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By

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Introduction

Soybean has been used particularly in our country for many foods, and soybean proteins have been prepared for commercial food purposes. It is of necessity to investigate the soybean proteins to provide a scientific basis for its utilization as a food. Soybean contains about 40% protein on a dry weight basis. Among those, for instance, glycinin is fairly well characterized, and it is designated for a protein fraction which precipitates when a solution of soybean proteins in 10% of sodium chloride is dialyzed against water, and is recognized as a homogeneous and a major component among soybean proteins (1). Where as many other protein fractions in soybean have not characterized yet.

In the ultracentrifugal studies (2, 3, 4), it was revealed that sobyean proteins consist of at least four components having $S_{20,w}$ of 2, 7, 11 and 15S respectively, of which the 11S component is the major part. It has been observed (5) that cooling a concentrated aqueous extract of defatted soybean meal results in precipitation of CIF (Cold Insoluble Fraction) which contains about 80% of this 11S component and is electrophoretically homogeneous but heterogeneous in the phase-rule solubility test. More detailed studies (5, 6, 7, 8, 9) of the CIF by the ultracentrifugation have revealed that the 11S component is capable of polymerization through the formation of disulfide bonds and dissociating into subunits in low pH and low ionic strength.

Water extracted proteins and CIF have been fractionated by means of gel filtration with Sephadex G–200, and the 11S component has been purified by chromatography of calcium phosphate gel (10, 11). In electrophoretic studies (12), however, it has been reported that soybean proteins consist of two major components and three minor components.

Heterogeneity of soybean proteins demonstrated by electrophoresis has no obvious correlation with the ultracentrifugal components.

Starch gel electrophoresis was used to analyze the components of the soybean

proteins. In this work, a high concentration of urea was used in the gel medium to dissolve the proteins, because of its high resolving power. Gel electrophoresis in high concentration of urea is shown to be useful in separating the components of proteins. By this technique, the presence of at least fourteen components was demonstrated and discussions were made on the difference and correlation of each protein fraction obtained.

Experimental

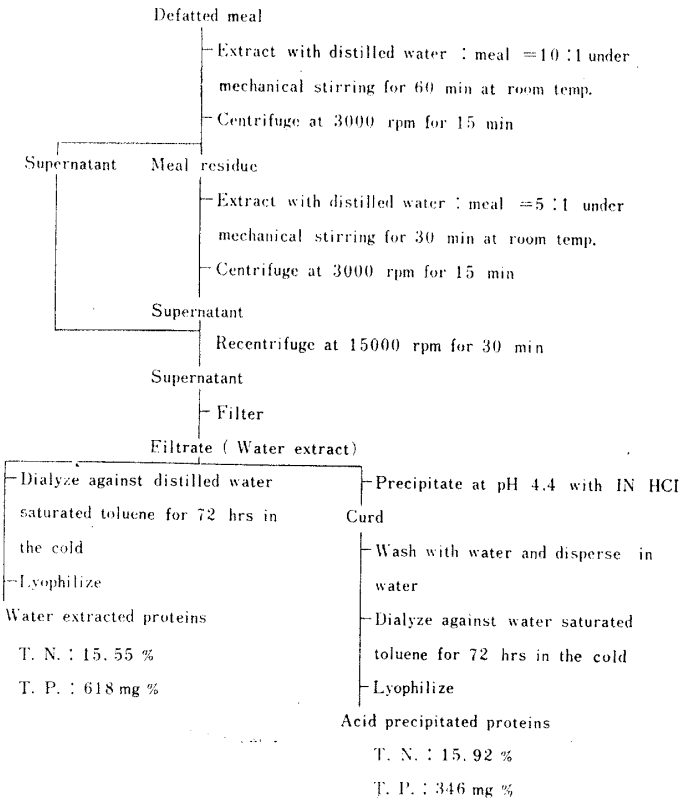
Soybean (Species, Nemasirazu) harvested in 1963 grown at Iwanuma, Miyagi, Japan, were stored at 5°C until used. As described by Wolf and Briggs (3), defatted meal was prepared from the beans by grinding coarsely in a coffee mill, then extracted with peteroleum ether (boiling point 30–60°C) in a Soxhlet extractor for 5 hours, followed by grinding finely in a coffee mill and re-extracting with peteroleum ether for 20 hours.

The extracted meal contained 7.40% nitrogen.

1. Preparation of soybean proteins

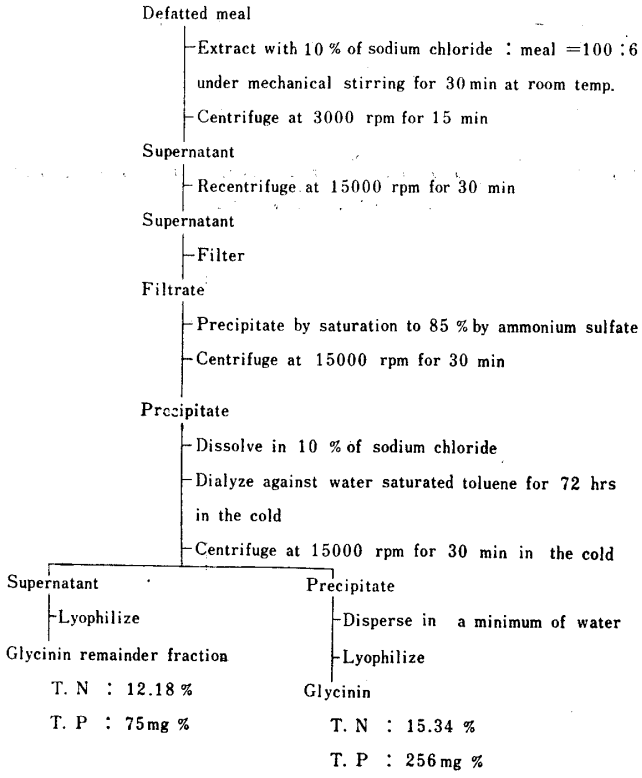
Buffer extracted proteins, water extracted proteins, acid precipitated proteins,

Table 1. Preparation of water extracted proteins and acid precipitated proteins



cold insoluble fraction, glycinin and ammonium sulfate fractions were prepared by the method as described in Table 1, 2, 3, 4 and 5.

Table 2. Preparation of glycinin and its remainder fraction



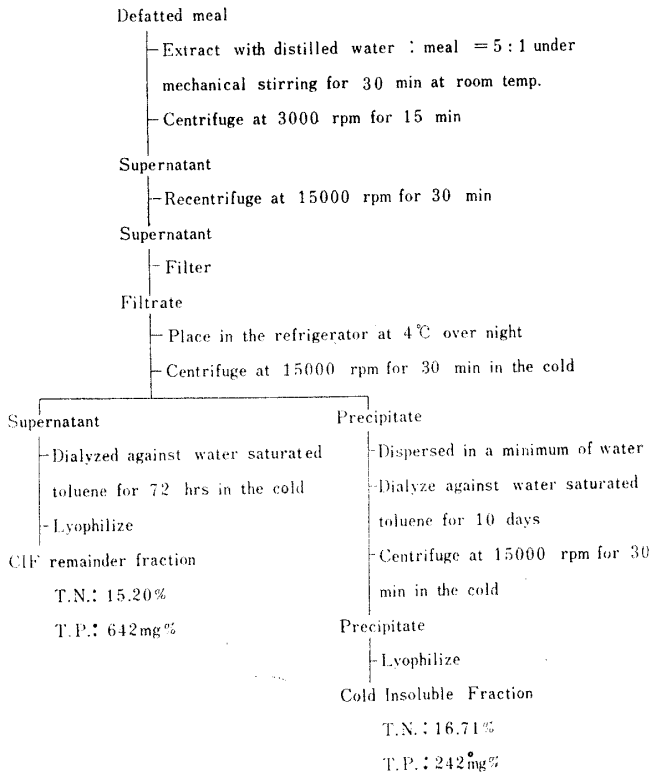
2. Starch gel electrophoresis

The apparatus was similar to that described by Smithies (13) for horizontal electrophoresis. The plastic tray used in the present work had the internal dimensions of $6 \times 80 \times 240$ mm. To slice gel, both sides of the tray were made from two sections. The lower one was cemented to the bottom of the tray while the top section was removable. Either layer could be used for staining.

Potato starch was hydrolyzed by the method of Smithies (13). The following procedure, described by Wake (14), was used to prepare the gel, which had a final composition of 11.9% of hydrolyzed starch, 7M urea and 0.02 M mercaptoethanol (just using only 11.9% of hydrolyzed starch and 0.02M mercaptoethanol, no urea) in the tris-citrate buffer described by Poulik (15). To a 500 ml beaker were added 21.0 ml of stock solution of tris-citrate buffer (0.76 M tris, titrated to pH 8.6 with citric acid), 123.0 ml of water, 25.0 g of hydrolyzed starch and 0.3 g of mercaptoethanol. The mixture was heated in a boiling water bath and stirred until the starch granules had ruptured. At this stage, 68.6 g of urea were added slowly and dissolved while stirring and heating. Air bubbles were removed by application of a negative pressure. The gel was poured onto the plastic tray and

slotformer was placed in position. 550mm from one end. It consisted of apiece of 245 ×85 mm plastic onto which were cemented, in a row, 6 pieces of 2×5×9 mm plastic which will form slots. The gel was allowed to set overnight at room temperature. The slot-former was carefully removed and the sample solutions (approxi. 0.1 ml, 0.5–3.0% proteins, 7M urea and 0.02M mercaptoethanol in the tris-citrate buffer) were introduced with fine-tipped pipette. The gel was then covered with poly-ethyelene films or Saran Wrap.

Table 3. Preparation of Cold Insoluble Fraction and remainder fraction



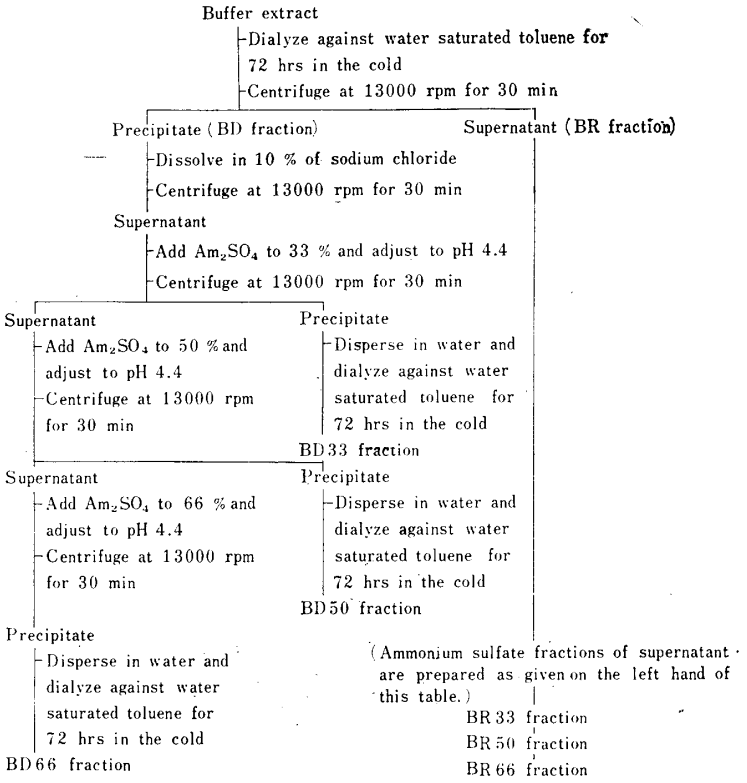
The electrophoresis was carried out at 5°C. Both compartments of the electrode vessels were filled with saturated solution of sodium chloride which were connected with bridge solution (consist of 3 M urea in 0.3M borate buffer of pH 8.6). Electrical contact between the electrode solution and the gel was made by filter papers. Platinum plate was used for the electrode, of which the positive electrode was furthest from the starting slots, and 175V were applied across the system for about 17 hours. At the end of the experiment the polyethylene film and top section of the tray sides were removed. The gel was sliced with the fine wire and the slice which had freshly cut surface was placed on a tray contained the Amide Black 10B staining solution described by Smithies. The gel was stained and washed with the usual solution which consisted of acetic acid: water: methanol=1: 5: 5.

Result

It seems that the subunits of soybean proteins have been dissociated in terms of gel electrophoresis in high concentration of urea. Gel electrophoresis in high concentration of urea would be useful in separating the subunits of proteins. It is usually understood that urea causes associated protein to come apart into its subunit due to breakage of H-bond or hydrophobic bond. Also mercaptoethanol prevents the formation of polymerization through -SS- linkage and is capable to depolymerize its disulfides.

The reproducibility of electrophoretic patterns, although varies by pH, voltage, temperature and starch concentration, is very good when condition are carefully controlled. The resolution of electrophoretic bands is found to be remarkably improved by addition of low concentration of mercaptoethanol to the protein solution and gel medium.

Table 4. Preparation of ammonium sulfate fractions

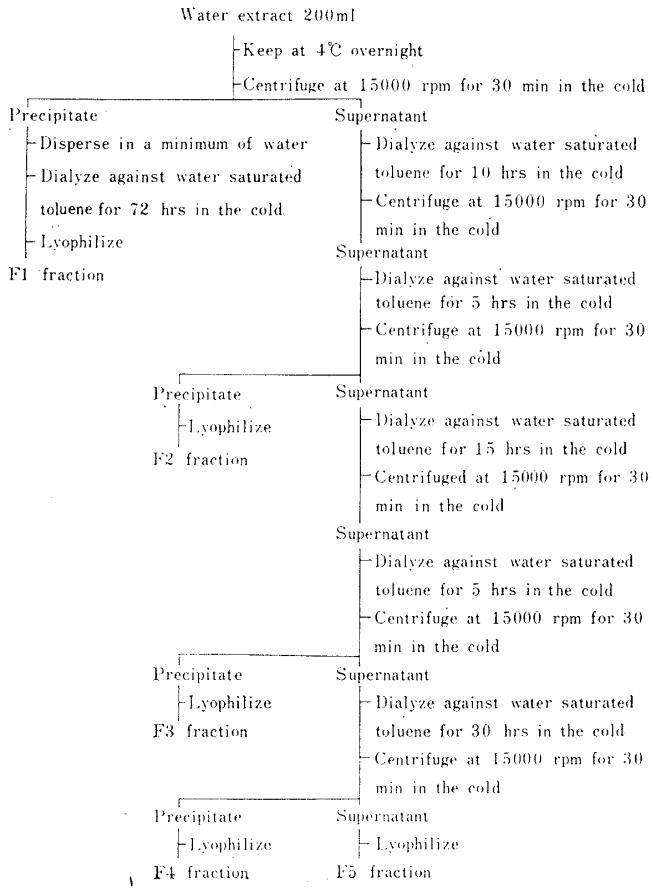


Nearly 90% of the nitrogen of original soybean defatted meal was extracted with a tris-HCl buffer (pH 7.6, μ=0.5) instead of water as a solvent in Table 1. In Fig. 1 are shown the starch gel electrophoretic patterns of buffer extracted proteins, and also their schematic diagrams are presented to define more clearly the number and relative intensities of the electrophoretic bands. The minor components can be seen better at high concentration of smaple but the bands of major components

then spread out and sometimes run together.

In their patterns at least 11 protein bands, 1 to 11, toward the possitive electrode migrate, essentially a few of material remaining in the starting slots. Bands, 1 to 10, are clearly resolved, band 10 is the most prominent, and bands, 7, 8 and 9, are

Table 5. Fractionation of water extract by cooling and dialyzing



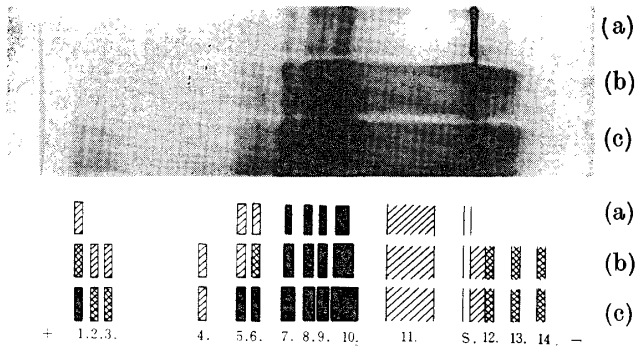


Fig. 1. Starch gel electrophoretical patterns and schematic diagrams of buffer extracted proteins: (a) protein conc. 0.5%, (b) 2.0%, (c) 4.0% using tris-citrate buffer, pH 8.6, containig 7 M urea and 0.02 M mercaptoethanol, voltage 175 V, time 17 hrs, temp. 5°C (S; starting slot, +; positive electrode, −; negative electrode)

next in the intensity.

It has not been characterized about bands, 12, 13 and 14, to migrate toward negative electrode which has fairly high isoelectric point in soybean proteins. And this alkaline proteins can only be seen when urea was used. It may be due to CNO^- from urea which react with the polar sites of protein or mercaptoethanol and then change a net charge of the protein molecule. Better separation is obtained by dissolving freeze-dried protein samples only a few hours before electrophoresis.

A few of the bands are faint and can be just seen on the gel or a photographic negative. After repeated experiments, identity is conformed on the gel between bands of water extracted proteins and several different protein preparations.

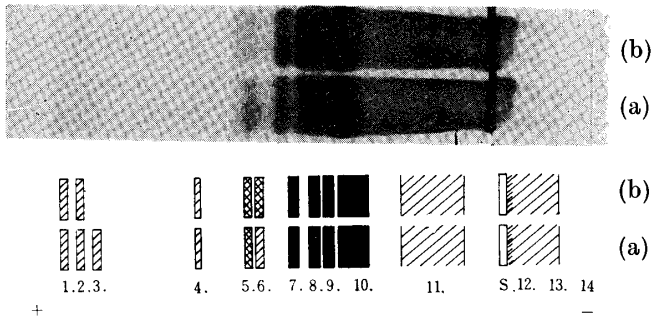


Fig. 2. Starch gel electrophoretical patterns and schematic diagrams of water extracted proteins (a) and acid precipitated proteins (b), using tris-citrate buffer, pH 8.6, containing 7 M urea and 0.02 M mercaptoethanol, voltage 175 V, time 17 hrs, temp. 5°C, protein conc. 2.0% (s; starting slot, +; positive electrode, −; negative electrode)

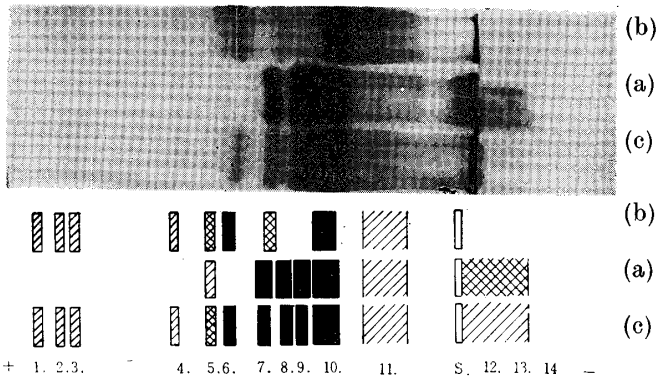


Fig. 3. Starch gel electrophoretical patterns and schematic diagrams of glycinin (a), its remainder fraction (b) and water extracted proteins (c) as contrast, using tris-citrate buffer, pH 8.6, containing 7 M urea and0.02 M mercaptoethanol, protein conc. 2.0%, voltage 175 V, time 17 hrs., temp. 5°C (s; starting slot, +; positive electrode, −; negative electrode)

In Fig. 2 are shown the electrophoretic patterns of water extracted proteins (a) and acid precipitated proteins (b). Their patterns are very similar except that band 3 is missing in the pattern of acid precipitated proteins. No difference was

observed on gel patterns of buffer extracted proteins (Fig. 1) and water extracted proteins.

The glycinin, having been known as major protein of soybean, was named for the protein fraction which precipitated when a solution of soybean proteins in 10% of sodium chloride was dialyzed against water (1). In Fig. 3 are presented electrophoretic patterns of glycinin (a), its remainder fraction (b) and water extracted proteins (c) as contrast. A comparison of electrophoretic patterns in Fig. 3 indicates the presence of bands in glycinin having counterparts with identical mobilities in its remainder fraction and water extracted proteins except bands, 1 to 4 and 6, which are missing in glycinin. In glycinin fraction, it appears more alkaline components compared with the remainder fraction or water extracted proteins.

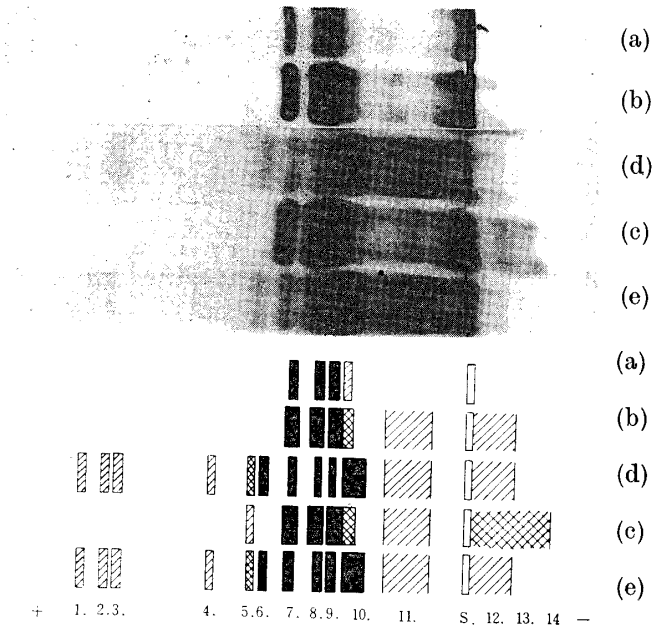


Fig. 4. Starch gel electrophoretical patterns and schematic diagrams of CIF, its remainder fraction and water extracted proteins as contrast (a) CIF, protein conc. 0.5% (b) CIF, 11.0% (c) CIF, 2.0% (d) its remainder fraction, 2.0% (e) water extracted proteins, 2.0%, using tris-ctrate buffer, pH 8.6, containing 7 M urea and 0.02M mercaptoethanol, voltage 175V, time 17 hrs., temp. 5°C.

In Fig. 4 is shown the electrophoretical patterns of CIF (a,b,c,) its remainder fraction (d) and water extracted proteins (e) as contrast. A comparison of electrophoretic patterns in Fig. 4 indicates the presence of bands in CIF having counterparts with identical mobilities in its remainder fraction and water extracted proteins except bands, 1 to 6 and 10. But a few bands toward negative electrode and bands, 7, 8 and 9, are as intense as in the pattern of glycinin (Fig. 3).

The ammonium sulfate fractionation of soybean proteins has been used by

many investigators (2, 11, 18) years ago, and by this method Naismith has obtained a partially purified preparation of the 11S component. As described in Table 4, ammonium sulfate fractions are prepared and in Fig. 5 are presented their electrophoretic patterns except BD33, BR, BR50 and BR66 fractions. In the patterns of BR, BR50 and BR66 fractions, all gel patterns are alike; bands, 1 to 6, 10 and 11 can be found in those patterns, particularly bands, 1, 6 and 10, being dominant and 2, 3 and 5 faint. A conparison of the patterns in Fig. 5 indicates the presence of bands in BD (c), BD33 (b) and BD 66 (a) fractions having counterparts with identical mobilities in buffer extracted proteins except bands, 1, 2, 4, and 6, but in the patterns of BD, BD 33 and BD 50, band 10 are less prominent, and a few bands migrating toward negative electrode and bands, 7, 8 and 9, more prominent than in the pattern of buffer extracted proteins. BD 66 fraction is quite similar to pattern of CIF; in pattern BD 66 fraction, band 10 is faint but bands, 7, 8 and 9, more intense.

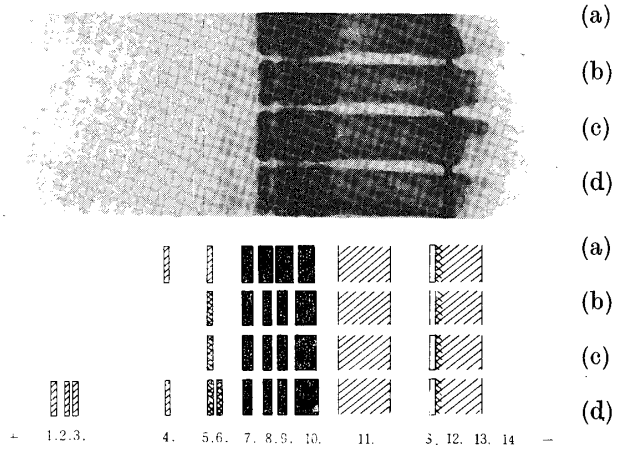


Fig. 5. Starch gel electrophoretical patterns and schematic diagrams of ammonium sulfate fractions: (a) BD66, (b) BD50, (c) BD, (d) buffer extracted proteins, using tris-citrate buffer, pH 8.6, containing 7 M urea and 0.02 M mercaptoethanol, proteiin conc. 2.0%, voltage 175V, time 17 hrs., temp. 5°C.

As described in Table 5, by the precipitation of soybean proteins which rises during cooling and dialyzing water extract are fractionated into F 1, F 2, F 3, F 4 and F 5 fractions. In Fig. 6 are presented the patterns of their fractions. In the pattern of fraction, bands, 7, 8, 9, 12, 13 and 14, are as prominent as in the patterns of glycinin and CIF and band 10 is faint, but bands, 12, 13 and 14, cannot be found in the patterns of F 3, F 4 and F 5 fractions; components corresponding to bands, 7, 8, 9, 12, 13 and 14, are contained in precipitate which rises during cooling and in the first of dialyzing water extract. Top group bands, 1, 2 and 3, are found in the patterns of F 3, F 4 and F 5 fractions, and the most prominent in the pattern of F 6 fraction; components corresponding to top group bands are contained in supernatant free from any precipitate which rises during cooling and

dialyzing, and so soluble in water. Band 10 is extremely prominent in the patterns of F 3 and F 4 fractions and band, 1 and 6, prominent in the pattern of F 5 fraction, in which a new band between band 7 and 8 can be seen and one between 3 and 4 can just be seen on the gel or on a photographic negative.

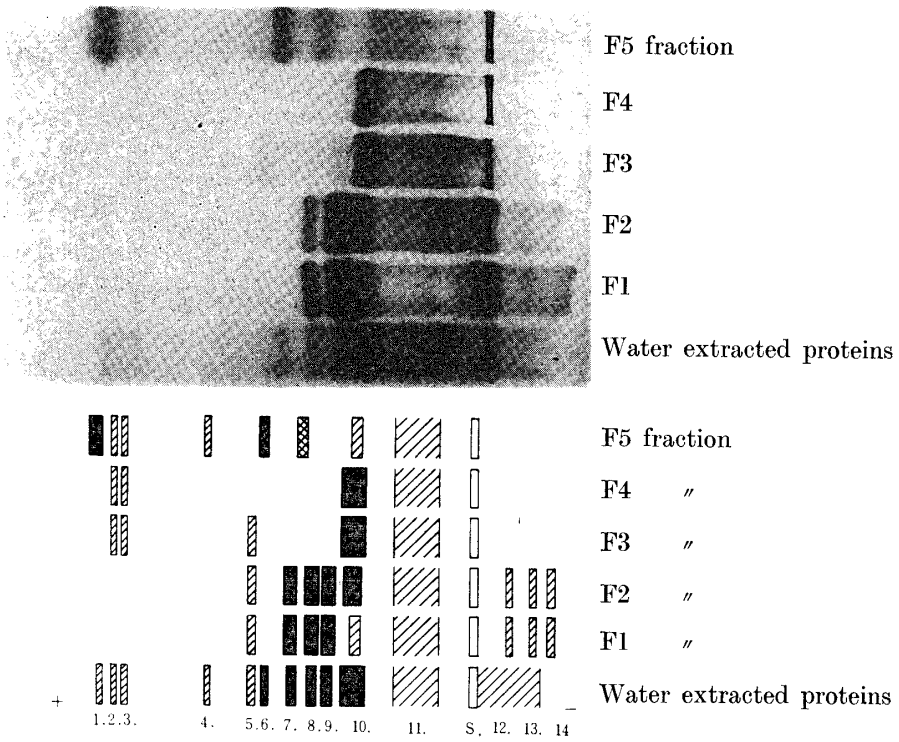


Fig. 6. Starch gel electrophoretical patterns and schematic diagrams of fractions by cooling and dialyzing, using tris-citrate buffer, pH 8.6, containing 7 M urea and 0.02 M mercaptoethanol, protein conc. 2.0%, voltage 175 V, time 17 hrs., temp. 5°C.

To isolate components and to re-examine to see if the bands run true, the sides of the tray are made from three sections, each 2 mm in depth. The lowest one is cemented to the bottom of the tray while the two upper sections are removable. A water extracted proteins sample is applied to separate as usual on a gel and the top most layer is stained to show the position of protein bands. After correcting for shrinkage of the stained slice, the lower is cut into fractions.

Each of these is homogenized with 5 ml of the tris-citrate buffer (pH 8.6 containing 7M urea and 0.02M mercaptoethanol). After centrifuging to remove the insoluble gel, the protein is extracted and applied to a fresh gel and alongside the original.

In Fig. 7 are shown their electrophoretic patterns. Bands, 7, 8 and 9, appear in exactly the same position from which they were obtained. Such a result about other components is obtained. Thus the bands represent components which are distinct from one another.

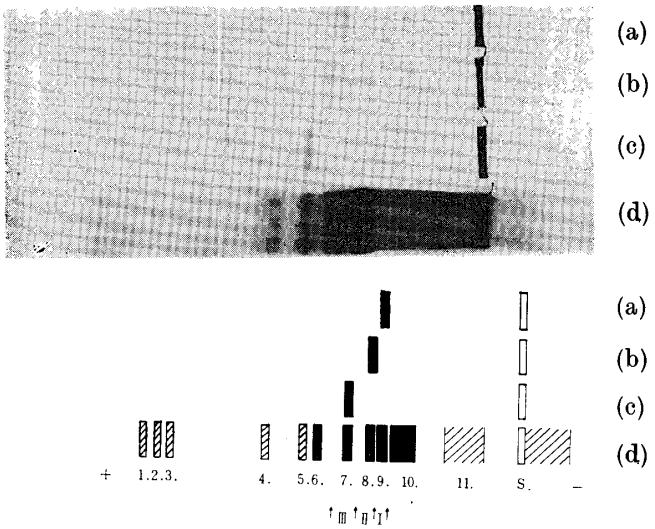


Fig. 7. A test for artifacts of analysis; sections labeled 1, 11, 111 were cut out; (a) section 1, (b) section 11, (c) section 111, (c) water extracted proteins, using tris-citrate buffer, pH 8.6, containing 7M urea and 0.02M mercaptoethanol, voltage 175V, time 17 hrs., temp. 5°C.

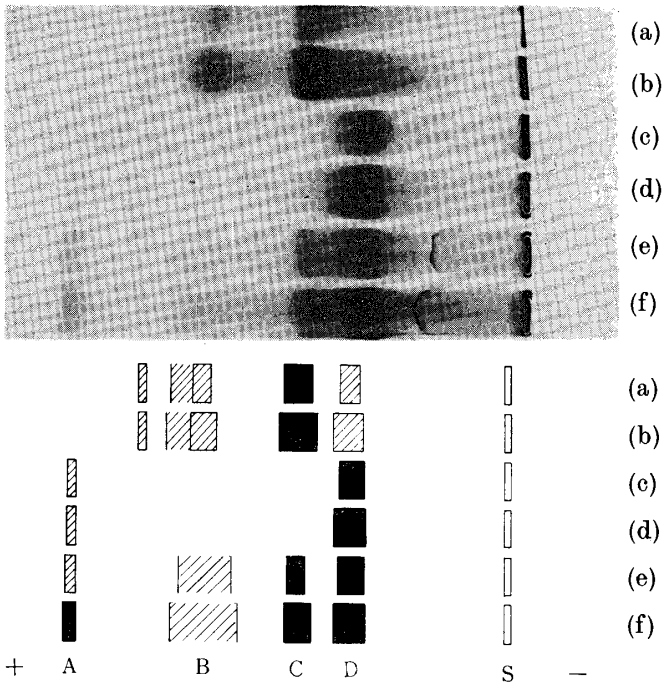


Fig. 8. Starch gel electrophoresis in no urea; (a) CIF, protein conc. 1.0%, (b) CIF, 2.0%, (c) F4, 1.0% (d) F4, 2.0%, (e) water extracted proteins, 1.0%, (f) ibid, 2.0%; using tris-citrate buffer, pH 8.6, containg 0.02M mercaptoethanol, voltage 175V, time 17 hrs., temp. 5°C.

In Fig. 8 is shown the patterns of water extracted proteins, CIF and F 4 fractions without urea both in sample solution and medium. At least four bands, A, B, C and D, migrate toward the positive electrode of water extracted proteins. Bands,

A, C and D, are clearly resolved, band D is the most domimant, but band B is relatively diffused out. In the pattern of CIF, band C is prominent, and in the pattern of F4 fraction, band D is prominent.

Discussion

By ultracentrifugal studies (2, 4, 8, 17), it has been reported that glycinin, acid precipitated proteins and water extracted proteins from soybean are quite similar and that then have at least four different sedimenting components, of which 11S makes up the major part, and in CIF the 11S, more abundant than the glycinin, but some Japanese soybeans have 2 : 1 ratios of 7S to 11S components (4). In the starch gel electrophoresis without urea, at least four bands A, B, C and D, are observed, band D is the most prominent and band C is next intensity in the pattern of water extracted proteins (Fig. 8), whereas band C is the most prominent in CIF. With 7M urea, band 10 is the most prominent among buffer extracted proteins, water extracted proteins and acid precipitated proteins (Fig. 1, 2), which is as prominent as bands, 7, 8 and 9, in the pattern of glycinin (Fig. 3) but faint in the pattern of CIF (Fig. 4). It is suggested that soybean proteins are separated into their subunits by urea and mercaptoethanol, and the subunit corresponding to band 10 is a major in most of the preparations, however, which mentions faint in CIF. It has been reported that CIF is electrophoretically homogeneous but heterogeneous by phase-rule solubility test (5), and that the 11S component has ability to undergo conformational changes probably of dissociation into subunits (8), involving rupture of weak secondary forces, such as hydrogen bonds and van der Waal's interaction (7, 16). Also it has been reported (10) that there are three kinds of N-terminal amino acids in purified 11S component. This is because of the three bands of CIF, 7, 8 and 9, (Fig. 4). It is suggested that the 11S component may be composed of bands 7, 8, 9, 12, 13 and 14 or band C.

Since CIF, glycinin, F 1 and F 2 fractions are precipitate during cooling or dialyzing and similar in their patterns except band 10 (Fig. 3, 4, 7), it may be regarded that components corresponding to bands, 12, 13 and 14, may be undergo binding with components corresponding to bands, 7, 8 and 9, during cooling and dialyzing or by intermoleculer disulfide exchange.

Summary

By starch gel electrophoresis using tris-citrate buffer (pH 8.6) containing 7M urea and 0.02M mercaptoethanol in sample solution and medium, at least fourteen components were demonstrated in whole soybean proteins; bands, 1 to 11, migrated toward positive electrode and bands, 12 to 14, toward negative electrode, of which band 10 was the most prominent and bands, 7 to 9, were more prominent. Without urea four components were demonstrated in whole soybean

proteins; band D was the most prominent and band C was more prominent. In CIF and glycinin, which precipitated during cooling and dialyzing, bands, 7 to 9 and 12 to 14, for urea system, or band C for no urea system were seen. A fraction was obtained rich in component corresponding to band 10 or band D.

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