Immunochemical Study of Changes in Reserve Proteins of Germinating Soybean Seeds

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Abstract. Changes in the reserve proteins of soybean seeds (Glycine max) were investigated by the techniques of disc electrophoresis and disc immunoelectrophoresis. Three different antisera were used in these studies, an anti-whole soybean extract serum 129, an anti-11S soybean protein monospecific serum 102, and an anti-7S soybean protein monospecific serum 132. At least 6 antigenically distinct components were found to be present in the proteins of the isolated soybean protein bodies. These components are metabolized at different rates during germination. The major soybean protein (11S component) is found to be present even after 16 days of germination, whereas the 7S component disappears after the ninth day. Histochemical observations of cotyledon sections during germination are also reported.

Experimental Procedures

Germination of Soybean Seeds. The soybean seeds (Glycine max) used in this investigation were Harosoy 63 variety grown in 1964 and stored at 25°. The seeds were soaked in distilled water for 4 hours, placed in a humid chamber on top of expanded vermiculite and covered with wet paper towels at the initial stages of germination. The towels were removed later to allow free development of the shoot. The germination was carried out in the dark at 25° for periods of 3, 6, 9, 13, and 16 days. The germinating seeds were sprayed daily with distilled water and abnormal seedlings were discarded. At the end of each germinating period, using mainly the length of shoot as a criterion, a sample of seedlings was collected and combined for analysis. After removal of the seed coat and seedling axis, the cotyledons were ground and dried at room temperature.

Protein Bodies. The protein bodies were isolated from the soybean meal by density gradient centrifugation at 24,000 × g as described by Tombs (16). Purity of the protein bodies was determined by optical microscopy. The isolated bodies were dialyzed against water to remove sucrose and precipitate the protein. The precipitated protein was removed by centrifugation, dissolved and dialyzed against pH 7.6 phosphate buffer made 0.4 M in NaCl, and 0.01 M in 2-mercaptoethanol, designated standard buffer (17). No protein was detected in the supernatant after precipitation of the globulins.

Protein Samples. The ground dry cotyledons were defatted with hexane in a Soxhlet apparatus for 4 to 5 hours. Residual solvent was removed by evaporation at room temperature. A crude
reserve protein preparation was obtained by extraction of the defatted cotyledons with 10% (w/v) sodium chloride (cotyledons: 10% sodium chloride ratio, 1:10) at room temperature for 2 hours. The extracts were squeezed through Miracloth (Calbiochem) and centrifuged at 10,000 × g for 30 minutes to clarify the supernatant liquor. The clear extracts were then dialyzed against water until chloride negative. The resulting precipitate was washed several times with water, dissolved and dialyzed with standard buffer.

The 11S component of soybean proteins was isolated from the cold insoluble fraction by ammonium sulfate fractionation and DEAE-Sephadex A-50 ionic strength gradient elution as described previously (5). A 7S component of soybean proteins was prepared as described by Koshiyama (8).

Preparation of Antisera. Young adult white rabbits were immunized by 3 intraperitoneal injections at 7 day intervals of 2% (w/v) antigen solution (whole soybean extract of ungerminated cotyledons or 11S soybean protein component) in standard buffer mixed and homogenized with an equal volume of Freund's complete adjuvant (Difco). The antigen dosage was 1 ml the first week, 2 ml the second week, and 3 ml the third week. After a rest period of 4 weeks, the animals were given a 5 ml booster injection of antigen by the same route and bled after 2 weeks. The sera were collected and stored at 4° after filter sterilization and addition of 1:10,000 merthiolate.

Disc Electrophoresis. Polycrylamide gel columns were prepared as described by Ornstein (11) and Davis (6). Electrophoresis using a 0.2 to 0.4 mg protein sample was usually carried out for 30 minutes in tris-glycine buffer (ionic strength 0.01; pH 8.3) with a current of 5 mA per gel column. Detection of the separated protein components was achieved by staining the columns for 1 hour with Amido-Schwartz dye followed by electrical destaining, as described by Davis (6). Densitometer tracings were performed with a Canalco Model F microdensitometer.

Disc Immunoelectrophoresis. The samples were first subjected to disc electrophoresis as described above. The unstained columns were then entirely embedded in a gel medium which consisted of 1% "Ionagar" No. 2 (Oxoid) in pH 8.8 tris-barbital - sodium barbital, 0.05 ionic strength, buffer (Gelman). After solidification of the agar, trenches were cut parallel to the line of columns and filled with the antiserum. The reactants were allowed to diffuse at room temperature, namely at 24° to 26°, for 3 to 5 days in a moist chamber. Precipitin lines appeared usually in 1 to 2 days. Results were recorded photographically.

Optical Microscopy. Defatted cotyledon samples were fixed in a mixture consisting of 1% chromic acid, 10% acetic acid, formaldehyde, and water (30:25:5:40 ratio). The samples were dehydrated with progressively increasing concentrations of ethyl and tertiary butyl alcohols, and imbedded in paraffin. After sectioning, the samples were immersed in ethyl alcohol and then, successively, in a series of aqueous ethyl alcohol solutions of increasing water content, and finally in water. The sections were then stained with Safranin O. The protein under these conditions is stained red. After this step, the sections were immersed in aqueous solutions of increasing concentrations of ethyl alcohol and stained with Fast green. Cellulosic material is stained blue-green.

Results and Discussion

Histological observations on cotyledonary sections in the quiescent and germinating states of the seed were performed by light microscopy. The cellular protein bodies in the quiescent state exhibit a dense granular appearance. On germination, the large granules disintegrate to smaller particles scattered within the cells. Disintegration of protein bodies occurred faster in the cotyledonary area.

![Fig. 1. Longitudinal section of soybean seed. A) cotyledonary area proximal to the adaxial surface of the cotyledon. B) cotyledonary area proximal to the abaxial surface of the cotyledon.](image-url)
proximal to the adaxial surface of the cotyledon (A, fig 1). A typical example was seen at the sixth day of germination when cotyledonary cells near the adaxial surface contained disintegrated protein bodies whereas cells distal to the adaxial surface (B, fig 1) were still filled with a large mass of protein bodies which appear to coalesce. Eventually, this mass also disintegrated to smaller particles. The disintegrated protein bodies in the cells proximal to the abaxial surface of the cotyledon appeared to line up at the perimeter of the cells and accumulate at the end of the cell facing the adaxial surface. These observations were consistent and reproducible in all experimental sections. Recently, Tombs (16) showed typical photomicrographs and electron micrographs of disintegrating soybean protein bodies. Since our results are very similar to Tombs we have avoided duplication of these pictures in the present report.

From the histochemical observations, it appears that the protein bodies of the soybean, as has been generally assumed, contain reserve protein which is metabolized during germination of the seed. Saio and Watanabe (14) attempted to fractionate the reserve protein of the protein bodies by Sephadex G-200 gel filtration and acrylamide gel electrophoresis. They found several components to be present which were not further characterized. However, they observed that the protein in the protein bodies became largely soluble by extraction with 10 % sodium chloride solution. This observation is in agreement with our results indicating that extraction of defatted cotyledons with 10 % sodium chloride solution produces a crude reserve protein fraction which contains all the protein components present in the protein bodies. Thus, changes in reserve protein during germination can be followed in whole cotyledon extracts.

Figure 2 shows a typical and reproducible pattern obtained by subjecting the proteins of the isolated protein bodies to disc electrophoresis on polyacrylamide gel and subsequent diffusion of the separated components against anti-whole soybean extract serum 129. The major stained band appears at an approximate relative mobility value (Rm) of 0.30. The scale of the relative mobility values has been arbitrarily assigned taking the separating line of the stacking and separating gels as the “0.00 point” and the position of the fastest moving band as “1.00”. Although it appears that this major stained band represents 1 component, immunodiffusion against antiserum 129 reveals 2 concentric precipitin arcs, c and e. Immunodiffusion of the electrophoresed proteins of the protein bodies against the monospecific anti-11S soybean protein serum 102 (3,4) and anti-7S soybean protein serum 132 (unpublished results) showed that precipitin arc e represents the 11S component of soybean proteins, and arc d the 7S component. In the absence of more specific nomenclature, the 7S component described in this report is the one isolated by the method of Koshiyama (8). This distinction is made since the presence of other 7S components in soybean proteins is possible (7,10). These results illustrate that overlapping of 2 of the major soybean protein components occurs in 7 % polyacrylamide gels and that superior resolution is obtained by the technique of disc immunoelectrophoresis. Preliminary results have shown that the 11S component is the major protein of the protein bodies and that the 7S component is present in smaller amounts.

Other protein components present in the protein bodies and separated by disc electrophoresis were
arbitrarily designated a, b, c, f, and g. Components a and b present at the anodic end of the column are antigenic and produce 2 corresponding immunoprecipitin bands, a and b. Component b is found in larger amounts than component a and forms an intense precipitin band. Because of its lower concentration, component a produces a weak precipitin arc. Component c exhibits higher electrophoretic mobility than the 11S and 7S components (band d, e) and forms an immunoprecipitin line c, the center of which has higher mobility in comparison to the centers of the arcs, d and e. Component g represents a polymer of the 11S component. This component appears when the purified 11S soybean protein is electrophoresed without mercaptoethanol in the gel column (5). Component g also gives a precipitin band with anti-11S soybean protein serum 102 which is an extension of the line formed by the 11S component ("reaction of identity") and indicates immunochemically identical protein molecules. This line on prolonged diffusion is split in 2 (fig 4B) which may be an indication of 2 different polymeric forms. Component f near the cathodic end of the column is also antigenic and produces the corresponding immunoprecipitin arc f. In conclusion, the proteins present in the protein bodies consist of at least 6 immunochemically different components 2 of which, the 11S and 7S components, have been isolated and found to be homogeneous by several criteria (3, 4, 5, 8). N-Terminal amino acid analysis of the 11S component (5) indicated that the protein contains 12 polypeptide chains, 8 of which end in glycine, 2 in phenylalanine, and 2 in either leucine or isoleucine. Koshiyama (9) found that the N-terminal residues of the 7S component, in moles per 180,000 molecular weight, were as follows; aspartic acid, 1; alanine, 1; glycine, 1; valine, 1; serine, 2; tyrosine, 1; glutamic acid, 1; and leucine (iso-leucine), 1. The difference in N-terminal amino acid residues of the 2 proteins rules out the possibility of these being 2 polymeric forms of the same protein. Thus, Tombs (16) believe that the different bands obtained by polyacrylamide gel electrophoresis of the protein bodies represent the monomer, dimer and polymers of the same protein "glycinin" does not appear to be valid.

The disc electrophoresis pattern of the crude reserve protein preparation (see Methods) of whole cotyledons is shown in figure 3. This fraction exhibits an essentially identical pattern to that obtained from the protein bodies. The disc immunoelectrophoresis pattern of the crude reserve protein fraction (fig 4), developed with anti-whole soybean extract serum 129, anti-11S soybean protein serum 102, and anti-7S soybean protein serum 132, is also similar to that obtained with the protein bodies. Only component a appears to be at a higher concentration level in the crude reserve protein fraction than in the protein bodies (fig 3). Disc electrophoresis patterns of the isolated 11S and 7S components is also shown in figure 3. It
was noted that the 7S component exhibits a little higher electrophoretic mobility in the isolated form than in mixture with the other soybean proteins. Probably during the isolation procedure, substances bound to the protein are removed and thus its mobility is altered. However, the molecule maintains its antigenicity against both the anti-whole soybean extract serum 129 and the monospecific anti-7S soybean protein serum 132.

Changes in reserve proteins during germination were investigated by the disc electrophoresis and disc immunoelectrophoresis techniques. Figure 5 shows electrophoretic patterns, and figure 6 microdensitometer tracings of the stained bands at 0, 3, 6, 9, 13, and 16-day germination periods. General observations from these patterns indicate the following changes: formation of subunits or fragments with Rm values between 0.5 and 1.0 starting at the 6-day germination period and progressively increasing in intensity as the germination proceeds; formation of aggregation products near the cathodic end of the column reaching a maximum at the 13-day period; and decrease in the intensity, accompanied by higher electrophoretic mobility, of the main bands (Rm 0.3-0.5) in relation to the bands at the anodic end of the gels.

The formation of fast moving components (Rm 0.5-1.0) and aggregation products (probably subunits aggregated at random) was previously demonstrated when the 11S component was dissociated by 6 M guanidine hydrochloride containing 0.2 M mercaptoethanol (5). Dissociation of soybean proteins into subunits under various experimental conditions has also been shown by starch gel electrophoresis (13, 15). It is, therefore, possible that dissociation of the reserve proteins into subunits precedes final metabolic utilization of these complex molecules by the germinating seed. Random attack of enzymes on the intact reserve proteins can produce a multiplicity of fragments some of which may retain their antigenic sites at some unfragmented part of the molecule. Such fragments should then produce multiple immunoprecipitin bands as visualized by immunoelectrophoresis. Such precipitin bands were not found.

**Fig. 6.** Microdensitometer tracings of disc electrophoresis bands obtained from crude reserve protein preparations at 0-day (A), 3-day (B), 6-day (C), 9-day (D), 13-day (E), and 16-day (F) germination periods.

**Fig. 7.** Disc immunoelectrophoresis patterns, developed with anti-whole soybean extract serum 129, of crude reserve protein preparations at 0-day (A), 3-day (B), 6-day (C), 9-day (D), 13-day (E), and 16-day (F) germination periods. Arrows indicating immunoprecipitin bands do not point at any specific position in the bands.
Although, some observations as reported here appear to be in agreement with the proposed metabolic mechanism of dissociation into subunits, this work does not present conclusive evidence. Further investigations will be necessary to clarify the mechanism by which these proteins are utilized by the germinating seed.

Ordinary disc electrophoresis provides certain information about changes in protein components during germination but identification of individual protein components is very uncertain. However, disc immunoelectrophoresis provides more meaningful information on the fate of these components. Figure 7 and 8 show results obtained at different stages of germination using anti-whole soybean extract serum 129, anti-7S soybean protein serum 132, and anti-11S soybean protein serum 102. The 11S component (e) acquires higher electrophoretic mobility during the first 3 days of germination. After this period, its mobility is relatively unchanged, and it is found to be present even up to 16 days of germination, although the intensity of the immunoprecipitin band is decreased after the ninth day. The 7S component (d) exhibits increasing electrophoretic mobility up to the ninth day of germination when it suddenly is reduced to a very small amount (note 13-day and 16-day germination periods). Component f disappears after the ninth day. Component c shows little change in electrophoretic mobility. The intensity of its precipitin band is slowly decreased but is present during the entire 16-day germination period. Components a and b do not appear to change much. A new antigenic component, h, appears at the sixth day of germination with an approximate Rm value of 0.75. This component probably represents an antigenic subunit which does not react with anti-11S or anti-7S sera.

It has been shown that 4 of the protein components (c, d, e, and f) present in the reserve protein of the soybean seed are actively metabolized during the first 16 days of germination. Two of the components (a and b) are either metabolized at a later period or have a biochemical role other than that of the other components. The increased mobility acquired by the 11S and 7S components during germination can be explained by deamidation of the asparagine and glutamine residues of these proteins producing an increase in negative charges by exposure of carboxyl groups. Panalaks et al. (12) reported that ammonia does not accumulate during germination of etiolated soybean seedlings. However, the released ammonia could be transferred by some metabolic pathway to free asparagine in the cotyledons which in turn is transported to the plant axis.

It would be interesting to follow the fate of the individual components by producing antisera to the subunits. This approach may provide additional information about the mechanism of degradation of the reserve protein of seeds. This preliminary investigation shows that immunochemical methods can be effectively utilized in such studies.

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Literature Cited