

Affinity Chromatography of the Major Seed Protein of the Bean (*Phaseolus vulgaris* L.)¹

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ABSTRACT

The major globulin of the French bean (*Phaseolus vulgaris* L.) undergoes a reversible pH-dependent polymerization. At pH values above 6.5, the monomeric form of the protein predominates; and at pH values below 6.5, the protein occurs as a polymer, probably a tetramer. At extremes of pH, the protein dissociates further into peptides. The reversible pH-dependent interaction between globulin subunits is used in this report as the basis for an affinity chromatography procedure for isolation of the globulin. The major globulin from several genetic variants can be obtained in gram quantities and does not indicate the presence of any impurities on discontinuous sodium dodecyl sulfate gel electrophoresis.

tetrameric species (18.2S) predominates at lower pH values (18). At extremes of pH, the globulin dissociates into even lower mol wt peptides (18). The availability of a solubility-based isolation procedure for G1 protein and the existence of this pH-dependent equilibrium suggested the possibility of developing an isolation procedure for the G1 protein based on the affinity of the monomer species for other monomer molecules. Monomers of the G1 protein which are covalently coupled to a solid support should interact specifically with other monomers at a pH value where polymerization is favored and thus allow separation of the G1 protein from other proteins incapable of polymerization with immobilized monomer. Changing the pH to a value where dissociation is favored should release the isolated G1 protein. The present report demonstrates the feasibility of this approach to isolation of legume seed globulins.

Legume seed globulins are, on a global scale, an important food protein resource. Manipulation of the genetically controlled composition of these proteins has taken a high priority in efforts to increase the over-all nutritional value of legume seeds (1). Legume globulins provide, in addition, a particularly interesting system for the study of protein synthesis in plant tissues since rapid synthesis of these proteins occurs at well defined stages in the development of the seed (12). The major obstacle in the study of legume seed globulins has been the difficulty encountered in obtaining homogeneous preparations of these proteins. Most published methods for isolation of legume seed globulins are separations based on differential solubility of the seed proteins in various solvents. The majority of these separations are differential salt solubility methods based on the very early observations of Osborne and Campbell (13) whose nomenclature (legumin, vicilin) is, in many cases, still retained. It is difficult for any solubility-based isolation technique to give homogeneous protein preparations, and most preparations of legume seed globulins isolated by these methods show minor bands of contaminants after electrophoresis under dissociating conditions. The partial success achieved with solubility-based separations is due mainly to the fact that the major storage globulins are present in high concentrations in the seed. Until preparations representing single molecular species can be easily isolated, the study of these important proteins will not progress rapidly.

The globulins of *Phaseolus vulgaris* L. cv. Tendergreen seeds have received considerable attention, and an ascorbic acid-NaCl extraction technique has been shown to separate effectively the high salt-soluble globulin (G1)² from the low salt-soluble fraction (11, 17). The G1 protein is a glycoprotein which undergoes a reversible pH-dependent association. The monomer (7.1S) is the predominant species at pH values above 6.5, whereas the

MATERIALS AND METHODS

Plant Material and Extraction Procedure. Seeds of *P. vulgaris* L. cv. Improved Tendergreen were obtained with Northrup-King Seed Co., Madison, Wisconsin (lot No. 35110-04900). Seeds of a low methionine strain of *P. vulgaris* (PI 229,815) were grown locally. Isolation of the G1 globulin was accomplished using the procedure described by Sun *et al.* (18). Seeds were ground in a Wiley cutting mill and the flour extracted by stirring for 1 hr (4 C) in 0.5 M NaCl, 0.25 M ascorbate. A 7-fold dilution of the supernatant from this extraction resulted in precipitation of the high salt-soluble protein. This precipitate was extracted with 0.5 M NaCl, and the high salt-soluble protein collected. Protein obtained by this procedure was stored at 0 C until use. Protein concentrations were estimated using 3.3×10^5 for the molar extinction coefficient of the G1 protein in 0.5 M NaCl at 280 nm.

Preparation of G1 Coupled to Sepharose. G1 protein from *P. vulgaris* L. cv. Improved Tendergreen was coupled to Sepharose 4B by the procedure described by Cuatrecasas and Anfinsen (5). About 0.5 g of G1 protein in 0.1 M NaHCO₃ adjusted to pH 9.5 was mixed with 200 ml of Sepharose 4B which had been activated with 30 g of cyanogen bromide. After 20 hr of gentle mixing at 4 C, the coupled product was extensively washed with 0.1 M NaHCO₃, pH 9.5, then with 0.5 M NaCl, pH 8.5, then with 0.5 M NaCl, pH 4.5, and finally again with 0.5 M NaCl, pH 8.5. The coupled G1-Sepharose was stored at 4 C.

Isolation of G1 by Affinity Chromatography. Approximately 7 g of seed were ground and extracted with 100 ml of 0.5 M NaCl, pH 8.5, for 30 min. The extract was centrifuged at 15,000 rpm in a SS34 rotor of a Sorvall RC-5 refrigerated centrifuge. The supernatant solution was mixed with the G1-Sepharose in a 600-ml coarse fritted disc Büchner funnel and the pH of the slurry was adjusted with 0.1 N NaOH to pH 8.5. After thoroughly mixing the crude extract with the G1-Sepharose, the slurry was adjusted to pH 4.5 with 0.1 N HCl. After allowing the mixture to stand at pH 4.5 for 20 min, the G1-Sepharose was

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² Abbreviation: G1: high salt-soluble fraction.

washed with several liters of 0.5 N NaCl, pH 4.5, in 300-ml increments until the absorbance of the washes was less than 0.01 at 280 nm. The coupled Sepharose was then mixed with 300 ml of 0.5 M NaCl, pH 8.5, adjusted to pH 8.5 with 0.1 N NaOH, and allowed to stand for 15 min. Collection of this and subsequent washes by filtration removed the retained G1 protein.

Sephadex G-200 Gel Filtration. Gel filtration of the G1 protein was done in a column of Sephadex G-200 (1.6 × 52 cm) equilibrated with 0.01 M citrate, 0.5 M NaCl adjusted to pH 4.5 or 7.2. The flow rate of the column was controlled at 13.7 ml/hr using a Gilson HP4 pump. Fractions of 0.45 ml (2 min) were collected using a Gilson FC-80H micro-fractionator and the absorbance of the fractions at 280 nm was measured using a Beckman 25 spectrophotometer.

SDS Gel Electrophoresis. The discontinuous SDS gel electrophoresis system of Laemmli (10) was modified to give a 200:1 ratio of acrylamide-*bis* acrylamide (9). Electrophoresis was performed in thin layer slab gels (1.6 mm thick) in an apparatus similar to that described by Studier (16).

RESULTS

Gel Filtration of G1. Gel chromatography of G1 demonstrates the presence of at least three forms of the protein (Fig. 1). The relative amount of each form present in solution is dependent upon the pH. At pH 7.2 (Fig. 1), the monomeric form of the protein is predominant, but the presence of a higher mol wt polymer which elutes more rapidly is clearly evident. At pH 4.5, the rapidly eluting polymeric species is predominant, and a low mol wt form of the protein which elutes more slowly than the monomer (presumably dissociated peptides) can also be seen. This behavior is consistent with the previously reported sedimentation behavior of this protein except that no reproducible dissociation into peptides at low pH was obtained from centrifugation studies (18).

Affinity Chromatography of G1. The elution of a 0.5 M NaCl extract of *P. vulgaris* cv. Tendergreen seeds from a G1-Sepharose resin is shown in Figure 2. Elution at pH 4.5 with 0.5 M NaCl results in removal of most of the protein. Changing the elution pH to 8.5 releases the G1 protein which was retained on the column. Increasing the amount of seed meal extracted into 100 ml of 0.5 M NaCl and then mixed with the G1-Sepharose increased the yield of G1 protein to a maximum of about 1 g (Fig. 3).

Discontinuous SDS gel electrophoresis patterns of G1 isolated in this fashion from two different genetic variants are shown in Figure 4. The electrophoretic mobility of G1 subunits varies for different hybrid seed lines, and the genetic dependence of the banding pattern has been shown to occur in a manner consistent with that expected for control by a single Mendelian gene (15). As shown in Figure 4, affinity isolated G1 from *P. vulgaris* L. cv. Improved Tendergreen shows three distinct bands on SDS gel electrophoresis (corresponding to the three subunits of the pro-

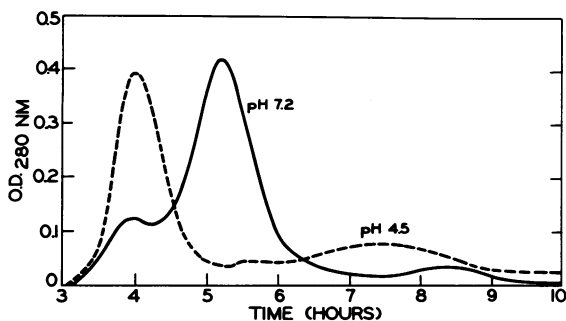


Fig. 1. Sephadex G-200 chromatography of G1 at pH 7.2 and pH 4.5.

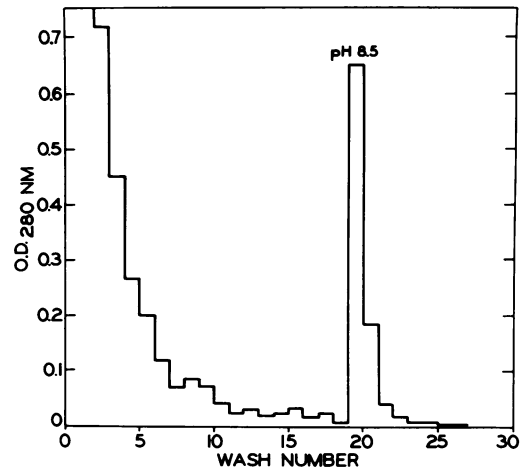


Fig. 2. Elution of a crude 0.5 M NaCl extract of *P. vulgaris* L. cv. Improved Tendergreen seeds from a G1-Sepharose 4B column. Coupled G1 was washed with 300-ml increments of 0.5 M NaCl, pH 4.5, until absorbance at 280 nm was less than 0.01. Subsequent elution was with 300-ml increments of 0.5 M NaCl, pH 8.5.

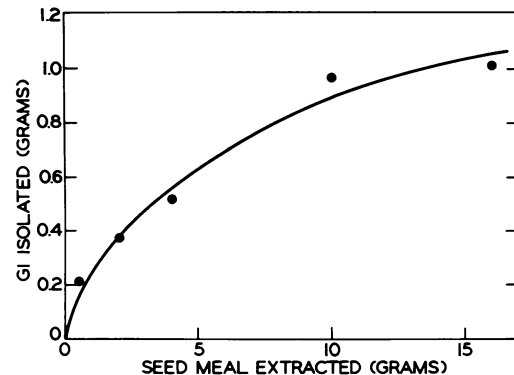


Fig. 3. Effect of increased sample size on amount of G1 obtained by affinity isolation. Seed meal was extracted with gentle stirring in 100 ml of 0.5 M NaCl for 30 min. The supernatant of this extract was incubated with the G1-Sepharose resin for 30 min at pH 4.5. Elution of extract was as described in text.

tein). The affinity-isolated G1 shows no trace bands of the other major globulin fraction (G2) (18) or of other impurities in this gel system even at high loading of the gel (Fig. 4). G1 from *P. vulgaris* (PI 229,815) can also be isolated by affinity chromatography on a G1-Sepharose resin made with G1 isolated from the Tendergreen cultivar. Affinity-isolated G1 from *P. vulgaris* (PI 229,815) shows three bands on discontinuous SDS gel electrophoresis, but the subunit of highest mol wt migrates more rapidly than does the corresponding subunit of the Tendergreen cultivar. In this case as well, no minor bands are present in the affinity-isolated G1.

DISCUSSION

The majority of affinity isolation procedures utilize the affinity of macromolecules for low mol wt substances. However, any specific interaction of sufficient stability may be used as the basis for isolation of the components involved. An affinity isolation procedure similar in principle to that reported here is the preparation of aldolase by Chan and Mawer (4). These workers used the reversible urea-induced dissociation of aldolase tetramers as the basis for isolation of the enzyme. Unfortunately, the urea concentration which resulted in dissociation of the tetramer was higher than that required for denaturation of the monomeric

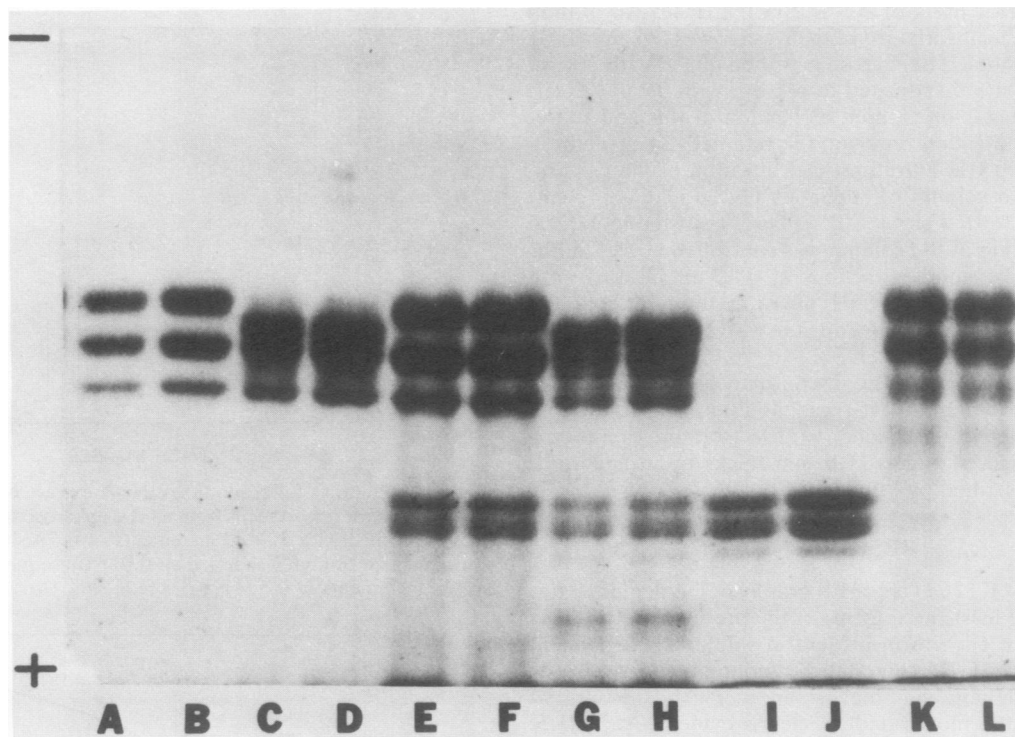


FIG. 4. Discontinuous SDS slab gel electrophoresis of G1 fractions. A, B: Affinity-isolated G1 from *P. vulgaris* L. cv. Improved Tendergreen; C, D: affinity-isolated G1 from *P. vulgaris* (PI 229,815); E, F: supernatant from crude 0.5 M NaCl extract of *P. vulgaris* L. cv. Improved Tendergreen; G, H: supernatant from crude 0.5 M NaCl extract of *P. vulgaris* (PI 229, 815); I, J: G2 from *P. vulgaris* L. cv. Improved Tendergreen isolated by solubility procedures (18); K, L: G1 from *P. vulgaris* L. cv. Improved Tendergreen isolated by solubility procedures (18).

species. The method, therefore, was not a practical isolation procedure, since recovery of active aldolase from an aldolase monomer-Sepharose resin requires not only dissociation of the tetramer, but also renaturation of the isolated monomers. Finally, mention should also be made of the isolation of α -gliadin reported by Bernardin and co-workers (2). α -Gliadin undergoes a reversible pH-dependent aggregation resulting in precipitation of high mol wt polymers, and collection of this high mol wt aggregate by ultracentrifugation was shown to be a useful step in its isolation. Although this is a solubility-based rather than affinity isolation, the use of a pH-dependent subunit \times subunit interaction is similar in principle to the isolation reported here.

The major globulin of the French bean, *P. vulgaris* L., was reported by Sun *et al.* to undergo a reversible pH-dependent monomer-tetramer equilibrium (18). The existence of this equilibrium is confirmed in this paper by both the pH-dependent gel filtration behavior of the G1 protein and by the successful isolation of this protein using a procedure dependent upon this equilibrium. The monomeric form of the protein appears to predominate at pH values above 6.5 but below pH 11 where dissociation into peptide subunits occurs. Below pH 6.5, the tetrameric form of the globulin predominates. The gel filtration results suggest that at lower pH values, dissociation into peptides can occur; however, no evidence of this low pH dissociation was observed in sedimentation experiments (18).

Covalent attachment of the G1 globulin to Sepharose allows the specific isolation of this protein from crude extracts of the whole seed. At pH 4.5, the G1 protein in a crude extract will interact with the immobilized G1 to form a tetramer. Subsequent washing of the G1-Sepharose resin at pH 4.5 removes all proteins except the G1 which has formed tetramers which include a G1 subunit covalently attached to the Sepharose. Changing the pH of elution to pH 8.5 causes dissociation of the tetramers and the free G1 can be removed, leaving behind only

the covalently attached G1 molecules. This procedure gives a preparation of G1 protein with no indication of minor impurities on SDS gel electrophoresis. A complete isolation of G1 protein from *P. vulgaris* seeds can be conveniently done in about 2 hr and the resulting preparation is of higher purity than is obtainable by more time-consuming solubility-based methods.

The application of affinity procedures to the purification of the globulins of other legume species should in some cases be possible. Several legume seed globulins are known to undergo either pH- or salt-dependent reversible equilibria (3, 6-8, 14) and should, therefore, be susceptible to similar kinds of isolation procedures.

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