catalyst. Skellon and Taylor (8) isolated 8% dihydroxybehenic acid by oxidation of brassidic acid in presence of uranium catalyst. This suggested that the peroxide value of soybean oil autoxidized in the presence of cobalt was due to cyclic peroxide. Methyl oleate autoxidized similarly in the presence of cobalt also did not give the hydroperoxide wave. However attempts to concentrate the peroxides from this sample by the solvent extraction method and by the urea complex method were not successful.

Summary

The polarograms of autoxidized soybean oil indicated the presence of a peroxide, which was formed in considerably larger proportion in soybean oil than in methyl esters of similar composition. This peroxide was reduced at more positive potentials than the hydroperoxide at the dropping mercury electrode. In solvent fractionation, using mixtures of Skellysolve F and acetone, this peroxide associates with the polymerized fraction. Hydrogenated soybean oil samples and olive oil also showed the presence of this peroxide in considerable concentrations in the period that they began to give turbid solutions in Skellysolve F. Soybean oil oxidized in the presence of cobalt catalyst contained negligible proportions of peroxides normally reduced at the dropping mercury electrode. Attempts to concentrate the peroxides from methyl oleate oxidized in a similar manner did not succeed.

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Soybean Protein Fractions and Their Electrophoretic Patterns[1]

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MEISSL AND BÖCKER (11) were the first to publish investigations on the isolation and fractionation of soybean protein. However, the work by Osborne and Campbell (14) has been more frequently quoted to describe the early work.

Osborne and Campbell used salt-extraction and precipitation methods to separate and identify four different proteins in the soybean. The principal fraction was a globulin which they named glycinin and defined as "the salt-solution soluble globulin which separates after dialysis." They found small quantities of a second globulin, more soluble than glycinin, which they called phaseolin because of its resemblance to phaseolin from the pea; small amounts of an albumin-like protein (1.5%); and a proteose.

Jones and Csonka (7) precipitated five fractions from the salt-solution soluble protein by various concentrations of ammonium sulfate ranging from 38 to 69% saturation. The fraction precipitated at 55% saturation had an isoelectric point at pH 5.2 and most nearly resembled Osborne and Campbell's glycinin. Ryndin (15) and others (6, 19) have studied soybean protein fractionation by salt-solution methods.

Smith, Circle, and Brother (17), Smith and Circle (18), and Smiley and Smith (16) published detailed information on the effects of neutral salts and various acid and alkaline solutions on dispersion and precipitation characteristics of the soybean protein. While their work was intended as a guide in protein isolation on a commercial scale, it revealed valuable fundamental information on dispersion characteristics of soybean protein.

Briggs and Mann (3) and Mann and Briggs (10) have been the only investigators to publish information on an electrophoretic method of identifying soybean protein fractions. Using a phosphate buffer at pH 7.6, they identified seven different electrophoretic protein fractions in the water extract from fat-free soybean meal. They also isolated and purified a fraction from a 10% salt extract of the meal which they called glycinin, and for which they obtained three Tiselius boundaries, thus indicating the electrophoretic inhomogeniety of the Osborne glycinin. However they did obtain an electrophoretically homogenous pattern for a fraction separated by cooling a water extract of the meal. They did not find any of their fractions homogenous by the phase-rule solubility test.

The present investigation continues the work on separation and electrophoretic characterization of proteins from a water extract of fat-free soybean meal. A Perkin-Elmer Tiselius instrument[4] was used for the electrophoretic part of the work. In preliminary studies, patterns on our instrument were compared with patterns obtained from three different Klett-type instruments, and results in all cases were equivalent.

Initial Sample Preparation

Preparation of the Undenatured Soybean Meal. The soybean meals were prepared for fat extraction by cracking the beans between corrugated rolls and removing hulls by aspiration. Water was added to the grits to give a moisture of about 16%, and, after standing overnight in a cold room, the grits were flaked between smooth rolls, air-dried at low temperatures, and then extracted with a hexane-pentane (b.p. 30°-60°C.) mixture.

Dispersion of the Protein in Water. Somewhat more than 90% of the nitrogenous components of the meal prepared as described above will disperse in water; the dispersion has a pH in the range of 6.5 to 6.7.

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[4] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

Smith, Circle, and Brother (17) showed that water at room temperature disperses more of the proteins from soybean meal than neutral salt solutions, and, if the water is made alkaline, the dispersibility of the nitrogen can be readily increased to about 98%.

Several explanations have been offered for the dispersibility of soybean meal protein in water. Osborne and Campbell thought it was due to the potassium phosphate in the meal, others to the sum total of dialyzable salts, and still others to the dispersing action of the phosphatides.

The salt theory is unsatisfactory because the effect of low concentrations of salts (17) is to precipitate the protein. Osborne's potassium phosphate theory is also unsatisfactory because Earle and Milner (4) and Fontaine *et al.* (5) have shown that only very small amounts of the phosphorus of the soybean are inorganic phosphates and that most of it is phytin phosphorus. A simpler and more satisfactory explanation for water solubility of the meal protein is that it is due to alkalinity of the meal in relation to the isoelectric values of the proteins. In our work we have found that the proteins which are extracted from the meal with water at pH 6.7 and recovered by precipitation with acid at pH 4.5 may be almost completely redispersed by suspending in water and adding sodium hydroxide to about pH 7.0. The redispersion is more readily accomplished if the protein curd is carefully handled in its recovery, *i.e.*, after acid precipitation of the curd it should be removed from suspension by gravity settling or very low gravitational force in the centrifuge. Heavily pressed curd is difficult to redisperse because of its strong cohesive nature. Considerable mechanical action, such as vigorous stirring or shaking with pebbles, is necessary for this redispersion at a neutral pH.

Acid-Precipitated Protein

About 88% of the total nitrogen compounds in the water or alkaline extract of the meal are readily precipitated and recovered as protein by adjusting the pH of the dispersion to the range of 4.6 to 4.0. The 12% of the nitrogen remaining in solution is about equally divided between soluble protein and non-protein nitrogen. The acid-precipitated protein thus obtained, after varying degrees of chemical treatment, is a commercial product sold under several different trade names. Present production capacity of soybean protein is estimated to be in excess of 45 million lbs. a year.

Effect of Purification Treatments on Tiselius Patterns of Acid-Precipitated Protein. Preliminary experiments were carried out to determine the effect on Tiselius patterns of a) washing and reprecipitating the curd and b) pH of the protein extraction.

a) Washing and Reprecipitation—Three samples of protein were prepared by extracting 150 g. of defatted meal for 1 hr. with 2,500 ml. of water. The extract was clarified in the International Centrifuge, and the protein was precipitated with sulfuric acid at pH 4.5. The precipitate was allowed to stand for 1 hr. and was then recovered by centrifugation. This curd, containing about 66% water, was taken without washing as Sample 1. Sample 2 was a portion of the same curd, which was washed twice by shaking in liberal amounts of water. Sample 3 was the remaining part of the curd further purified by redispersing at pH 9.0, clarifying in the centrifuge, reprecipitating at pH 4.5, and washing twice with water. All three samples were dried by lyophilization.

Tiselius patterns were made of the three samples at a protein concentration of 0.6% in a phosphate buffer at pH 7.6 and 0.1 ionic strength. All three samples showed three components having equivalent patterns within the limits of the experimental method. Mobilities for the major component in each sample were 8.39, 8.51, and 8.36 $\times$ 10^{-5} Cm^2 $volts^{-1}$ sec^{-1} as measured on the ascending boundary. Contrary to the accepted practice of measuring the descending boundary (9), the ascending boundary was measured because of its greater clarity.

Preliminary experiments were carried out at 0.6% protein concentration because the solutions were too opaque at higher concentrations. It was then discovered that centrifuging the solution for a few minutes at 16,000 $\times$ G in a Servall centrifuge improved the clarity of the solution and patterns could be made at 1.2% concentration. Figure 1, showing

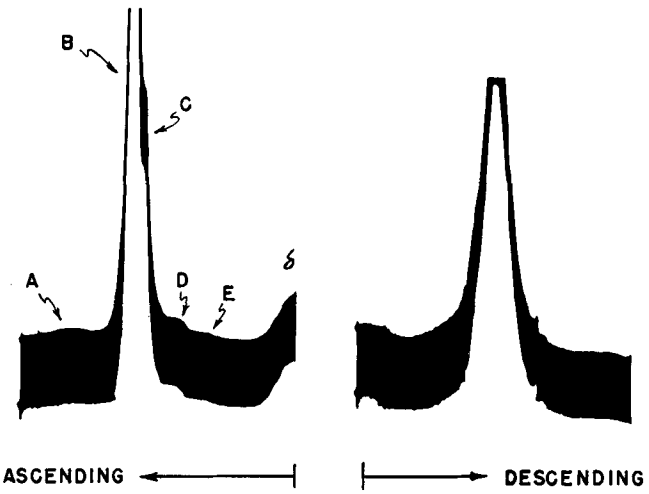

FIG. 1. Electrophoretic pattern for acid-precipitated soybean protein at 1.2% concentration. Pattern after 120 min. in pH 7.6 phosphate buffer at 0.1 μ.

five components in the ascending pattern, is for acid-precipitated protein at 1.2% concentration prepared without washing or reprecipitation. However when patterns were made at this concentration on washed, acid-precipitated protein, the components D and E did not appear.

b) Effect of pH of Extraction—In a similar manner Tiselius patterns were compared for Samples 4 and 5, which were isolated by extracting the meal at different pH values and with different ratios of meal to dispersing agent. Sample 4 was prepared by a single extraction of the meal with distilled water at a meal-to-water ratio of 1:10 and resulting pH of 6.5. Sample 5 was prepared by making two successive extractions of the meal at ratios of 1:10 and 1:5, respectively, with both extractions at a controlled pH of 7.5, and the two extracts were combined. The protein was precipitated at pH 4.5, washed, and dried by lyophilization. Yields for Samples 4 and 5 were 27 and 42 g. of protein, respectively. Patterns for Samples 4 and 5 were made at 0.6% protein concentration by the same procedure as for Samples 1, 2, and 3, and they gave equivalent patterns. Mobilities for the major component in each sample were 8.57 $\times$ 10^{-5} Cm^2 $volts^{-1}$ sec^{-1}.

From these preliminary tests it was concluded that the various protein components of the meal were extracted in a fairly constant ratio to each other in the pH range of 6.5 to 7.5 and over a wide ratio of meal to dispersing agent. However washing and reprecipitation of the protein removed or at least reduced the concentration of components D and E so that they were not apparent in Tiselius patterns made at 1.2% protein concentration.

Patterns in Figure 1 were made in a 6-ml. cell with a pH 7.6 phosphate buffer of 0.1 ionic strength after 120 min. of migration. The five electrophoretic fractions shown in the ascending side have been designated in the order of their decreasing rate of migration as A, B, C, D, and E. The relative amounts of the fractions as calculated from planimeter readings were: A = 3.8%, B+C = 88.0%, D = 3.7%, and E = 1.9%. The B and C proteins migrated at nearly the same rate, and separate measurement of their relative values was not practical on this pattern.

Samples of material responsible for opacity of protein solutions were collected in both the International and Servall centrifuges and analyzed for nitrogen. Nitrogen values on a dry basis were 14.27% and 14.09%, respectively, showing that proteinaceous materials were mainly responsible for the cloudy solutions. Further testing demonstrated that centrifuging for 30 min. or more at top speed in the Servall not only clarified the solution but caused the C fraction to disappear. Figure 2 shows a pattern for acid-precipitated protein after centrifuging at 16,000 × G for 30 min. The picture was taken in phosphate buffer at pH 7.6 and 1.0% protein concentration. With the exception of component C the mobilities and concentrations of the remaining fractions did not change substantially. However, it should be remembered that clarity of the solution is also influenced by pH and the presence of salts. Dispersions prepared from acid-precipitated protein can be clarified by raising their alkalinity above pH 10.0, indicating that one of the protein fractions is not well dispersed until a high pH is reached.

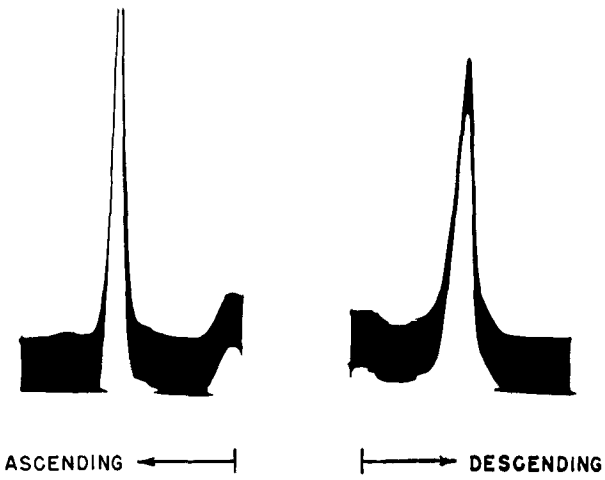

FIG. 2. Electrophoretic pattern for acid-precipitated protein after centrifuging in buffer solution at 16,000 × G for 30 min. Taken at 1% protein concentration after 120 min. in pH 7.6 phosphate buffer at 0.1 μ.

Salt-Solution Soluble Protein. Osborne and Campbell (14) defined glycinin as the "salt solution (10% NaCl) soluble protein" extracted from the defatted meal and precipitated by dialysis. Briggs and Mann (3) found that protein prepared by this procedure is electrophoretically inhomogenous. Their pattern showed one major and two minor components. The name glycinin has been used extensively in the soybean literature. There would be some advantage in continuing the use of the name glycinin if it could be given a well-characterized component of the soybean. Therefore another approach was made to the extraction of glycinin.

In reviewing the conditions of Osborne and Campbell's extraction of glycinin it was found that their salt-solution extraction of the meal had a pH of approximately 6.0. This pH is alkaline to the isoelectric point (pH 4.2) of the acid-precipitated proteins which contain Osborne's glycinin. Therefore, in preparing glycinin according to Osborne's definition, two protein-peptizing factors, alkali as well as salt, are acting on the protein.

In order to minimize the alkaline effects protein was prepared by a double extraction of defatted soybean meal with water, removing the insoluble residue in a centrifuge, adding NaCl to 0.5 N concentration, and HCl to pH 4.2, the apparent isoelectric point of the mixture of acid-precipitated proteins (18). After allowing time for equilibrium, the solution was clarified in a centrifuge and the dissolved protein was precipitated by dilution and dried by lyophilization. This protein was further purified by redispersal in 0.5 N NaCl at pH 4.2 and reprecipitation. For an electrophoresis pattern the protein was dispersed in 0.1 ionic strength, pH 7.6 phosphate buffer, clarified, adjusted to 1.2% concentration, and run 120 minutes in the 6-ml. cell. The pattern of this preparation is shown in Figure 3.

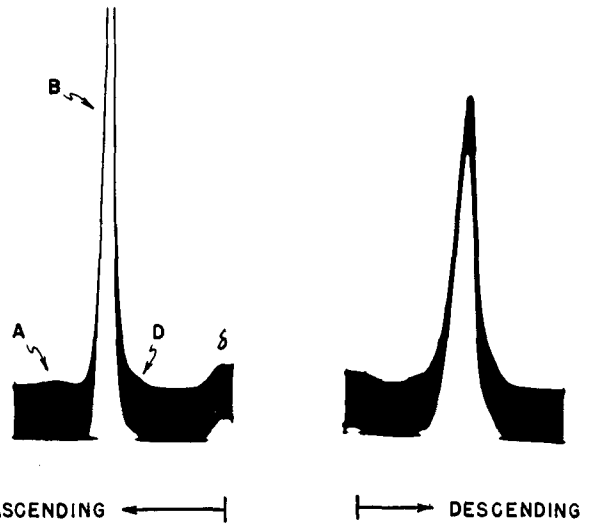

FIG. 3. Electrophoretic pattern for protein soluble in 0.5 N NaCl at pH 4.2. Pattern at 1.2% protein concentration, after 120 min. in pH 7.6 phosphate buffer at 0.1 μ.

At 1.2% concentration the ascending pattern shows three components having migration rates of 11.85, 8.8, and 6.1 × 10^{-5}, respectively. The descending pattern shows an additional slow-moving component which is defined better in the descending pattern than in the ascending pattern. The relative proportions for the three components are estimated as 2.5%, 96%, and 1.5%. The mobility for the major component corre-

sponds to component B and the slow fraction to components H and D. However the mobility of 11.85 for the fast-moving component is higher than any of those in Figures 1 and 5 and may be a component which did not appear in the acid-precipitated or whey patterns.

Soybean Whey

The solution recovered from acid-precipitated protein is known as soybean whey. Although it has a different composition from that of milk whey, the two products have many characteristics in common. For example, if a water extract of soybean meal is prepared for making isolated protein and this solution is allowed to sour through the fermenting action of wild yeast, the resulting acids precipitate the protein, and removal of the curd leaves soybean whey. Soybean whey may be obtained also by extracting defatted meal in a pH range of 4.5 to 4.0.

The soybean whey contains the heat-coagulable protein which Osborne and Campbell (14) likened to albumin as well as small amounts of other proteins, proteoses, and non-protein nitrogen. It also contains trypsin inhibitor, Liener's soyin (8), sucrose, raffinose, stachyose, salts, a number of enzymes including the amylases (13), proteases (12), lipoxidase (1), and other minor constituents of the soybean.

Nearly one-third of the meal used in soybean protein isolation may be recovered from the whey by evaporation. The whey solids are low in protein, and their recovery by evaporation for feed is not economically feasible. The whey therefore presents a disposal problem and an added cost to the isolated protein. The amount of solids recoverable from the whey will vary with the conditions of extraction, such as water-to-meal ratio, pH of the extracting solution, number of extractions of the meal, and method of preparing the flakes. Table I shows the percentage

TABLE I
Percentage of Original Meal Carried Through to Whey in Protein Isolation for Two Different Meals[a]

pH of meal extraction	Percentage of original meal found in whey from	
	Petroleum-ether extracted meal	Ethanol-extracted meal
	%	%
6.6	22.6	15.6
7.3	27.4	11.7
9.5	28.5	24.8
10.4	36.2	28.5

[a] Meal prepared by petroleum-ether extraction and ethanol extraction. Data obtained in pilot-plant operation: Belter, P. A., Beckel, A. C., and Smith, A. K., Ind. Eng. Chem., *36*, 799-803 (1944).

of original meal found in the whey in isolating protein from two different soybean meals. No. 1 meal had its oil extracted with petroleum ether, and No. 2 with ethanol. Acid-precipitable protein was isolated from both meals by extracting at the indicated pH, precipitating the protein at pH 4.2, and removing it by centrifugation.

The following tabulation shows the composition of whey solids on a moisture-free basis for two whey solutions obtained by different methods. The whey solutions from which the solids were obtained are not the same as those reported in Table I.

	Method 1	Method 2
	%	%
Nitrogen	2.44	3.61
Total sugars	35.6	27.5
Reducing sugars	15.2	3.4
Ash	21.1	17.0

Data in Method 1 were obtained by extracting meal with a 0.2% solution of sodium hydroxide at a ratio of 1:20, and precipitating the protein with sulfuric acid. Total solids in this whey amounted to 3.5%. The high ash is due to sodium sulfate. Since there is very little reducing sugar present in the original soybeans, the 15.2% reducing sugars must have been derived from the 35.6% of total sugars.

Whey solids in Method 2 were obtained by extracting defatted soybean flakes at a water-to-meal ratio of 10:1 after adjusting the pH of the system to 4.6. This method leaves the acid-precipitable protein in the residue. Whey solids can be recovered by spray-drying or by lyophilization, yielding a product which is quite hygroscopic.

Whey prepared by either of the two methods is unstable. Apparently instability is due to a slow reaction of protein with a phosphorus material, presumably phytic acid or phytin (as indicated by phosphorus analysis) to form an insoluble reaction product. Therefore to study proteins of the whey it is necessary to eliminate the "phytic acid" reaction. "Phytic acid" compounds are removed by adjusting the pH of the whey to 8.0 and removing the resulting precipitate in a centrifuge. The whey then remains clear for an indefinite period.

Electrophoretic Patterns of the Soybean Whey Proteins

From data on whey composition it is apparent that the protein concentration in the normal whey is too low for satisfactory Tiselius patterns. For example, in estimating protein concentration from the nitrogen value of the whey solution, it is important to remember that Becker, Milner, and Nagel (2) have demonstrated that approximately 50% of the nitrogen in the whey is protein and 50% non-protein nitrogen. Thus a whey solution with 3.5% total solids of which 3.6% is nitrogen would have a protein concentration of approximately 0.4% or only one-third the concentration used for a good pattern of the acid-precipitated protein.

To concentrate whey proteins and remove extraneous components, two procedures were used. In the first the protein was adsorbed from the whey by a mixture of equal parts of bentonite and analytical-grade diatomaceous filter aid, eluted with pyridine, and dialyzed against pH 7.2 phosphate buffer of 0.1 ionic strength. In this procedure it is not necessary to remove the phytic acid compounds from the whey solution.

The second procedure was to remove the phytic acid compounds by adjusting the pH of the whey to 8.0; the resulting precipitate was removed in a centrifuge. The centrifugate containing the proteins was adjusted to pH 7.2 and dried by lyophilization. Whey solids were then dissolved in pH 7.6 phosphate buffer of 0.1 ionic strength and dialyzed. Electrophoretic patterns were made from protein solutions prepared according to these two procedures in a 6-ml. cell at 1.2% protein concentration. Results of the first and second procedures are shown in Figures 4 and 5, respectively.

The ascending patterns of Figures 4 and 5 show four and five components, respectively. Apparently the adsorption method of concentrating proteins (Figure 4) failed to remove one of the components in high enough concentration to give a pattern. In Figure 5 the five components have been designated as F to J

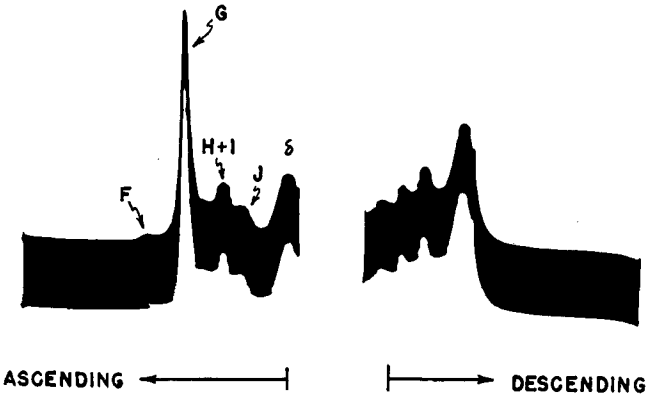

FIG. 4. Electrophoretic pattern of whey protein at 1.2% concentration. Proteins were concentrated from whey by adsorption on mixture of bentonite and analytical-grade diatomaceous filter-aid and eluted with pyridine. Pattern at 120 min. in pH 7.2 phosphate buffer at 0.1 μ.

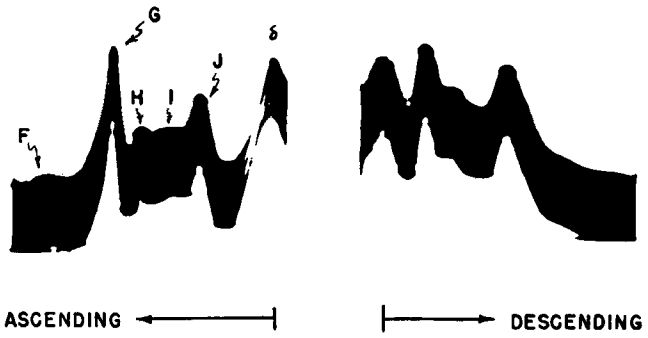

FIG. 5. Electrophoretic pattern of whey protein at 1.2% concentration. Phosphorus compounds were removed by precipitating at pH 8.0; solution was readjusted to pH 7.0 and lyophilized. Pattern after 120 min. in pH 7.6 phosphate buffer at 0.1 μ.

in the order of their decreasing rates of migration. The 10 components indicated in the combination of Figures 1 and 5 are shown in Table II with the mobilities and relative percentages. The data show that fractions D and E have the same mobilities as H and I, respectively. This equivalence in mobilities is another indication that two components may be common to both acid-precipitated and whey fractions. Tentatively, accepting this assumption, the water extract of defatted soybean meal has a minimum of eight components. Undoubtedly there are other protein components present, but their concentrations are too low for showing in these patterns.

The water-soluble proteins of the soybean were divided into soluble and insoluble fractions at pH 4.5. Electrophoretic patterns for each of these fractions

TABLE II

Relative Percentage Composition and Mobilities of Various Components in Acid-Precipitated and Whey Fractions of Soybean Protein

Protein [a]	Composition, %	Mobilities [b]
Acid-precipitated		
A	3.8	9.1
B	88.8	8.6
C		8.1
D	3.7	6.2
E	1.9	4.8
Whey		
F	3.0	10.5
G	41.0	7.8
H	12.0	6.2
I	16.0	4.8
J	28.0	3.5

[a] Letters refer to electrophoretic fractions described in text.
[b] Mobilities as measured on the ascending boundary should be multiplied by -1×10^{-5} for Cm^2 $volts^{-1}$ sec^{-1}.

had five identifiable components. However there were indications that two components were common to both fractions. Assuming this to be true, there are a minimum of eight components electrophoretically identifiable in the soybean. The most homogenous fraction was isolated by extracting the acid-precipitated protein at pH 4.2 with 0.5 N sodium chloride.

A method of stabilizing whey protein preparations so that satisfactory electrophoretic patterns may be obtained is described.

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