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Isolation and Purification of 7S and 11S Globulins from Broad Beans and Peas

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A simple and effective preparation technique for isolation of 7S and 11S globulins from broad beans and pea seeds has been developed. It is based on differential solubility of 7S and 11S globulins in sodium chloride solution at pH 4.8 and at 5 and 20 °C. The method makes it possible to isolate 6 g of 11S and 1 g of 7S globulins from 100 g of broad bean meal or 1.5 g of 11S and 2 g of 7S globulins from 100 g of pea meal, with only low centrifugation. If necessary, the admixture of 15S component in 11S globulin preparations can be removed by gel chromatography on Sepharose CL-6B.

Preparative isolation of 7S and 11S globulins from legume seeds presents a certain difficulty. A number of techniques are known to be effective only in obtaining enriched globulin fraction: salting out by ammonium sulfate (Danielsson, 1949), isoelectric precipitation (Derbyshire et al., 1976), gel chromatography (Gwiazda et al., 1980). The method of selective thermal denaturating (Varfolomeeva et al., 1985) makes it possible to obtain a precipitate of homogeneous 11S globulins, but with denaturation and loss of considerable amount of 11S and all 7S globulins. Centrifugation in a sucrose gradient (Mori and Utsumi, 1979) yields a homogeneous globulin fraction, but this method cannot be suggested as a preparational one. A successful solution of the problem of preparational isolation of 7S and 11S globulins was achieved by zone precipitation (Scholz et al., 1974; Wright and Boulter, 1974), which combines the principle of isoelectric precipitation with that of gel chromatography. The main drawback of this method is the need to use multiple chromatography techniques to achieve complete separation of components. Thus, none of the methods listed above has proved to be effective in obtaining homogeneous preparations of 7S and 11S globulins in the quantities necessary for the studies of different physicochemical properties of concentrated protein solutions.

7S and 11S globulins are known to have different solubility in solution with low ionic strength (Osborne and Campbell, 1898a–c). 7S globulins are more soluble than 11S globulins. These data formed the basis for developing a simple method of isolating 7S and 11S globulins from broad beans and pea seeds.

MATERIALS AND METHODS

Sorted seeds of broad beans (*Vicia faba* L.) of Fribo variety and those of peas (*Pisum sativum* L.) of Orlovsky-3 variety were ground in a laboratory cyclone mill (UDY Corp., Fort Collins, CO). The flour was sifted with the sieve of 0.3-mm cell size.

Isolation of 11S Globulins. A 100-g portion of meal was dispersed in 900 mL of distilled water and the resultant mixture titrated with 0.5 M solution of NaOH to pH 8.0 and then mixed at 50 °C for 1 h. The suspension was centrifuged at 5000*g* for 30 min (K-70D, MLV, GDR). Dry NaCl was added to the supernatant to 0.5 M. The solution was titrated to pH 4.8 with 0.1 M HCl containing 0.5 M NaCl. The suspension obtained was centrifuged at 5000*g* for 30 min, and supernatant was diluted with distilled water to 0.3 M NaCl concentration. It was centrifuged at 1000*g* for 10 min. After centrifugation the system separated into two liquid phases: 1, lower phase; 2, upper one. The lower liquid phase with a protein concentration of about 50% and 0.3 M NaCl concentration was the fraction enriched with 11S globulin. Dry NaCl was added to this concentrated liquid phase to 0.6 M. The obtained concentrated solution was diluted with 0.6 M NaCl solution to about 5% protein concentration. Then, it was centrifuged at 5000*g* for 30 min. The supernatant was diluted with distilled water to 0.3 M NaCl. The system separated into two phases when kept for 10–15 min. The lower concentrated protein phase contained 11S globulin with negligible admixture of 15S globulin. All the operations were made at 20 °C. Protein yield was 6 g from broad beans and 1 g from pea seeds per 100 g of meal.

Isolation of 7S Globulins. The upper liquid phase was cooled to 5 °C and exposed to this temperature for 1 h. The precipitate obtained (fraction enriched with 7S globulin) was separated by centrifugation at 1000*g* for 15 min at 5 °C. The supernatant with 0.3 M NaCl concentration was diluted with 5 °C cool distilled water to 0.15 M NaCl concentration. The precip-

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itate (pure 7S globulin) was separated by centrifugation at 1000*g* for 15 min at 5 °C. The protein yield was 1.5 g from broad beans and 2 g from pea seeds per 100 g of meal.

Sedimentation analysis was carried out in standard phosphate buffer (0.02 M NaH_2PO_4 + 0.0266 M Na_2HPO_4 + 0.4 M NaCl) at pH 7.6 at 260 000*g* in an MOM 3170B ultracentrifuge (Budapest, Hungary) according to Claesson and Moring-Claesson (1961).

Gel chromatography was carried out on a 2 cm × 50 cm column of Sepharose CL-6B (Pharmacia, Uppsala, Sweden) equilibrated with phosphate buffer (0.02 M NaH_2PO_4 + 0.0266 M Na_2HPO_4 + 0.4 M NaCl), pH 7.6. A 5-mL portion of 5% 11S globulin solution was applied to the column and eluted at 0.6 mL/min. Gel chromatography of 7S globulins was performed on a 4 cm × 50 cm column equilibrated with 0.5 M NaCl solution. A 10-mL portion of 7S globulin solution was applied to the column and eluted at 2 mL/min. The protein content in the eluate was measured at 290 nm on UV detector PUM-2 (SKB BP AN USSR, Puschino, USSR).

Polyacrylamide gel electrophoresis without sodium dodecyl sulfate according to Reisfeld et al. (1962) was performed on vertical plates with polyacrylamide concentration equal to 5%. Electrophoresis was performed at 40 mA/gel (1 mm × 115 mm × 115 mm) for 2 h at 20 °C. The samples were stained for 1 h in 0.04% Coomasie G-250 solution, containing 3.6% $HClO_4$. Excess dye was removed by triple washing with a solution containing 7.5% acetic acid and 5% ethanol. The reagents for electrophoresis gel were provided by Reanal (Budapest, Hungary).

Globulin solubility in NaCl solutions at pH 4.8 was determined in the following way: A 1-mL portion of 1.5% globulin solution at pH 4.8 containing 1 M NaCl (in the case of 11S globulins) or 0.5 M NaCl (in the case of 7S globulins) was diluted with distilled water to different concentrations of NaCl. The mixtures were exposed to a given temperature (20 and 5 °C) for 2 h and then centrifugated at 1000*g* for 10 min (K-23, MLV, GDR). The supernatants were diluted 2-fold with 8 M urea solution and kept for 24 h. Protein concentration was determined by optical density at 278 nm on a Specord UV–vis spectrophotometer (Karl Zeiss, Jena, GDR). A 4 M urea solution was used as the control.

Extinction Coefficients. Initial protein solution at 1% concentration was prepared in 0.5 M NaCl solution at pH 4.8. Precise protein concentration was determined on the basis of nitrogen content in accord with Kjeldahl (N × 6.25) (Perrin, 1953). Initial protein solution was diluted with 0.5 M NaCl solution to different protein concentrations (in the range of 0.02–0.5%). Solutions obtained were diluted 2-fold with 8 M urea solution and kept for 24 h. Optical density of this solutions was determined at 278 nm on a Specord UV–vis spectrophotometer (Karl Zeiss, Jena, GDR). The extinction coefficients of proteins were calculated as a slope of the plot of the optical density versus protein concentration in accord with the least-squares fitting method. The extinction coefficients of 11S globulin from broad beans and peas at 278 nm in 4 M urea solution were 7.58 and 7.94, respectively, while the extinction coefficients of 7S globulins were 6.06 and 6.5.

RESULTS AND DISCUSSION

In order to establish the optimal conditions for separation of 7S and 11S globulins, the effects of ionic strength and the pH of precipitation of globulins from broad beans and peas on the yield and proportion of 7S and 11S components were studied. Protein yield was determined in accord with Kjeldahl, and fractional composition was estimated by sedimentation analysis. Globulin yield amounted to 7% of the initial broad beans meal at pH 4.8–5.0 and 0.3 M NaCl. The content of 11S globulin in the preparation amounted to 80% according to the data of sedimentation analysis. When the pH was reduced to 4.5 at the same NaCl concentration, globulin yield decreased to 2.5% while the content of 11S component decreased to 50%. With pH increasing up to 5.5, globulin yield was sharply reduced (<0.5% of meal content). Therefore, to obtain preparations enriched with 11S component, precipitation should be run at pH 4.8. Figure 1 presents sedimentograms of preparations, enriched with 11S globulins, that were thus obtained. Taking into account better solubility of 7S globulins in NaCl solution, reprecipitation of preparations enriched with 11S globulin from 0.6 M NaCl solution was carried out by diluting with distilled water up to 0.3 M NaCl concentration at pH 4.8, since diluting up to the concentration lower than 0.3 M NaCl did not result in complete removal of 7S globulins. Figure 2 presents sedimentograms of reprecipitated preparations of 11S globulins. One can see that reprecipitation results in complete removal of 7S globulins. The obtained preparations contained <5% of 15S globulin, which is the dimer of 11S globulin (Mori

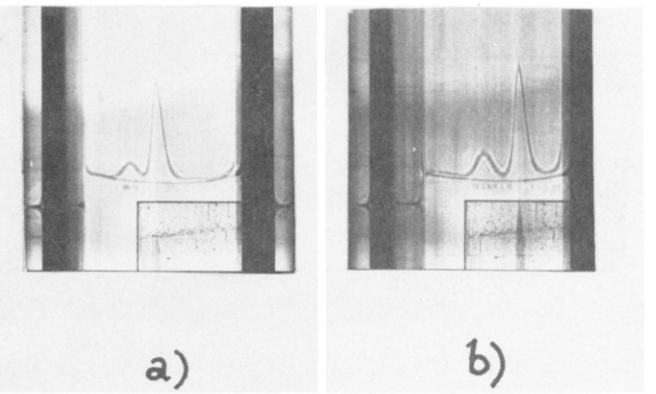

Figure 1. Sedimentograms of globulin solutions, obtained at pH 4.8, 0.3 M NaCl, and 20 °C from broad beans (a) and peas (b).

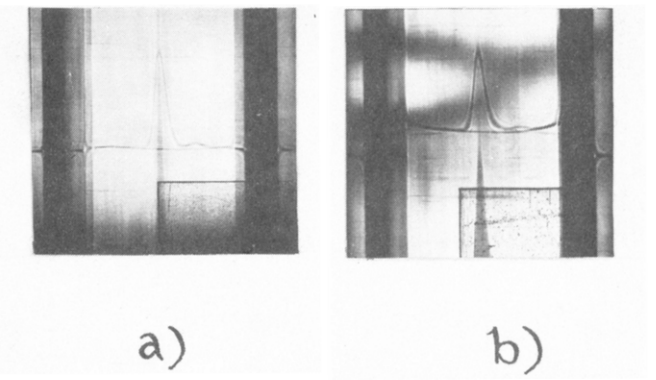

Figure 2. Sedimentograms of solutions containing 11S globulin preparations obtained after reprecipitation at pH 4.8, 0.3 M NaCl, and 20 °C.

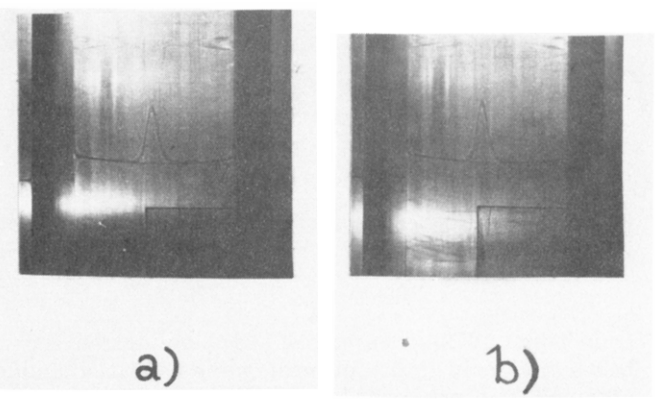

Figure 3. Sedimentograms of 11S globulin solution obtained from broad beans (a) and peas (b) after gel chromatography on Sepharose CL-6B.

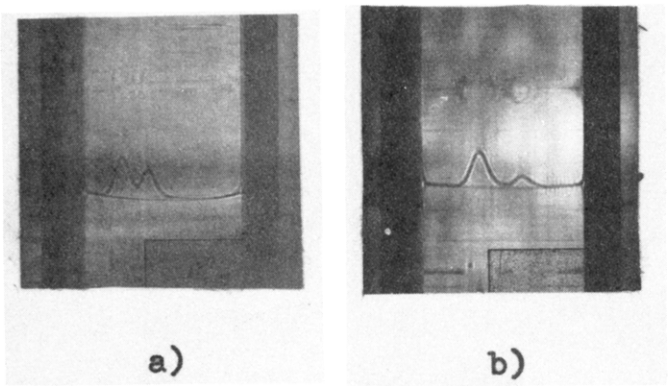

Figure 4. Sedimentograms of fraction solutions, enriched with 7S globulins, obtained at pH 4.8, 0.3 M NaCl, and 5 °C.

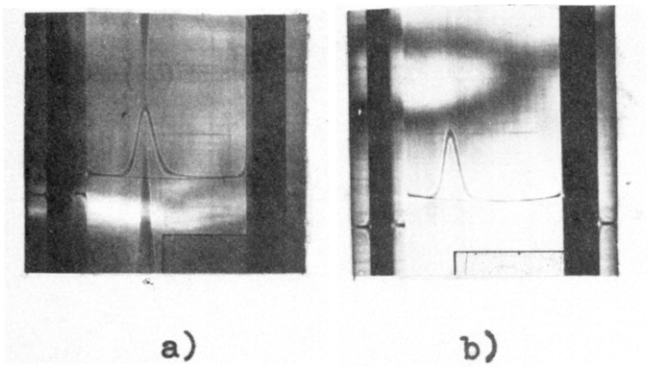

Figure 5. Sedimentograms of 7S globulin solutions before gel chromatography from broad beans (a) and peas (b).

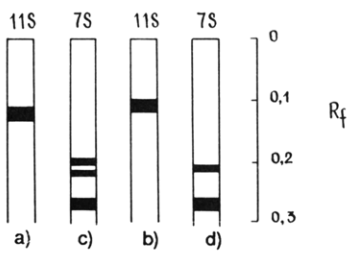

Figure 6. Electropherograms of 11S globulins obtained from broad beans (a) and peas (b) and 7S globulins obtained from broad beans (c) and peas (d).

and Utsumi, 1979). The yield of 11S globulins from broad beans amounted to 6% of meal, while for 11S globulin obtained from pea seeds it amounted to 1.5%.

To remove the admixture of 15S component, gel chromatography on Sepharose CL-6B was performed. Figure 3 presents sedimentograms of chromatographed preparations of 11S globulins from broad beans and pea seeds. It is shown that the preparations have only one component. The sedimentation constants of 11S globulins from broad beans and pea seeds in standard phosphate buffer were equal to 10.8 and 11.2 S, respectively.

The supernatant obtained after separating the fraction enriched with 11S globulin contained 7S and 11S globulins, albumins, and low molecular weight substances. To remove 11S globulin remaining in the solution, the supernatant with the 0.3 M NaCl was cooled to 5 °C. As a result, the system separated into two phases. The upper phase contained 7S globulins, albumins, and low molecular weight substances, while the lower phase contained 7S and 11S globulins (Figure 4). When the upper phase was diluted with distilled water to 0.15 M NaCl, 7S globulin precipitated. The yield of 7S globulin from broad beans was 1%, while that of pea seeds was 2% of the meal. To isolate 7S globulins from low molecular weight admixtures, one could run gel chromatography on Sephadex G-50. Figure 5 shows sedimentograms of 7S globulin preparations from broad bean and pea seeds in standard phosphate buffer. The sedimentation constants of 7S globulins from broad beans and pea seeds were equal to 6.05 and 6.2 S, respectively.

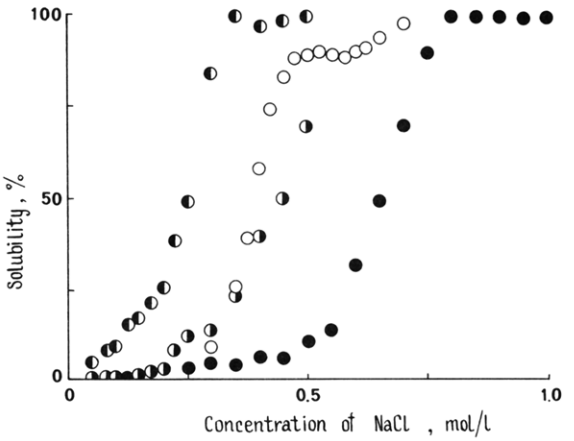

Figure 7. Solubility curves of 7S and 11S globulins from broad beans at pH 4.8: ○, 11S globulin, T = 20 °C; ●, 11S globulin, T = 5 °C; ◐, 7S globulin, T = 20 °C; ◑, 7S globulin, T = 5 °C.

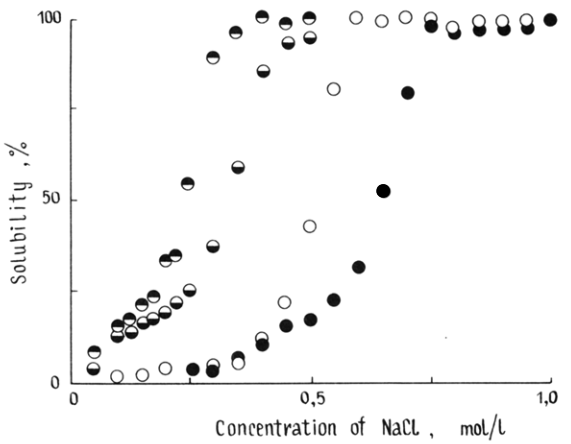

Figure 8. Solubility curves of 7S and 11S globulins from peas at pH 4.8: ○, 11S globulin, T = 20 °C; ●, 11S globulin, T = 5 °C; ◓, 7S globulin, T = 20 °C; ◒, 7S globulin, T = 5 °C.

Electrophoresis in polyacrylamide gel also showed homogeneity of chromatographed 11S globulin preparations (Figure 6). The 7S component is represented by a number of streaks caused by genetic variants of these proteins (Casey and Domoney, 1984; Schlesier, 1984).

Dependence of 7S and 11S globulin solubility on NaCl concentration was studied at pH 4.8 at 20 and 5 °C. The solubility curves have S-like shapes (Figures 7 and 8). For both broad beans and peas the solubility of 11S globulins is approximately 5–10% at 20 °C and 0.3 M NaCl, while the solubility of 7S globulins amounts to 90%. It is this difference in the solubility of 11S and 7S globulins under the given conditions that makes it possible to isolate 11S globulins from 7S globulin admixtures. The 11S globulins are practically insoluble at 5 °C and 0.3 M NaCl, which makes it possible to isolate 7S globulins from 11S globulin admixtures. The solubility of 7S globulins at 5 °C and 0.15 M NaCl is <5%, which ensures isolation of 7S globulins from albumins. These results confirm that the conditions chosen for the isolation of 7S and 11S globulins are correct.

The suggested method makes it possible to effectively isolate homogeneous preparations of 7S and 11S globulins from broad beans and pea seeds. It is characterized by high yield and makes these proteins available for extensive research on concentrated protein systems.

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Characterization of Pure Proanthocyanidins Isolated from the Hulls of Faba Beans

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Extraction of the hulls of faba beans with 70% aqueous acetone containing 2.8 mmol/L ascorbic acid proved to be a very efficient solvent system for the extraction of phenols. Treatment of the concentrated extract with ethyl acetate and liquefied phenol and batch adsorption on Sephadex LH-20 freed the isolated crude proanthocyanidins (PA) from small molecular weight UV-absorbing and protein contaminants. Gel filtration of the purified extract on Sephadex LH-20 yielded pure PA and showed homogeneous band during the elution at 275.2 nm (maximum). The overall yield was 12%; the preparation contained six flavan-3-ol units/molecules.

The hulls of faba beans are rich in proanthocyanidins (condensed tannins) (Martin-Tanguy et al., 1977; Cansfield et al., 1980; Griffiths and Moseley, 1980; Hewitt and Ford, 1982; Cabrera and Martin, 1986), yielding anthocyanidins after hydrolysis with strong mineral acid (Freudenberg and Weinges, 1958). These phenolic compounds, PA, were found to inhibit many enzymes in vitro, including digestible enzymes such as trypsin and α-amylase (Griffiths and Moseley, 1980).

In humans, the consumption of diets based on faba beans rich in PA was found to reduce the digestibility of protein (Hussein and Abbas, 1985a) and sulfur-containing amino acids (Hussein and Mottawei, 1985b).

Only few reports dealt, however, with the extraction, purification, isolation, and characterization of the PA.

The present work deals with the purification and characterization of the PA isolated from the hulls of faba beans.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were reagent grade. The origins to all the listed chemicals were Pharmacia (Uppsala, Sweden) and E. Merck (Darmstadt, West Germany).

The seeds Giza 3 were from the Department of Legumes of the Egyptian Ministry of Agriculture.

Extractions. The hulls (seed coats) were removed from the beans with a pair of scissors, ground in an electric mill, and extracted in one of the following solvents to which was added 2.8 mmol/L ascorbic acid as an antioxidant: (1) water; (2) 10% acetic acid; (3) 1% HCl in MeOH; (4) 10% tartaric acid in methanol; (5) 70% aqueous acetone, at a ratio of 1:20 (w/v). After filtration, the residue was reblended four more times with 150 mL of the same solvent system, and the combined filtrates were

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