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ISOLATION OF THE ACIDIC AND BASIC SUBUNITS OF GLYCININ

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Abstract—The acidic and basic subunits of glycinin—the major storage protein of soybean seeds (*Glycine max*)—were isolated by a simple ion exchange chromatography method involving a two-step pH change of the elution buffer. Abnormal behaviour of the subunits in dodecyl sulfate polyacrylamide gel electrophoresis is suggested.

INTRODUCTION

Biochemical studies on the structure and properties of the storage proteins of soybean have important implications in technological and genetic engineering developments in regard to food protein supply. It was originally reported [1, 2] that glycinin, the major storage protein of soybean seeds, is composed of acidic and basic subunits. Subsequent studies have indicated that the acidic subunits have higher MW than the basic subunits [3–5] and that some degree of homology exists among the acidic subunits [6]. However, there is no general agreement on the number of acidic subunits. Catsimpoolas [1] reported the presence of three acidic and three basic subunits and his findings were later confirmed by Badley *et al.* [5]. Kitamura and Shibasaki [4] consider glycinin to be composed of four acidic and three basic subunits. The fourth acidic subunit of MW 45000 was previously rejected by Catsimpoolas *et al.* [3] as being part of the structure of glycinin because of its low concentration in relation to the rest of the acidic subunits. These authors considered the fourth component to be either an association product or a contaminant. Some discrepancy also exists concerning the MW of the subunits. For the acidic subunits, MWs of 37200 [3], 34800 [5], 37000 [4], and 45000 [4] have been found. The basic subunits appear to have MWs of 22300 [3], 22500 [4], and 19600 [5]. The amino acid sequence of individual polypeptide chains is not known.

In view of the importance in understanding the multisubunitary structure of storage proteins, further studies on the subunits of glycinin are necessary to establish the complete chemical and physical structure of the protein. The first step in such work is the isolation of the individual basic and acidic subunits. This has been accomplished in the past by isoelectric focusing [1] and DEAE-Sephadex A-50 chromatography [4]. In attempts to develop a simple procedure for the initial isolation of the two types of subunits (i.e. acidic and basic), we adopted the ion exchange chromatography method of Wright and Boulter [7]. This report describes the successful separation of the acidic and basic subunits of glycinin in sufficient amounts to permit further fractionation within each group by other methods. MWs were determined by the use of a discontinuous buffer system in sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

Glycinin purified by ammonium sulfate fractionation [8] and subjected to DEAE Bio-Gel chromatography exhibits the typical elution pattern shown in Fig. 1. The protein (400 mg) was eluted with pH 7.6 phosphate buffer containing 0.01M mercaptoethanol and a gradient of NaCl concentration from 0.1 to 1.0 M. The solid line

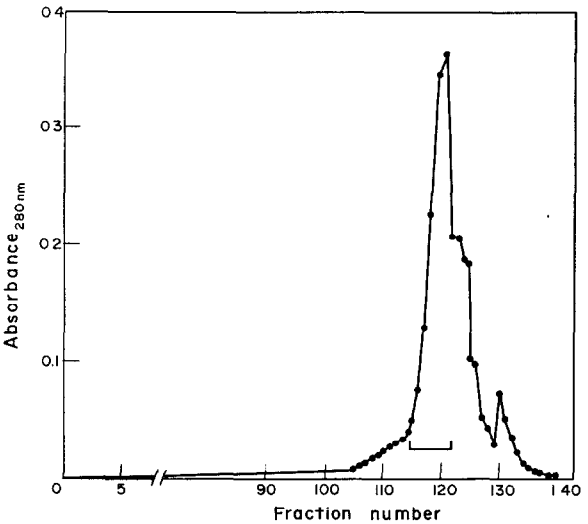

Fig. 1. Purification of glycinin by DEAE Bio-Gel ion exchange chromatography. Column size: 40 × 4 cm. Sample: 400 mg of glycinin in 15 ml of pH 7.6 phosphate buffer containing 0.1 M NaCl and 0.01 M mercaptoethanol. Elution: Same buffer containing NaCl in gradient concentration of 0.1 M to 1.0 M. Flow rate 25 ml/hr. Solid line: UV absorbance at 280 nm. Barred line: pooled fractions containing pure glycinin.

in Fig. 1 represents UV absorbance and the barred area indicates the pooled fractions that consist of homogeneous glycinin as determined by electrophoresis. The use of DEAE Bio-Gel instead of DEAE-Sephadex A-50 [9] for the final step in the purification of glycinin has the advantage that regeneration of the Bio-Gel column is more convenient since it does not have to be repacked after each experiment. The disulfide bonds of the purified protein were reduced with mercaptoethanol in the presence of 6 M guanidine hydrochloride and the liberated sulfhydryl groups were blocked with iodoacetamide. The dissociated protein was subsequently subjected to ion exchange chromatography on a Dowex AGI(X2) resin in the presence of 6 M urea. The first elution was carried out with pH 8 Tris–acetate buffer in 6 M urea and the final elution was done with pH 4.5 acetate buffer in 6 M urea. Figure 2 shows the separation

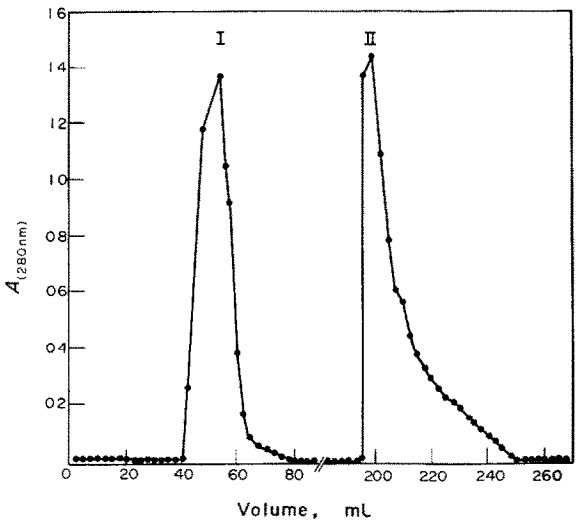

Fig. 2. Ion exchange chromatography (Dowex AGI (X2) resin) of purified and carboxymethylated glycinin. Column size: 20 × 1.5 cm. Sample: 250 mg modified glycinin in 10 ml of 50 mM Tris–acetate buffer (pH 8) containing 6 M urea. Elution: First elution: in above buffer; second elution in 50 mM acetic acid containing 6 M urea adjusted to pH 4.5 with NaOH. Flow rate: 20 ml/hr. Fraction I: basic subunits of glycinin. Fraction II: acidic subunits of glycinin.

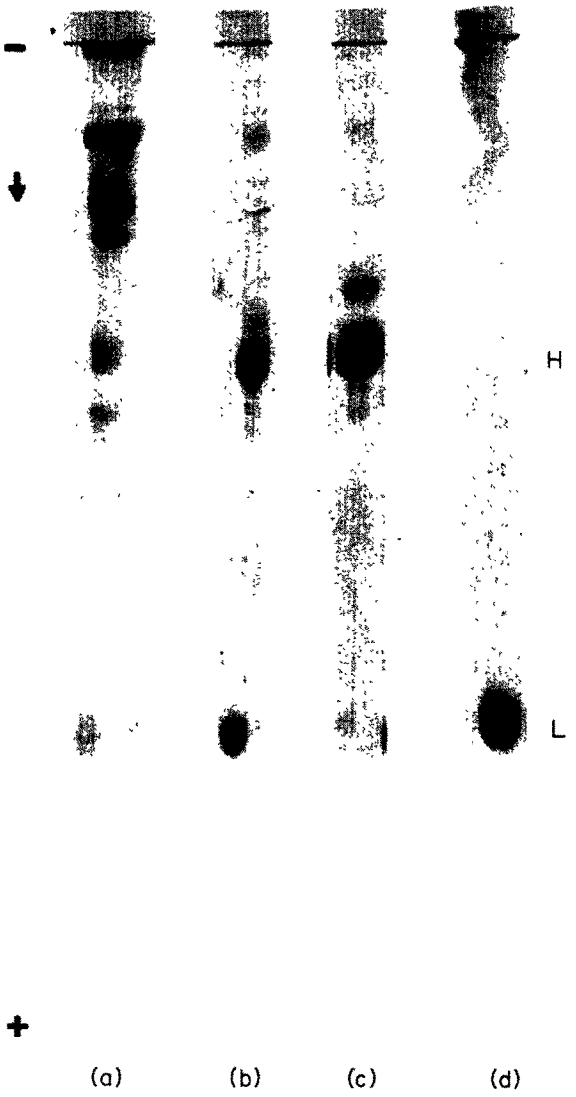

Fig. 3. SDS electrophoresis patterns of: ammonium sulfate purified glycinin (a); DEAE–Biogel purified glycinin (b); isolated basic subunits (Fraction I) of glycinin (c); and isolated acidic subunits (Fraction II) of glycinin (d). H and L indicate the position of the heavy (acidic) and light (basic) subunits of glycinin, respectively.

of two types of subunits. At pH 8 the basic subunits which are not retained by the resin are eluted first (peak I). Change of the pH of the elution buffer to 4.5 causes the elution of the acidic subunits (peak II). Figure 3a shows the electrophoretic (SDS–PAGE) pattern of glycinin before purification by column chromatography. Several impurities can be distinguished that do not correspond to H and L components discussed below. Final purification of glycinin from DEAE-Biogel chromatography exhibits a SDS–PAGE pattern as shown in Fig. 3b. Two groups of components are distinguishable in regard to a molecular size and these are designated as H and L denoting heavy and light chains, respectively. One L component and major and minor H components can be seen. From the comparison of the relative mobility (R_f) values of these bands to those of standard proteins, the MW of the major H component was estimated to be 42 000 and that of the minor H component 45 000. The L component has a MW of 19 000. Isolated basic subunits are shown in Fig. 3c and correspond to peak I of Fig. 2. The basic subunits correspond to the L component of purified glycinin. Isolated acidic subunits shown in peak II of Fig. 2 exhibit a PAGE pattern shown in Fig. 3d. The acidic subunits correspond to the H component.

DISCUSSION

We have demonstrated that the use of ion exchange chromatography on Dowex AGI(X2) column involving a two step pH change of the buffer provides a simple means for the isolation of the acidic and basic subunits of glycinin in bulk quantities (up to 250 mg). The column can be used repetitively after *in situ* regeneration. Thus,

sufficient quantity of the two types of subunits can be obtained for further fractionation and characterization.

The use of a discontinuous buffer system in SDS-PAGE was used to allow high resolution separation of the subunits. However, the MW of the L component is slightly lower than that reported by Catsimpoolas *et al.* [3] and Kitamura and Shibasaki [4] using a continuous pH buffer system and in closer agreement to that reported by Badley *et al.* [5]. The major H component exhibits much higher MW by the present technique than previously reported [3–5]. However, the MW of the minor H component is in agreement with previously reported values [3,4]. These results suggest that the subunits of glycinin behave abnormally in SDS–PAGE and therefore MW estimation can be considered at best very approximate. This condition does not allow simple arithmetic determination of the number of polypeptide chains from the MW of the protein [5, 10] and its subunits. Other methods will have to be employed for comparison. Further work is also necessary to clarify the nature of the minor H component which Kitamura and Shibasaki [4] consider it to be one of the acidic subunits of glycinin.

EXPERIMENTAL

The soybeans used were Corosoy variety grown in 1973 and stored at 25°. Seeds were cracked, dehulled, and ground. Material was defatted with hexane (bp 70°) in a Soxhlet apparatus for 48 hr and subsequently dried at room temp. Glycinin was prepared as described in ref. [8]. Briefly, this consisted of extraction of defatted flakes with H_2O (flake: H_2O ratio—1:5) at 25°. Subsequently the protein was precipitated in the cold for 18 hr (4°) and centrifuged to obtain the cold insoluble fraction. The protein in this fraction was precipitated twice with $(NH_4)_2SO_4$ (pH 7.6) between 51 and 66% of saturation followed by acid precipitation at pH 4 and further precipitation between 26 and 40% saturation with $(NH_4)_2SO_4$. Chromatographic fractionation of the purified protein was performed on a 4 × 40 cm DEAE–Biogel anion exchange column (100–200 mesh) equilibrated with a pH 7.6 Pi buffer (32 mM K_2HPO_4) made 0.1 M in NaCl and 0.01 M in mercaptoethanol. Ionic strength elution was carried out by addition of 1 M NaCl to a mixing chamber containing 400 ml of the standard buffer. Detection of the protein fractions was by *A* at 280 nm. The pooled glycinin fractions were dialyzed against H_2O and freeze-dried. The purified protein (250 mg) was dissolved in 25 ml of 1 M Tris–HCl, pH 8.7 made 6 M in guanidine HCl and 1 mM EDTA. The soln was flushed with N_2 for 15 min, then 0.25 ml mercaptoethanol was added. The soln was incubated at 37° for 5 hr. After the addition of iodoacetamide (1.2 g) the reaction was allowed to proceed in the dark. When a negative nitroprusside test was obtained (about 15 min), the protein was dialyzed against H_2O and freeze-dried. Separation of the modified glycinin into acidic and basic subunits was performed on a Dowex AGI (X2) resin [7]. Briefly, the resin was washed exhaustively in a Buchner funnel with 2 N NaOH, H_2O, 4 N HOAc, H_2O and 0.05 M Tris–acetate buffer, pH 8. Just before use the resin was equilibrated on a 1.5 × 20 cm Pharmacia column with 0.05 M Tris–acetate buffer (pH 8) containing 6 M urea. The second soln used for elution was 0.05 N HOAc containing 6 M urea adjusted to pH 4.5 with NaOH. Fractions (2.5 ml) were collected at a rate of 30 ml per hr. The eluate was monitored at 280 nm and 254 nm. SDS slab gel electrophoresis in a discontinuous buffer system was performed according to the method described below. The polyacrylamide slab (10 × 14 × 1.5 mm) was prepared as follows: buffers used, 1) Zeta buffer (pH 8.89) containing 6.00 g glycine, 9.12 g Tris, 2.00 g SDS, to 2 l. with H_2O, 2) Gamma buffer (pH 8.92) containing 28.92 ml 1 N HCl, 11.47 g, to 100 ml H_2O, 3) Beta buffer (pH 6.44) containing 10.11 ml 1 M H_3PO_4, 1.92 g Tris, to 100 ml with H_2O. The acrylamide soln used for the separating gel contained 0.6 gm *N,N'*-methylenebisacrylamide (BIS), 11.4 g acrylamide (12% T, 5% C), to 50 ml H_2O. The stacking gel consisted of 0.313 g BIS, 2.817 g acrylamide (3.13% T, 10%C), to 50 ml with H_2O. The catalyst soln contained 0.03 g potassium persulfate, 1 mg riboflavin, to 100 ml with H_2O. Both separating and stacking gels contained 0.1% SDS. The samples were dissolved in a soln containing 6 M urea, 0.3% SDS, and 10 mM dithiothreitol, and made to vol with Zeta buffer. Bromophenol blue was added as a tracking dye. Sample solns (1 mg/ml) were incubated at 50° for 30 min just prior to sample application. The separating gel was made by mixing 2 parts separating soln, 1 part Gamma buffer, and 1 part catalyst. This soln was degassed at 15 mm Hg for 10 min at which time SDS (0.1%) and 25 μl of Temed were added. The slab was poured and allowed to polymerize under uniform fluorescent light for 45 min. The stacking gel was made by mixing 2 parts stacking soln, 1 part Beta buffer, and 1 part catalyst. The soln was degassed at 15 mm Hg. SDS (0.1%) and 12 μl Temed were then added. Electrophoresis of 25 μg samples was carried out for *ca* 17 hr at 12 mA (constant) (20°). Prior to staining, the SDS was removed by fixation for 24 hr. The fixation soln consisted of equal parts of MeOH and 20% HOAc. Subsequently, the slab was stained with 1% Coomassie blue in 7% HOAc and destained by diffusion in 7% HOAc, and 30% MeOH. Zeta, Beta and Gamma buffers (system No. 2860) were designed by a computer program [1]. MW determination was performed by another computer system [12] from relative mobility (R_f) values of unknowns vs standard proteins.

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