Immunochemical Study of Soybean Proteins

N. Catsimpoolas and E. W. Meyer

Soybean protein fractions were analyzed by double gel diffusion and disc. immunoelectrophoresis with antiwhole soybean extract and anti-11S soybean protein antisera. At least five immunologically distinct components were identified in the whole soybean extract, cold insoluble fraction, and cold soluble fraction. Three precipitin bands were found in the isoelectrically precipitated globulins, two immunochemically distinct bands in a crude preparation of the 11S component, and one in the purified 11S protein fraction. Each of these fractions formed only one band when diffused against the anti-11S soybean protein specific antiserum. This band was identical to the band formed by the purified 11S component. The immunochemical method offers an additional physicochemical criterion for establishing homogeneity of isolated soybean proteins, and for the detection of specific homogeneous components. The isolated 11S component is the only immunochemically homogeneous protein described in the present study.

The immunochemical specificity of proteins, which is based on the steric configuration of certain antigenic groups in each molecule, has been utilized as an analytical tool to define protein components in a mixture and to prove their homogeneity. A recent example of the usefulness of such immunochemical studies as applied to the characterization of plant proteins was given by Grabar *et al.* (1965). In the present report immunodiffusion studies were used to define soybean protein fractions.

The antigenic properties of the soybean were first reported by Duke (1934). Ratner *et al.* (1955) concluded that the soybean was a weak antigen and that sensitization was enhanced by utilizing soybean extracts incorporated in Freund's adjuvant. Commercial soybean extracts were also studied by Crawford *et al.* (1965) using rabbit antisera in double gel diffusion and immunoelectrophoresis. In addition, the antigenic properties of soybean flour have been utilized in several serological methods for its detection in food products (Ferguson *et al.*, 1942; Glynn, 1939; Hale, 1945; Munsey, 1947).

The main objective of the reported investigations was to examine the antigenicity of soybeans by using whole, unfractionated soybean extracts which are commercially available. However, no attempt has been made to investigate the immunochemical properties of defined soybean protein fractions and especially of the soybean globulins which are used in their isolated form for commercial food purposes. Such a study could be useful in determining homogeneity of isolated soybean proteins, possible structural alterations during fractionation, their fate during germination of seeds, and their identification in food products.

EXPERIMENTAL

Protein Fractions. The soybeans used in this investigation were Harosoy 63 variety grown in 1964 and stored at 25° C. The seeds were cracked, dehulled, and flaked. The soybean flakes were defatted with pentane (b.p. 36° C.) in a Soxhlet apparatus for 4 to 5 hours. The extracted flakes were desolventized at room temperature.

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Whole soybean extract (WSE) was obtained by extraction of the defatted flakes with water (flake–water ratio, 1:10) at 25° C. for 1 hour and centrifugation at 10,000 r.c.f. for 30 minutes to clarify the supernatant liquor. For fractionation of the globulins by cooling (Wolf and Briggs, 1959), the defatted flakes were extracted with water (flake–water ratio, 1:5) at 25° C. for 1 hour, and the extract was clarified by centrifugation at 10,000 r.c.f. for 30 minutes. Subsequently, the protein extract was cooled overnight at 4° C. and centrifuged at 10,000 r.c.f. for 15 minutes at 4° C. The cold insoluble fraction (CIF) was obtained as a precipitate. The supernatant was designated cold soluble fraction (CSF). The cold insoluble fraction was then subjected to ammonium sulfate fractionation to obtain a crude preparation of the 11S component, Fraction X (X), as described by Wolf *et al.* (1962). The purified 11S component (11S) was prepared from Fraction X by ionic strength gradient elution on DEAE-Sephadex A-50 as reported by Catsimpoolas *et al.* (in press).

The isoelectric soybean globulins (ISG) were prepared from the whole soybean extract (WSE) by isoelectric precipitation at pH 4.5 with 5% HCl, washed free of whey constituents with water, and adjusted to pH 7.6 with 1*N* NaOH. The isoelectric globulins were then dialyzed against phosphate buffer, pH 7.6 made 0.4*M* in sodium chloride and 0.01*M* in mercaptoethanol, designated standard buffer (Wolf and Briggs, 1959). All the protein fractions described above were also dialyzed against the standard buffer to ensure identical conditions for gel diffusion.

Preparation of Antisera. Two different protein samples were used in the immunization procedure (described below): whole soybean extract and purified 11S component. The protein concentration of both solutions in standard buffer was 2%. Approximately 200 mg. of protein was required for immunization of one rabbit.

New Zealand white rabbits weighing 4 to 6 pounds were obtained from a commercial animal breeder and maintained on a typical medicated rabbit pellet diet. The rabbits were immunized by three intraperitoneal injections at 7-day intervals. Each injection contained 2% antigen solution in standard buffer mixed and homogenized with an equal volume of Freund's complete adjuvant (Difco). The immunizing dosage was 1 ml. the first week, 2 ml. the second week, and 5 ml. the third week. After a 30-day rest period, the animals were given a 5-ml. booster injection

of the antigen by the same route. The animals were then subjected to test bleeding from the marginal ear vein. If the concentration of precipitating antibody was adequate, the animals were exsanguinated and the sera from the separate bleedings were pooled and stored at 4° C. after filtration sterilization and addition of 1:10,000 merthiolate.

Double Gel Immunodiffusion. Double gel diffusion in agar was carried out in plates according to the method of Ouchterlony (1948). The gel medium consisted of a 0.85% Ionagar No. 2 (Oxoid) solution in pH 6.8 phosphate buffer prepared as follows: 0.77 gram of Na_2HPO_4, 0.38 gram of $KHPO_4$, and 10.02 grams of NaCl were dissolved in H_2O and made up to 1-liter volume after the addition of 1 ml. of 1% merthiolate. The reactants were allowed to diffuse at room temperature—namely, at 24° to 26° C.—for 2 to 3 days in a moist chamber. Results were recorded photographically.

Discontinued Immunoelectrophoresis. Polyacrylamide gel columns (7%) were prepared as by Ornstein (1964) and Davis (1964). Electrophoresis using a 0.2 to 0.4 mg. of 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. Preliminary 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 (1964). For immunodiffusion, the unstained columns were entirely embedded in the agar medium described above. After solidification of the agar, trenches were cut parallel to the line of the columns and filled with the antiserum. The reactants were allowed to diffuse at room temperature—namely, at 24° to 26° C.—for 3 to 5 days in a moist chamber. Precipitin lines appeared usually in 1 to 2 days, but in general, became more intense on longer standing. Also, certain bands became apparent only after standing for 3 to 5 days. Results were recorded photographically.

RESULTS AND DISCUSSION

Figure 1*A* shows representative and reproducible results obtained by double gel immunodiffusion of several soybean protein fractions against antiwhole soybean extract antiserum. The rabbits were immunized with the whole soybean extract in order to obtain a maximum number of antibodies. Since the response of the animals to immunization is not constant, several immune sera have to be tried and the number of antigenic components detected is to be considered as minimum (Grabar *et al.*, 1965).

Antigenic components present in different fractions (Figure 1*A*) and arbitrarily designated A, B, C, D, and E were traced by a combination of different double gel diffusion patterns and the results are shown in Table I. The numbers in Table I indicate band position within a fraction starting with 1 for the band closest to the antiserum well. For example, antigenic component A was found to be present in the whole soybean extract, cold insoluble fraction, and cold soluble fraction as the fastest moving band. Immunochemical identity of two antigens is indicated by the coalescence (looping, fusion) of the proximal band tips (Crowle, 1961).

The data in Table I show that the whole soybean extract, cold insoluble fraction, and cold soluble fraction exhibited at least five distinct antigenic components. Three precipitin bands were found in the isoelectrically precipitated soybean globulin fraction. The purified 11S protein shows only one precipitin line. The utilization of the immunochemical method in establishing

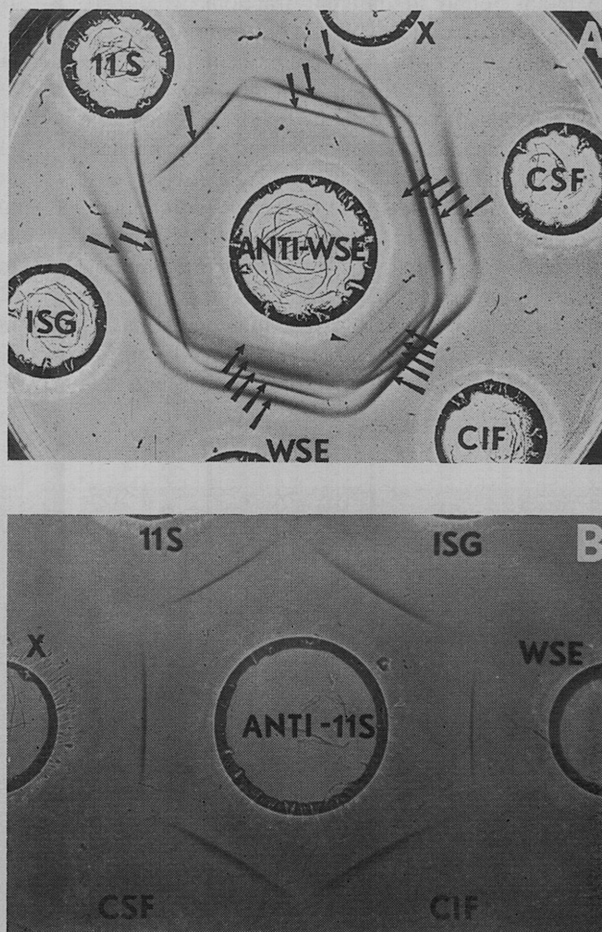

Figure 1. Double gel immunodiffusion of soybean proteins. *A.* Whole soybean extract (WSE), cold insoluble fraction (CIF) cold soluble fraction (CSF), 11S component purified by ammonium sulfate precipitation (X), 11S component purified by DEAE Sephadex chromatography (11S), and isoelectric soybean globulins (ISG) diffused against antiwhole soybean extract antiserum (Anti-WSE). *B.* Same fractions as above diffused against anti-11S soybean protein antiserum (Anti-11S)

Table I. Double Gel Immunodiffusion of Soybean Proteins

	Antigenic Components				
Fraction	A	B	C	D[a]	E
Whole soybean extract	1[b]	2	3	4	5
Cold insoluble fraction	1	4	3	2	5
Cold soluble fraction	1	2	3	4	5
Fraction X	...	...	1	2, 3[c]	...
Isoelectrically precipitated globulins	...	...	2	1	3
11S component	...	...	...	1	...

[a] Identical to the 11S component.
[b] Numbers indicate band position within a fraction starting with 1 for the band closest to the antiserum well.
[c] Higher polymer of the 11S component (see text).

homogeneity of isolated soybean proteins is thus immediately realized in the case of the purified 11S soybean protein. This protein was found to be homogeneous by disc. electrophoresis and DEAE-Sephadex chromatography (Catsimpoolas *et al.*, in press). The present immunodiffusion method adds an additional physicochemical criterion of purity.

All the bands found in different fractions which are listed as antigenic component D in Table I were immunochemically identical to the 11S component. The 11S protein purified by ammonium sulfate fractionation (Fraction X) forms three precipitin bands of which the main band (No. 2) is identical with the band of the purified 11S component. The very slow moving and slow appearing band (No. 3) may be identical to the 11S component, or could represent a higher polymer of the 11S protein. As the purified 11S protein was prepared from Fraction X by DEAE-Sephadex chromatography, it appears from the immunodiffusion examination that the contaminating components were effectively removed. Wolf *et al.* (1962) reported that the contaminating components exhibit a 15S peak by ultracentrifugation.

When the purified 11S component was injected into rabbits a specific antibody for the 11S component was produced (Figure 1*B*). The anti-11S soybean protein antiserum (Anti-11S) produced only one band when diffused against the whole soybean extract, cold insoluble fraction, cold soluble fraction, isoelectric globulins, and purified 11S component. The band formed with each fraction was identical to that of the pure 11S component.

Disc. electrophoresis in polyacrylamide gel has been successfully used for characterization of soybean protein components (Catsimpoolas *et al.*, in press). Figure 2*A* shows representative results obtained by disc. immunoelectrophoresis of whole soybean extract (WSE) against antiwhole soybean extract antiserum (A-WSE). In this method, the electrophoresced, but unfixed, gel column is immersed in agar, so that the specific antibodies can migrate toward protein bands which have already been separated. The lower part of the figure shows the relative electrophoretic mobilities of the stained protein components. The origin of the separating gel was designated "O" and the position of the fastest moving band of the whole soybean extract was designated "1." All other fractions were

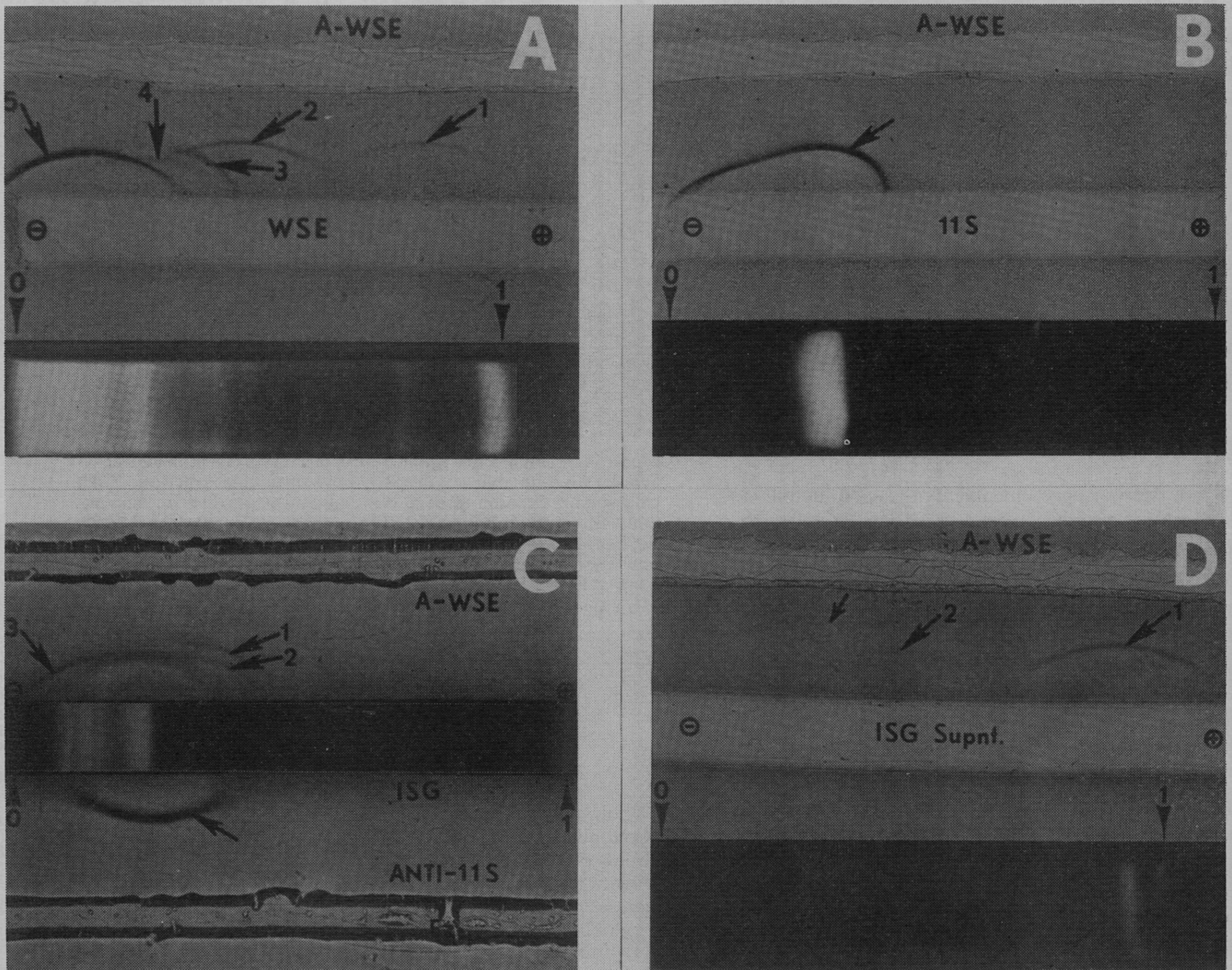

Figure 2. Disc. immunoelectrophoresis of soybean proteins. *A.* **Whole soybean extract (WSE) against antiwhole soybean extract antiserum (A-WSE).** *B.* **11S component purified by DEAE-Sephadex chromatography (11S) against antiwhole soybean extract antiserum (A-WSE).** *C.* **Isoelectric soybean globulins (ISG) against A-WSE and anti-11S soybean protein (Anti-11S) antisera.** *D.* **Supernatant fraction remaining after isoelectric precipitation of soybean globulins (ISG Supnt.) against A-WSE**

then compared with the immunoelectrophoresis pattern of WSE to establish relative mobilities and designation of bands. The upper part indicates the precipitin arcs formed by diffusion of the separated proteins against the antiserum trench. A minimum of five precipitin lines were visualized. The numbers indicate arc position within a fraction starting with 1 for the arc of the protein component with the highest electrophoretic mobility. The precipitin arcs exhibit various intensities and diffusion coefficients. The number of arcs obtained by disc. immunoelectrophoresis is the same as that indicated by double gel immunodiffusion. However, correlation of bands by these two techniques cannot be established without isolation of the antigenic components. It was observed that a larger number of stained protein bands is present in the disc. electrophoresis column as compared with the diffusion arcs against the antiserum. A possible explanation for this phenomenon is that, although certain protein components may exhibit different mobilities, and, thus, are separated by simple disc. electrophoresis, these components may have identical immunochemical specificity because of considerable similarities in structure. Thus, two or more separated bands spaced closely together because of a slight difference in electrophoretic mobility, will diffuse out of the column and behave immunochemically as a single component against a common specific antibody. Another possibility is that some of the stained bands represent protein subunits which are nonantigenic and, thus, fail to form a precipitin arc. Functionally, the results obtained by disc. immunoelectrophoresis can be more meaningful because they indicate proteins with structural differences. However, one cannot overlook the possibility that a given antiserum may not contain antibodies to all antigens in a complex mixture. Consequently, the observed number of arcs should be regarded as minimum.

The cold insoluble fraction (CIF) and cold soluble fraction (CSF) also exhibited five antigenically distinct components. The relative intensity of the arcs in these two fractions appears to be considerably different in comparison to those of the whole soybean extract fraction. In general, the relative electrophoretic mobility of the precipitin arcs in all three fractions (WSE, CIF, and CSF) remained about the same. The crude preparation of the 11S protein (X) exhibited two broad precipitin bands which appear to fuse at the ends, indicating two immunochemically similar components with different electrophoretic mobilities. The 11S component purified by DEAE-Sephadex (Figure 2*B*) exhibited only one precipitin arc when diffused against anti-whole soybean extract antiserum. This indicates that the 11S component is electrophoretically and immunochemically homogeneous.

Three distinct precipitin bands were found when the electrophoresced isoelectric soybean globulins (ISG) were diffused against the antiwhole soybean extract antiserum (A-WSE), Figure 2*C*. However, only one band was found when diffusion proceeded against the specific anti-11S soybean protein (Anti-11S) antiserum (Figure 2*C*). These results indicate that the isoelectrically precipitated soybean globulins consist of three immunochemically distinct components, one of which is identical to the 11S component isolated from the cold insoluble fraction.

The soybean proteins remaining in the supernatant (ISG Supnt.) after isoelectric precipitation of the globulins, exhibit mainly two precipitin arcs (Figure 2*D*) which are not present in the isoelectric globulin fraction. However, these two bands together with the three bands present in the isoelectric globulin fraction add up to a pattern similar to that obtained with the whole soybean extract.

The advantage of disc. immunoelectrophoresis over double gel immunodiffusion lies in the minimal probability that two arcs will coincide unless two substances have the same electrophoretic mobility and that their rates of diffusion as well as the antigen-antibody ratios are present such that precipitation occurs at the same line. However, the simultaneous fulfillment of all these conditions rarely occurs (Grabar and Burtin, 1964). The application of disc. immunoelectrophoresis in the general characterization of soybean proteins can be a valuable analytical tool in establishing homogeneity of isolated soybean protein fractions. The use of monospecific antisera would greatly facilitate the identification of each soybean protein component. The availability of the monospecific anti-11S soybean protein antiserum makes possible the identification of the 11S component in microgram quantities. Such monospecific antisera are under preparation in our laboratory by making use of the individual variations in antibody response of experimental animals and by techniques based on the absorption of polyvalent immune sera.

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LITERATURE CITED

Catsimpoolas, N., Rogers, D. A., Circle, S. J., Meyer, E. W., *Cereal Chem.*, in press.
Crawford, L. V., Roane, J., Triplett, F., Hannissian, A. S., *Ann. Allergy* **23**, 303 (1965).
Crowle, A. J., "Immunodiffusion," pp. 69–75, Academic Press, New York, 1961.
Davis, B. J., *Ann. N. Y. Acad. Sci.* **121**, 404 (1964).
Duke, W. W., *J. Allergy* **5**, 300 (1934).
Ferguson, C. S., Raciot, P. A., Rane, L., *J. Assoc. Offic. Agr. Chemists* **25**, 533 (1942).
Glynn, J. H., *Science* **89**, 444 (1939).
Grabar, P., Burtin, P., "Immuno-electrophoretic Analysis," pp. 17–18, Elsevier, New York, 1964.
Grabar, P., Escribano, M. J., Benhamou, N., Daussant, J., J. Agr. Food Chem. **13**, 392 (1965).
Hale, M. W., *Food Res.* **10**, 60 (1945).
Munsey, V. E., *J. Assoc. Offic. Agr. Chemists* **30**, 187 (1947).
Ornstein, L., *Ann. N. Y. Acad. Sci.* **121**, 321 (1964).
Ouchterlony, Ö., *Acta Path. Microbiol. Scand.* **25**, 185 (1948).
Ratner, B., Untracht, S., Crawford, L. V., Malone, H. J., Retsina, M., *Am. J. Diseases Children* **89**, 187 (1955).
Wolf, W. J., Babcock, G. E., Smith, A. K., *Arch. Biochem. Biophys* **99**, 265 (1962).
Wolf, W. J., Briggs, D. R., *Arch. Biochem. Biophys.* **85**, 186 (1959).

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