Separation of Soybean Whey Proteins by Isoelectric Focusing

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ABSTRACT

Soybean whey proteins were separated by isoelectric focusing in the region between pH 3 and pH 10, and by high-resolution electrofocusing in the range of pH 3 to pH 6, and pH 5 to pH 8. The separated proteins exhibited a spectrum of isoelectric points (pI) ranging from pH 3.38 to pH 10. The separated fractions were analyzed by disc electrophoresis in 11% polyacrylamide gels. The protein components from the different fractions migrated in the alkaline gel (stacks at pH 8.9, runs at pH 9.5) in a stepwise fashion starting from the anodic end of the gel column and progressively approaching the cathode as the isoelectric point increases. Proteins which exhibited isoelectric points higher than 6.80 did not migrate in the alkaline gel. However, these components were resolved by electrophoresis in acidic gels (stacks at pH 5.0, runs at pH 4.3). Carrier ampholytes (used to form the pH gradient) could not be removed from a number of fractions by extensive dialysis against water. The presence of ampholytes was demonstrated by disc electrophoresis, since these are stained by amido schwartz dye. Several of the fractions were isolated in better than 95% purity in one-step electrofocusing. Preliminary results show that multiple trypsin inhibitors and hemagglutinins are present in the soybean, in agreement with previous reports. The various components exhibiting trypsin inhibitory activity can be separated more effectively by electrofocusing in the region of pH 3 to pH 6, and components exhibiting hemagglutinating activity, in the region of pH 5 and pH 8. Since the latter components are very antigenic, this property could be utilized for their identification.

The soybean whey protein fraction (Wh) consists of water-soluble proteins remaining in the supernatant liquor after isoelectric precipitation (at pH 4.5) of the soybean globulins from a water extract of defatted soybean flakes, and subsequent removal of phytate salts and water-dialyzable compounds. This fraction contains proteins of biological interest such as trypsin inhibitors (1,2,3,4,5,6,7), hemagglutinins (2,8,9,10), and numerous enzymes. Although the function of the soybean globulins as "reserve proteins" has been examined (11,12), nothing is known about the role of trypsin inhibitors and hemagglutinins in the metabolic processes of the germinating soybean seed. Apparently these proteins are not toxic to the seed. It is also of interest to investigate the possibility of inhibitory effects of the various trypsin inhibitors on the proteolytic enzymes found in the soybean. The existence of soybean protease inhibitors has been suggested by the work of Weil et al. (13). These inhibitors may play a regulatory role in the digestion of the "reserve proteins" during germination. The study of the interrelationships of proteases and inhibitors in plants and animals will be greatly facilitated by the purification of the components involved. Fractionation of the soybean whey proteins and means of identification, in addition to the use of biological methods based on animal factors, may be helpful in establishing a point of reference in subsequent purification and metabolic studies.

Eldridge et al. (14) have shown that the ultracentrifugal pattern of soybean whey proteins exhibits only two peaks having sedimentation coefficients of 1.9 and 6.1 S. When the soybean whey proteins were analyzed by moving-boundary (Tris buffer, pH 8) and polyacrylamide gel (8M urea, pH 9.2) electrophoresis a minimum of 8 peaks and 24 bands were found, respectively. DEAE-Cellulose chromatography, applied initially to the fractionation of soybean whey proteins by Rackis et al. (2), has been the method most commonly used for the purification of

whey protein components. By this method, the whey solution was fractionated into 13 electrophoretically distinct components having $s_{20,w}$ values ranging from 0.99 to 6.14 S. In this report, separation of soybean whey protein by isoelectric focusing in stable pH gradients (15-16) is described. The separated fractions, which showed a spectrum of isoelectric points (pI) between pH 3 and pH 10, were then analyzed by disc electrophoresis (17,18) on 11% polyacrylamide gels in both basic and acidic buffers. By the combination of isoelectric focusing and disc electrophoresis, a great number of acidic and basic protein components were separated, indicating a much greater degree of heterogeneity than has been demonstrated by previous methods.

MATERIALS AND METHODS

Materials

Soybean whey proteins were prepared from defatted flakes of Harosoy 63 variety soybeans as described by Rackis et al. (2). Carrier ampholytes were obtained from LKB Instruments, Inc., Rockville, Maryland. All the other chemicals were of reagent grade.

Isoelectric Focusing

The principle and applications of this method have been described (15,16,19). The carrier ampholytes were selected to give pH gradients between pH 3 and pH 10, pH 3 and pH 6, and pH 5 and pH 8. An LKB 8102 electrofocusing column of 440-ml. capacity (LKB Instruments, Inc.) was used for these experiments. Stabilization against convection was achieved by using a density gradient prepared stepwise from one dense and one less dense solution (16). Preparation of the solutions and of the density gradient was performed as described in the preliminary instruction sheet and its addendum supplied by LKB Instruments. The procedure is identical to that described in detail by Vesterberg and Svensson (16) with the exception that the volumes of the solutions have been modified to fit the 440-ml. column. The anode solution was placed at the bottom of the column and the cathode solution at the top. The sample was prepared by dissolving 250 mg. of the lyophilized soybean whey protein in 13.8 ml. of the less dense solution and centrifuging at 10,000 X g for 15 min. to remove insoluble material. The protein sample then replaced the less dense solution in the preparation of the fractions Nos.23, 24,and 25 (between 189- and 216-ml. volume) of the density gradient.

After focusing for 48 hr. with a final potential of 500 v. (at 10°), the contents of the column were drained slowly through the bottom tubing into a fraction collector. Fractions (4.5 ml.) were collected and the absorbance of each fraction at 280 mμ was determined using a 1-cm. cell, with a Beckman DU spectrophotometer equipped with thermospacers and photomultiplier attachment. The pH of each fraction was also measured at 25°C. with a Beckman Expandomatic pH meter equipped with a Beckman Expandomatic range selector.

Selected fractions were pooled and dialyzed against several changes of water at 5°C. for 5 days to remove sucrose and ampholytes. The dialyzed material was then lyophilized to provide samples for disc electrophoresis and other analyses.

Disc Electrophoresis

These analyses were performed essentially as described by Davis (17) using equipment manufactured by Canal Industrial Corporation. The gels were 11% in

respect to acrylamide and were polymerized with riboflavin and light instead of ammonium persulfate catalyst (18). Two types of gels were used, one alkaline (stacks at pH 8.9, runs at pH 9.5) and one acid (stacks at pH 5.0, runs at pH 4.3). The gels were prepared according to formulation described in technical literature distributed by Canal Industrial Corporation (Chemical Formulation for Disc Electrophoresis, April, 1965).

Immunochemical Methods

Anti-soybean protein sera were prepared as described previously (18,20,21). Immunoelectrophoresis in agar gel was carried out by the general procedure described by Grabar and Williams (22) as modified by Scheidegger (23). The gel medium, buffers, and electrophoretic conditions as applied to immunoelectrophoresis of soybean proteins have been described previously (24).

Other Methods

Proteolytic activity was measured under optimum conditions, as described by Weil et al. (13), using casein as substrate and pH 5.5 buffer at 50°C. for 2.5 hr. However, the incubation mixture was modified to contain: 1 ml. of 1% casein solution, 1 ml. pH 5.5 citric acid-sodium citrate buffer (0.1M) and 0.2 ml. of aqueous protein solution (approximately 0.1 mg. protein) from each fraction isolated by electrofocusing. After incubation, 3 ml. of a 5% trichloroacetic acid solution was added to each tube. The solutions were mixed thoroughly and allowed to stand for a minimum of 30 min. at room temperature. After centrifugation at 10,000 X g for 30 min., the absorbance of the clear supernatant at 280 mμ was determined using 1-cm. cells in the Beckman DU spectrophotometer. The increase in absorbance of the trichloroacetic acid-soluble extract (over that of the casein control) served as a measure of proteolysis.

Trypsin-inhibitory activity was measured according to the casein digestion method of Kunitz (25). Hemagglutinating activity was determined according to the method of Liener (26).

RESULTS AND DISCUSSION

Electrofocusing in the Region between pH 3 and pH 10

Separation of soybean whey proteins in the pH 3 to pH 10 region was performed in order to obtain a complete pI spectrum of both acidic and basic proteins. Although high resolution is not achieved over such a wide range of pH values, the information obtained is valuable in determining the narrow pH range necessary for high-resolution experiments.

Figure 1 shows the isoelectric focusing spectrum of soybean whey proteins between pH 3 and pH 10. The proteins in the isolated fractions were detected by measuring the absorbance at 280 mμ. The carrier ampholytes, being low-molecular-weight aliphatic polyamino-polycarboxylic acids, do not interfere with the absorption of proteins at this wave length (16). It may be seen that the bulk of these proteins have isoelectric points between pH 3 and pH 8. A minor amount of protein is of a strongly basic nature and is focused in the region of pH 8 to pH 10. Some of the protein precipitates during electrofocusing and migrates in the acidic electrolyte (phosphoric acid) at the bottom of the column (see bar before fraction A in Fig. 1). A very minute amount of protein migrates in the basic electrolyte (ethylenediamine) at the top of the column (see bar after fraction O in Fig. 1).

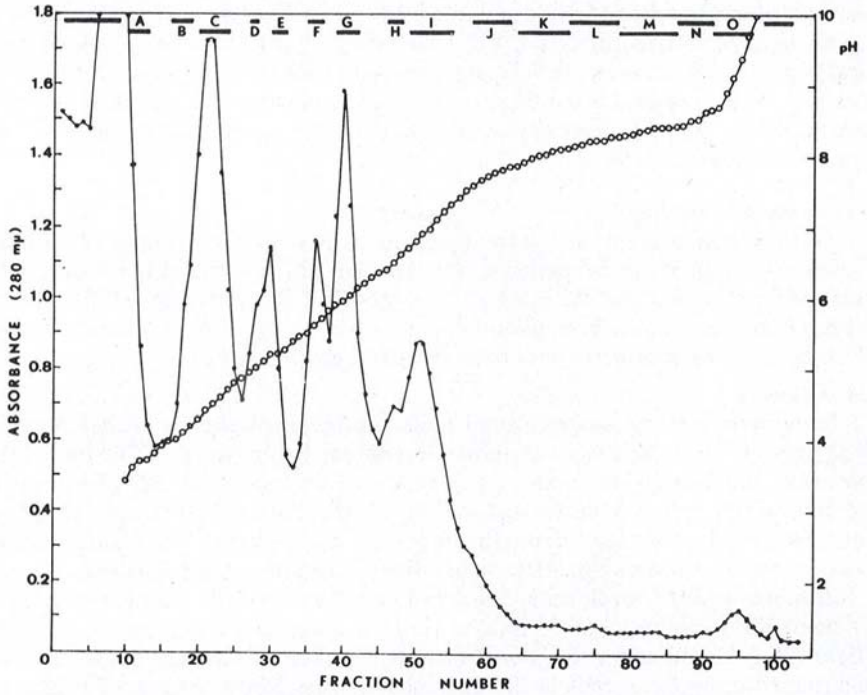

Fig. 1. Isoelectric focusing of soybean whey proteins in the region between pH 3 and pH 10. The solid circles represent the protein concentration as measured by absorbance at 280 mμ. Open circles represent the pH gradient. Solid bars in the upper part of the figure show the pH range of the fractions pooled.

Table I shows the pH range of pooled fractions which were designated with the letters A through O. Their positions in Fig. 1 are indicated by bars at the upper part of the diagram. The pH at the maximum point of each peak is given in Table I.

An analysis of the isolated fractions by disc electrophoresis in 11% alkaline and acidic gels (see "Methods") is shown in Fig. 2. The number of components found in each fraction by disc electrophoresis is indicated in Table I. In general, most of the fractions are heterogeneous by disc electrophoresis, and a great number of components was found to be present. Proteins which exhibited isoelectric points higher than 6.80 did not migrate in the alkaline gel; however, these components were resolved by electrophoresis on the acidic gels. Individual bands were numbered starting with 1 for the band with the highest relative mobility value within a gel. This system of identification facilitates discussion of the isolated components. Thus, "band C2 (alk.)" indicates a protein component with isoelectric point in the range of pH 4.21 to pH 4.68 (Table I) which migrates as the second fastest band in the alkaline gel, whereas a band with the designation "(ac.)" migrates in an acidic gel. Some of the components may migrate in both the alkaline and acidic gels.

The protein components from the different fractions seem to migrate in the alkaline gels in a stepwise fashion, starting from the anodic end of the gel column and progressively approaching the cathode as the isoelectric point increases.

TABLE I. SOYBEAN WHEY PROTEINS SEPARATED BY ELECTROFOCUSING BETWEEN pH 3 AND pH 10

Fraction	pH Range	pH Peak	No. of Components in Alkaline Gel	No. of Components in Acidic Gel
A	3.38-3.73	3.38	0	0
B	4.00-4.11	4.11	2	1
C	4.21-4.68	4.44	2	1
D	4.93-5.02	5.02	9	4
E	5.20-5.23	5.20	3	1
F	5.57-5.70	5.60	5	5
G	5.90-6.14	5.94	1	2
H	6.40-6.50	6.50	1	4
I	6.79-7.20	6.85	0	3
J	7.56-7.80	7.56	0	6
K	7.90-8.10	7.92	0	9
L	8.12-8.25	8.20	0	3
M	8.28-8.42	8.34	0	1
N	8.49-8.72	8.72	0	3
O	8.91-9.97	9.32	0	5

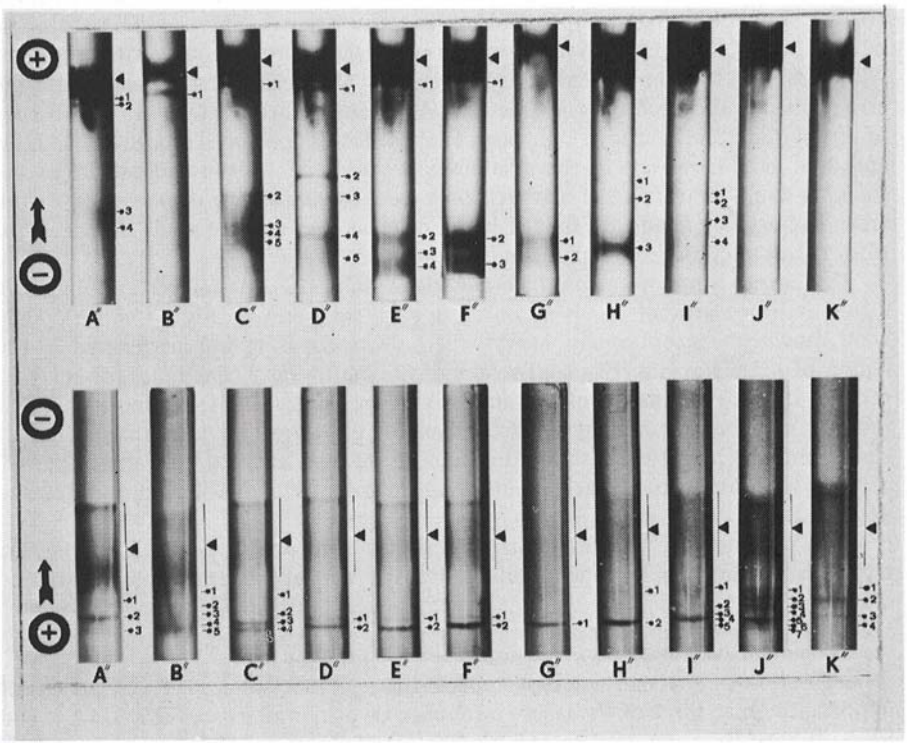

Fig. 2. Disc electrophoresis in 11% polyacrylamide gels of total soybean whey protein (Wh) and of soybean whey protein fractions separated by electrofocusing in the region between pH 3 and pH 10. Upper row, alkaline gels; lower row, acidic gels (see "Methods").

Overlapping of bands may occur, since a greater number of components is found after high-resolution isoelectric focusing. The number of protein components in the unfractionated whey protein fraction (Wh, Fig. 2) cannot be determined exactly because of the multiplicity and overlapping of bands. Only the approximate position of well-defined bands has been marked.

Carrier Ampholyte Disc Electrophoresis Artifacts

It is of significant interest that artifact bands were formed because of the presence of carrier ampholytes in certain protein fractions (see position of arrows in disc electrophoresis patterns). The carrier ampholytes have been reported to be removed easily by dialysis against water (16). However, in a more recent communication, Carlström and Vesterberg (27) demonstrated the presence of ampholytes (even after dialysis) in lactoperoxidase fractions separated by electrofocusing. The presence of ampholytes was shown by disc electrophoresis after amido schwartz staining. Unfortunately, pictures of the gels were not included.

We have repeatedly demonstrated the presence of ampholytes in certain fractions even after exhaustive dialysis against frequent changes of water for up to 10 days. When electrofocusing was performed in the region of pH 3 to pH 10, the artifact ampholyte bands appeared to contaminate the very acidic (isoelectric points below 4.10) and very basic (isoelectric points about 7.56) proteins. Although all the samples were subjected to identical dialysis conditions, the artifact bands appeared only in fractions A, B, and J through O. Note the absence of artifact bands in fractions C through I. These bands appeared in both the alkaline and acid gels. However, in the alkaline gels the artifacts migrated as fast as the tracking dye, whereas in the acid gels they were usually spread over a wider, diffuse area. The artifact bands are shaped like a concave disc and are easily distinguished from the characteristic compact bands of proteins.

The carrier ampholytes used for the high-resolution experiments are obviously different, since artifact bands appeared in each fraction (see Figs. 4 and 6). They were, however, exceptionally heavy when electrofocusing was performed in the region of pH 5 to pH 8 (Fig. 6). Because of the multiplicity of samples for analysis, other methods for removal of the ampholytes were not tried. It is conceivable that removal may be achieved by electrodialysis or gel filtration. It is suggested that electrophoresis in 11% gels should not exceed 30 min. at 5 ma. per current per gel column, because the artifact bands may migrate into the buffer and escape detection.

The presence of contaminant ampholytes did not appear to affect the trypsin-inhibitory or hemagglutinating activity of certain fractions, as will be discussed later.

High-Resolution Electrofocusing in the Region between pH 3 and pH 6

Separation of soybean whey proteins in the region between pH 3 and pH 6 is of importance, since three of the isolated soybean trypsin inhibitors (1.9S, STIAA, and STIA-2) have been reported to exhibit isoelectric points at pH 4.0, pH 4.2, and pH 4.5, respectively (see Yamamoto and Ikenaka (6) for references).

The isoelectric points (pI) spectrum of soybean whey proteins separated in the region between pH 3 and pH 6 is shown in Fig. 3. A more detailed description of

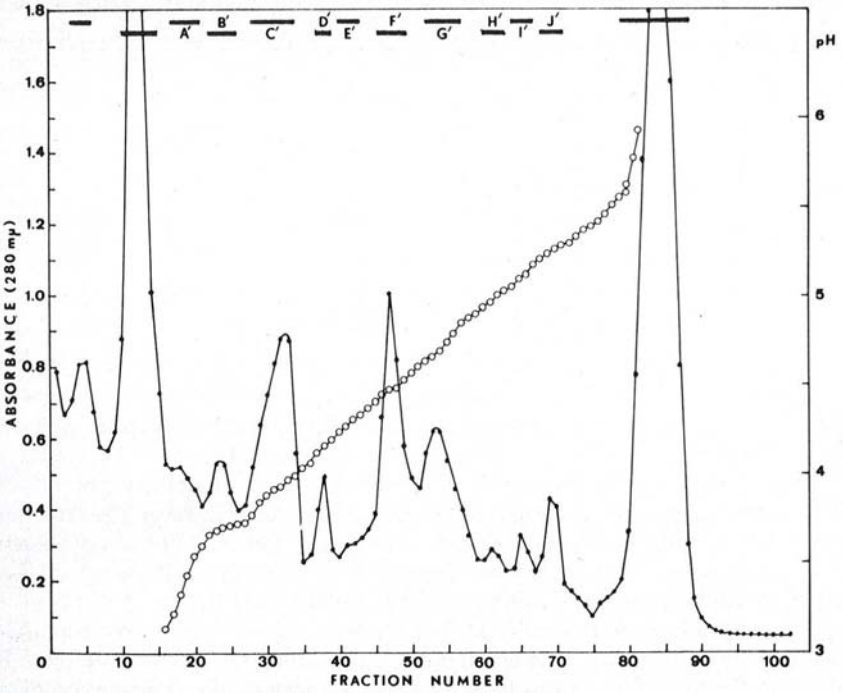

Fig. 3. Isoelectric focusing of soybean whey proteins in the region between pH 3 and pH 6. The solid circles represent the protein concentration as measured by absorbance at 280 mμ. Open circles represent the pH gradient. Solid bars in the upper part of the figure show the pH range of the fractions pooled.

the pH range and pH at the peak of the fractions pooled is given in Table II. A considerable amount of protein which was not focused in this pH region migrated into the electrolytes (see nonlabeled bars in Fig. 3). The absorption of each peak at 280 mμ is not always proportional to the amount of protein present in each fraction, since some of the fractions appeared slightly turbid. The turbidity exhibited by some of the peaks rendered them visible as separated bands during the electrofocusing experiment. The major amount of protein was found in fraction F′ which was separated between pH 4.45 and pH 4.53. The isoelectric point at the peak was found to be at pH 4.47. The major component of this fraction migrated as band F′1 (alk.) by disc electrophoresis in 11% alkaline gel (Fig. 4). The relative disc-electrophoretic mobility of this component coincided with the relative mobility of the major band of commercial soybean trypsin inhibitor (Mann) obtained according to the procedure of Rackis et al. (2). This component (SBTIA-2) has been assumed by several investigators to be identical with the classic inhibitor described by Kunitz (1). The immunochemical identity of component F′1 (alk.) and the major band of the commercial soybean trypsin inhibitor has been established (18). Work on further purification of component F′1 (alk.) (which was found to be 98% pure by densitometer tracing of the gel) and characterization by other means is in progress.

TABLE II. SOYBEAN WHEY PROTEIN FRACTIONS SEPARATED BY ELECTROFOCUSING BETWEEN pH 3 AND pH 6

Fraction	pH Range	pH Peak	No. of Components in Alkaline Gel
A′	3.20-3.54	3.32	1
B′	3.66-3.70	3.68	1
C′	3.77-3.99	3.93	1
D′	4.12-4.16	4.16	2
E′	4.24-4.33	...	4
F′	4.45-4.53	4.47	2
G′	4.63-4.84	4.67	9
H′	4.93-5.00	4.96	6
I′	5.05-5.12	5.09	9
J′	5.21-5.28	5.23	3

Fractions C′ through J′ exhibited trypsin-inhibitory activity. No activity was found in A′ and B′. These findings strongly suggest that the trypsin inhibitors present in soybeans represent a very heterogeneous group of proteins. Of course, all components within a fraction may not exhibit trypsin-inhibitory activity. In addition, contamination from adjacent fractions is possible. However, the technique of isoelectric focusing followed by disc electrophoresis appears to be a very valuable tool in determining homogeneity of isolated inhibitors. The availability of these two high-resolution methods will probably stimulate more extensive work on the purification of the various inhibitors. Since resolution of the disc electrophoresis bands is quite satisfactory, preparatory disc electrophoresis may be employed for further purification of electrofocusing fractions. At present, the disadvantage of the electrofocusing method is that only small amounts of protein can be isolated with the use of columns that are available at present.

Components A′l(alk.), B′l(alk.), and C′l(alk.) with isoelectric points at pH 3.32, pH 3.68, and pH 3.93, respectively, were found to be homogeneous (not contaminated by other proteins, but contaminated by ampholytes). Component C′l(alk.) exhibits trypsin-inhibitory activity. Yamamoto and Ikenaka (6) isolated

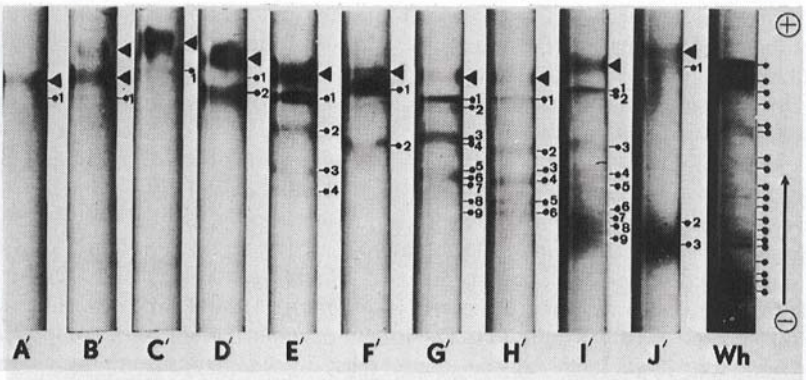

Fig. 4. Disc electrophoresis in 11% alkaline polyacrylamide gels (see "Methods") of total soybean whey protein (Wh) and of soybean whey protein fractions separated by electrofocusing in the region between pH 3 and pH 6.

the 1.9S inhibitor which has an isoelectric point at pH 4.0. The two proteins may be identical. The difference of 0.07 pH unit in isoelectric points may be due to different experimental conditions. Also, component D'2(alk.), isolated in better than 98% purity, and exhibiting isoelectric point at pH 4.16, may be identical to the STIA-1 inhibitor (4) with a reported isoelectric point at pH 4.2. The isoelectric points of the STIA-2 inhibitor (5) and inhibitors F-1 and F-2 (7) are not known. Therefore, any correlation of these with isolated fractions cannot be made.

High-Resolution Electrofocusing in the Region between pH 5 and pH 8

Electrofocusing in this region may be important in the isolation of hemagglutinins. Pallansh and Liener (8) estimated the isoelectric point of soybean hemagglutinin to be pH 6.1±0.1. Other soybean hemagglutinins (9,10) may also be focused in this region since all are very similar proteins.

The pI spectrum of soybean whey proteins subjected to electrofocusing between pH 5 and pH 8 is shown in Fig. 5. Several peaks were obtained. A detailed description of the pH range and pH at the peak of the fractions pooled is given in Table III. Diso-electrophoretic analysis of the fractions in both alkaline and acidic gels is shown in Fig. 6. Fractions C″, D″, E″, F″, G″, H″, and I″ exhibit hemagglutinating activity. No activity was found in fractions J″, K″. Minor activity was demonstrated in fractions A″ and B″. Thus, hemagglutinating activity is

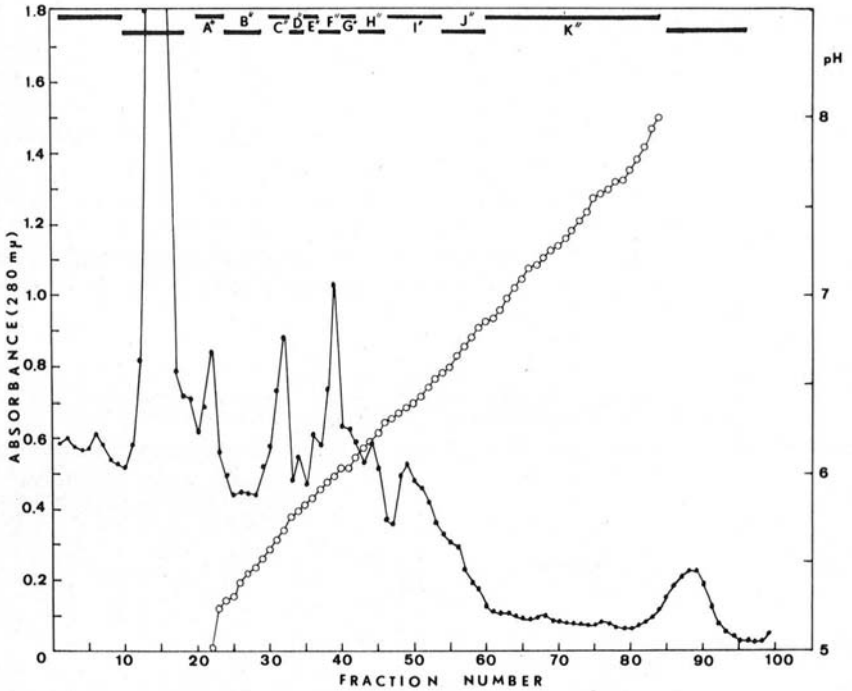

Fig. 5. Isoelectric focusing of soybean whey proteins in the region between pH 5 and pH 8. The solid circles represent the protein concentration as measured by absorbance at 280 mμ. Open circles represent the pH gradient. Solid bars in the upper part of the figure show the pH range of the fractions pooled.

confined mainly to the proteins with isoelectric points between pH 5.57 and pH 6.36.

Lis et al. (10) reported the purification of four soybean hemagglutinins by DEAE-cellulose chromatography. Although photographs of the gels were not included in the publication, they mentioned that the four hemagglutinins moved at the same velocity when submitted to electrophoresis on acrylamide gel at pH 4.5. This type of gel corresponds to the acidic gel in this study. In this respect, the major components C″4(ac.), D″2(ac.), E″2(ac.), F″2(ac.), G″1(ac.), H″2(ac.), and I″4(ac.) appeared to have the same relative mobility in the pH 4.3 polyacrylamide gel. Since nothing was said by Lis et al. (10) about electrophoresis in alkaline gels, comparison with our results cannot be attempted.

From the results described in this report, it is expected that isoelectric focusing in the pH region between pH 5 and pH 8, combined with other fractionation methods, will be very useful in the isolation and characterization of the multiple hemagglutinins present in soybeans. Again, correlation of these proteins with biological activity important to the metabolic processes of the seed will be desirable.

Antigenic Components

Fractions A″ through H″ contained several antigenic components when examined by immunoelectrophoresis in agar gel using a pooled antisoybean-water extract serum 123. This serum has been used in previous studies for the characterization of soybean globulins (11,24). Fractions B″, F″, and H″ exhibited one immunoprecipitin band each, and fractions A″, C″, D″, E″, and G″, two bands each. Fractions isolated in the regions of pH 3.0 to pH 4.67 and pH 6.36 and pH 9.32 did not form immunoprecipitin bands with antiserum 123, although several protein components were shown to be present.

However, it is conceivable that antiserum 123 (a composite of several antisera obtained by injecting total soybean water extract into rabbits) may not contain antibodies to all the potentially antigenic soybean whey proteins in a total soybean extract (which contains only 10% of whey proteins in relation to total protein

TABLE III. SOYBEAN WHEY PROTEIN FRACTIONS SEPARATED BY ELECTROFOCUSING BETWEEN pH 5 AND pH 8

Fraction	pH Range	pH Peak	No. of Components in Alkaline Gel	No. of Components in Acidic Gel
A″	4.88-5.22	4.99	4	3
B″	5.28-5.52	5.32	1	5
C″	5.57-5.76	5.68	5	4
D″	5.79-5.82	5.79	5	2
E″	5.86-5.91	5.86	4	2
F″	5.96-6.03	5.98	3	2
G″	6.03-6.09	6.03	2	1
H″	6.14-6.29	6.18	3	2
I″	6.31-6.56	6.36	4	5
J″	6.60-6.85	6.60	0	7
K″	6.87-8.02	...	0	4

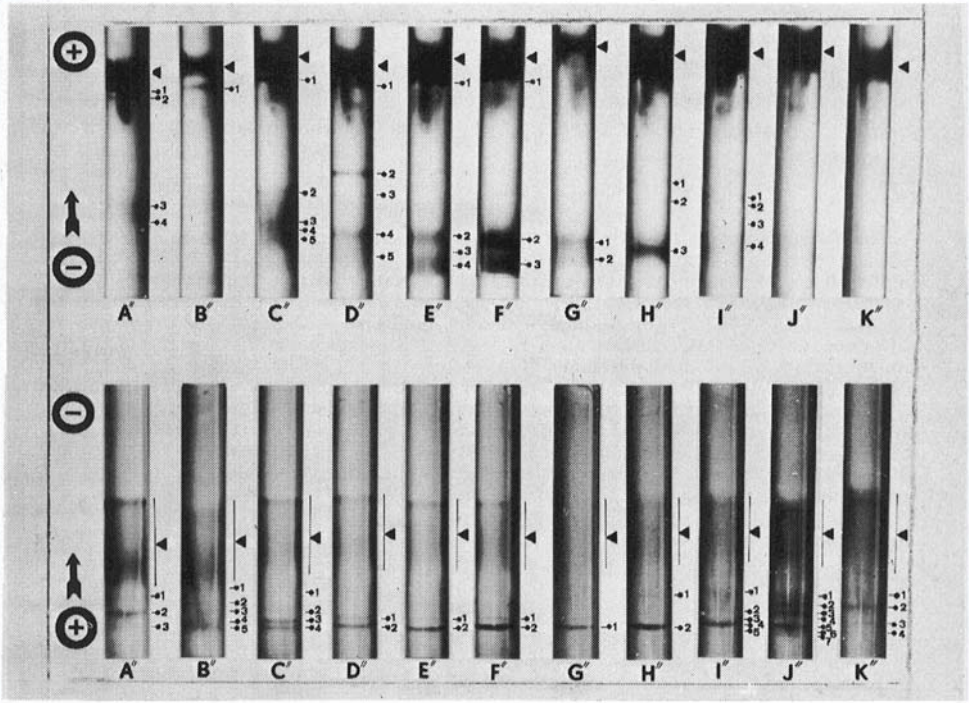

Fig. 6. Disc electrophoresis in 11% polyacrylamide gel of soybean whey protein fractions separated by electrofocusing in the region between pH 5 and pH 8. Upper row, alkaline gels; lower row, acidic gels (see "Methods").

present), since some proteins may be too low in concentration to elicit antibody response, especially if these proteins are not very antigenic. Therefore, immunochemical comparison of the isolated components should be done with antisera produced by injecting the whey protein fraction, thus ensuring a maximum number of specific antibodies. A more systematic immunochemical study of soybean whey proteins by using antisoybean whey protein sera will be reported in the future.

Proteolytic Activity

None of the fractions was able to digest casein under specified experimental conditions (see "Methods"). This may be attributed to several factors: (a) absence of proteolytic enzymes in the whey fraction because of precipitation of the enzymes with the globulins; Weil et al. (13) mentioned that 76% of the proteolytic activity was precipitated at pH 4.5; (b) incomplete extractability of the proteolytic enzymes under regular extraction procedures; (c) casein is not the proper substrate, and high amounts of the enzyme are required for a relatively small degree of digestion; and (d) enzymes and inhibitors are present in the same fraction.

CONCLUSIONS

Isoelectric focusing as described by Svensson (15), and Vesterberg and Svensson (16) is a very promising technique for the separation and identification of soybean

whey protein components. Certain of these components can be prepared in better than 95% purity in one-step fractionation. Other components cannot be isolated in homogeneous form by electrofocusing, but the method could be used for a preliminary crude fractionation. The rapidity and accuracy by which isoelectric points of proteins are obtained constitute one of the advantages of this new technique.

Note Added in Proof

Since submission of this work for publication, certain improvements in the isoelectric focusing technique for separation of soybean whey proteins have been realized. The ampholytes, which may cause artifact disc electrophoresis bands, are removed by extensive washing (overnight) of the electrophoresed gels with 12% trichloroacetic acid solution (Anal. Biochem. 26: 480-482; 1968). Staining with amido schwartz and electrical destaining are then performed as usual. Also, certain other factors should be taken into consideration to avoid contamination of adjacent fractions: The soybean whey protein must be clarified by centrifugation before electrofocusing. Overloading of the column with whey protein in excess of 200 mg. should be avoided, for improved resolution of separated components. The column must be drained slowly to avoid mixing of separated bands by swirling of the contents. Finally, small fractions (3 ml.) are recommended to be collected, for better separation of components. Under these conditions, whey proteins like trypsin inhibitors, hemagglutinins, and lipoxidase can be isolated in homogeneous form (unpublished results).

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