), 10% (w/v) NaCl (E), 10 mM EDTA + 10 mM EGTA (F), or 10 mM EDTA + 10 mM EGTA + 5 mM $CaCl_2$ + 5 mM $MgCl_2$ (G), as described under Materials and Methods. The sediments in the bottoms of the centrifuge tubes (pp) were dissolved and utilized to measure A_{280} or analyzed by SDS−PAGE (H). Markers and symbols: same as in the caption to Figure 4.

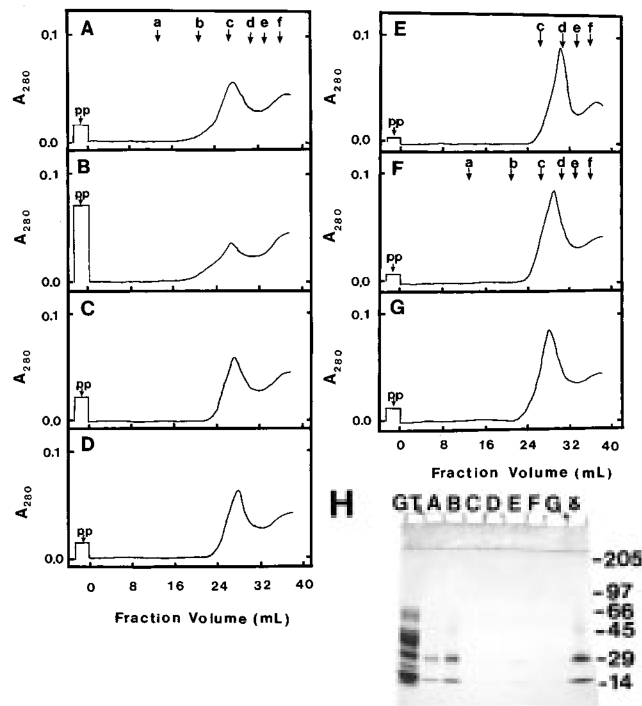

Figure 7. Effect of calcium, magnesium, and chelating agents on the sedimentation coefficient of *L. albus* γ-conglutin. γ-Conglutin (0.75 mg/mL; 2 mL) from *L. albus* was purified and subjected to ultracentrifugation in the presence of no additives (A), 1 mM $CaCl_2$ + 1 mM $MgCl_2$ (B), 5 mM $CaCl_2$ + 5 mM $MgCl_2$ (C), 10 mM $CaCl_2$ + 10 mM $MgCl_2$ (D), 10% (w/v) NaCl (E), 10 mM EDTA + 10 mM EGTA (F), or 10 mM EDTA + 10 mM EGTA + 2 mM $CaCl_2$ + 2 mM $MgCl_2$ (G), as described under Materials and Methods. The sediments in the bottoms of the centrifuge tubes (pp) were dissolved and utilized to measure A_{280} or analyzed by SDS−PAGE (H). Markers and symbols: same as in the caption to Figure 4.

4D) the protein components were moved to regions corresponding to higher sedimentation coefficients. The SDS−PAGE analysis of the precipitates formed in the bottom of the centrifuge tubes (Figure 4H) revealed that the highest Ca^{2+} + Mg^{2+} concentrations utilized caused the precipitation of most globulins, suggesting a cation-induced formation of very high order molecular aggregates—this effect was maximum for 5 mM Ca^{2+} + 5 mM Mg^{2+} (Figure 4C). Isopycnic ultracentrifugations performed in the presence of 10% (w/v) NaCl (Figure 4E), 10 mM EDTA + 10 mM EGTA (Figure 4F), or 10 mM EDTA + 10 mM EGTA + 5 mM Ca^{2+} + 5 mM Mg^{2+} (Figure 4G) caused much less migration of the proteins in the glycerol gradients. These results may be interpreted to mean that globulin molecules interact in a Ca^{2+}- or Mg^{2+}-dependent manner, existing as molecular aggregates. Aggregation of the seed globulins may be electrostatic in nature because these links are disrupted by high ionic strength. Furthermore, this process may involve association of identical or different globulin molecules. The order of these aggregates is directly correlated with the amounts of available calcium and/or magnesium. The use of chelating agents, which sequester most Ca^{2+} and Mg^{2+}, or the presence of high ionic strength, which breaks down the electrostatic interactions, dissociates the globulin aggregates into low sedimentation coefficient protein fractions. Thus, according to this hypothesis, the presence of a relatively high concentration of calcium and/or magnesium ions in the protein bodies of legume seeds promotes the aggregation of globulins into high-order macromolecular structures, allowing an efficient packaging of these proteins in a relatively small volume of space. From a theoretical point of view, protein bodies could be considered as macromolecular structures, composed of an amalgam or intimate mixture of monomers or subunits (the individual globulins), associated in a Ca^{2+}/Mg^{2+}-dependent manner. If this hypothesis is correct, the traditional 7 and 11 S protein components of legume seed proteins may reflect a preferential degree of globulin association under the conditions existing in vitro, with little in vivo significance in what concerns the packing of these proteins in the protein bodies. Nevertheless, this preferential degree of association depends heavily on the conditions utilized in vitro, being easily and extensively altered under some situations, particularly those affecting the availability of Ca^{2+} and/or Mg^{2+} or influencing electrostatic interactions (Figure 4A−G). It is tempting to speculate that solubilization of the globulins and their accessibility to proteases following germination could be easily promoted or facilitated by any mechanism that would absorb the divalent cations.

The experiments illustrated in Figures 5−7 are identical versions of the experiment presented in Figure 4 but performed with *L. albus* α-conglutin, β-conglutin, and γ-conglutin, respectively. Under the conditions utilized in our studies, ultracentrifugation analysis in the absence of additives revealed that α-conglutin (Figure 5A) is composed of two main fractions with

sedimentation coefficients of 11.5 and 6.2 S, β-conglutin (Figure 6A) contains one main fraction with a sedimentation coefficient of 14.2 S, and γ-conglutin (Figure 7A) occurs in two fractions, one with a sedimentation coefficient of 9.8 S and the other in the bottom of the centrifuge tube, with a very high sedimentation value. Ultracentrifugation of each of these conglutins in the presence of increasing concentrations of $Ca^{2+} + Mg^{2+}$ moved the protein fractions to regions of the glycerol gradients corresponding to higher sedimentation coefficients, leading, in all cases, to a considerable precipitation of the globulins in the bottom of the centrifuge tubes (Figures 5H, 6H, and 7H). Maximal precipitation was achieved at 10 mM Ca^{2+} + 10 mM Mg^{2+} for α-conglutin (Figure 5D), at 5 mM Ca^{2+} + 5 mM Mg^{2+} for β-conglutin (Figure 6C), and at 1 mM Ca^{2+} + 1 mM Mg^{2+} for γ-conglutin (Figure 7B), confirming the data presented in Figure 1A. Isopycnic ultracentrifugation of each of the conglutins in the presence of NaCl (Figures 5E, 6E, and 7E), chelating agents (Figures 5F, 6F, and 7F) or chelating agents + Ca^{2+} + Mg^{2+} (Figures 5G, 6G, and 7G) reduced considerably the migration of the proteins in glycerol gradients, confirming the results obtained for the total globulin fraction (Figure 4).

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; FPLC, fast protein liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; UV, ultraviolet.

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