[CONTRIBUTION FROM THE NORTHERN REGIONAL RESEARCH LABORATORY[1]]

Chromatography of Soybean Proteins. I. Fractionation of Whey Proteins on Diethylaminoethyl-cellulose[2-5]

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RECEIVED MAY 2, 1959

A method for the chromatographic separation of soybean whey proteins on a cellulose ion-exchange adsorbent using both analytical and preparative isolation procedures is described. Elution by stepwise and continuous increase in the salt concentration of the eluent was used. Effluent fractions were examined for biological activity and were characterized by electrophoretic and ultracentrifugal techniques. The whey solution was fractionated into 13 electrophoretically distinct components and 8 ultracentrifuge components having s_{20w} values between 0.99 and 6.14 S as compared to only 5 and 2 components resolved by conventional electrophoretic and ultracentrifuge methods, respectively. Good correlation was observed between the order of elution and the electrophoretic mobilities of the chromatographic fractions, indicating that ion-exchange had the dominant role. Two trypsin inhibitors of high purity were isolated directly from the whey and were separated simultaneously from each other. One of the trypsin inhibitors was chromatographically pure and homogeneous when subjected to electrophoresis and ultracentrifugation.

Introduction

Previous electrophoretic studies on soybean whey proteins have disclosed the presence of at least five electrophoretically distinct components.[6] Two resolvable peaks appear in ultracentrifuge patterns,[7] corresponding to components with s_{20w} values approximating 2 and 6S; however, these do not represent single proteins since biological tests indicate the presence of a heat-coagulable protein, trypsin inhibitor, hemagglutinin and a number of enzymes.[6,8] The whey protein system is much more complex than that revealed by electrophoresis and ultracentrifuge studies on the whole whey.

A promising advancement in the fractionation and purification of proteins in a complex mixture was made by the recent introduction of cellulose ion-exchange adsorbents by Peterson and Sober and their co-workers.[9,10] DEAE-cellulose is used in this Laboratory for the fractionation of soybean whey proteins by analytical and preparative procedures. This exchanger is also effective for the separation of several constituents heretofore unrecognized by conventional electrophoresis and ultracentrifuge methods, for the direct isolation of two trypsin inhibitors and for the isolation of specific fractions on a preparative scale.

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(2) Presented before the Division of Biological Chemistry at the 135th Meeting of the American Chemical Society in Boston, Mass., April, 1959.

(3) Reagent grade, diethylaminoethyl cellulose exchanger, Type 40, was purchased from Brown Company, Berlin, N. H. Two different lots, having an exchange capacity of 0.79 and 0.90 meq. per g., were used without any differences in their performance.

(4) Mention of trade or company names does not imply endorsement by the U. S. Department of Agriculture over similar products or firms not mentioned.

(5) Abbreviations used are: DEAE, diethylaminoethyl; SBTI, soybean trypsin inhibitor; SBTI (5X), 5X crystallized soybean trypsin inhibitor; SBH, soybean hemagglutinin.

(6) A. K. Smith, E. N. Schubert and P. A. Belter, *J. Am. Oil Chemists' Soc.*, **32**, 274 (1955).

(7) W. J. Wolf, Ph.D. Dissertation, University of Minnesota, 1956.

(8) S. J. Circle, "Soybeans and Soybean Products," Vol. I, K. S. Markley, editor, Interscience Publishers, Inc., New York, N. Y., 1950, Chapter VIII, pp. 275–370.

(9) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1957).

(10) H. A. Sober, F. J. Gutter, M. M. Wyckoff and E. A. Peterson, *ibid.*, **78**, 756 (1957).

Material and Methods

Preparation of Whey Proteins for Chromatography.—Adams soybeans, 1955 crop, were used throughout this study. Defatted meal was extracted twice, first with a 10:1 ratio of water to meal, then with a 5:1 ratio. After centrifugation the supernatants were combined and acidified to *p*H 4.4 with HCl and the precipitate discarded. The solution, or whey, was brought to *p*H 8.0 with sodium hydroxide, allowed to stand in the cold (4°) for 1 hour to precipitate the phytate salts, which were removed by centrifugation, the whey then was dialyzed against cold distilled water for 2 days and lyophilized.

Column Chromatography.—The anion-exchange adsorbent was equilibrated as follows: 30 g. of DEAE-cellulose was suspended in water, stirred well and titrated to *p*H 7.6 with a concentrated solution of potassium dihydrogen phosphate. The adsorbent was then washed several times on a büchner funnel with 0.01 M potassium phosphate, *p*H 7.6, the starting buffer and resuspended in 500 ml. of the same buffer; the fines were removed by decantation of the suspension after settling for 30 min. After equilibration overnight with starting buffer,[11] a slurry of the adsorbent was poured into the exchange column, which had a fine-fritted disk at the bottom, and was allowed to settle under gravity to constant height. Elution was carried out at room temperature using a flow rate of 30 ml. per hour for both the analytical and preparative columns.

Gradient elution with salt (0 → 0.3 M sodium chloride) in starting buffer was used to develop the chromatograms of the analytical column. Samples containing 5–20 mg. of protein in 5 ml. of starting buffer were pipetted on to the adsorbent, the walls of the column were washed down twice with 2 ml. of buffer and then 5 ml. of buffer was poured on top of the column before elution was started. One-ml. fractions were collected with an automatic fraction collector equipped with a drop-counting mechanism. Protein content of the effluent was determined with the phenol reagent described by Lowry, *et al.*[12] Phytate-free glycinin[13] was used as a protein standard.

For the preparative column, stepwise increases of sodium chloride concentration in potassium phosphate buffer (0.01 M, *p*H 7.6) was employed for elution. About 600 mg. of protein in 50 ml. of buffer was washed into the column with 2–5 ml. portions of starting buffer. Fractions of 10-ml. volume were collected with a constant-volume fraction collector at a rate of 30 ml. per hour. The optical density of each fraction was measured at 750 mμ. The starting buffer was run through the column and a large protein peak was eluted which contained those proteins having little or no affinity for the absorbent. When the optical density of the effluent returned to a base-line value, stepwise elution with

(11) For the analytical column, 2.8 g. of adsorbent settled under gravity to a height of 1.5 cm. × 17 cm., with a "hold-up" volume of 23 ml. For the preparative column, 11.0 g. of adsorbent settled to a height of about 2.2 cm. × 25.7 cm., with a "hold-up" volume of 89 ml. Starting buffer for both columns was 0.01 M potassium phosphate buffer, *p*H 7.6.

(12) O. H. Lowry, W. J. Rosebrough, A. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(13) A. K. Smith and J. J. Rackis, THIS JOURNAL, **79**, 633 (1957).

increased salt concentrations was introduced. Those fractions which comprised discrete peaks were combined, dialyzed against cold distilled water, lyophilized and subsequently analyzed in the electrophoresis apparatus, ultracentrifuge and for specific biological activity.

Specific Protein Analysis.—Trypsin inhibitor activity was determined according to Kunitz[14]; phosphatase, by measuring the liberation of inorganic phosphate from β-glycerol phosphate; β-amylase, by its hydrolytic activity on glycogen; and hemagglutinin, according to Liener.[15]

Electrophoretic and Ultracentrifugal Analysis.—The lyophilized fractions were dialyzed against potassium phosphate buffer, *p*H 7.6, 0.1 ionic strength, and were subjected to moving boundary electrophoresis at 2°. Mobilities were calculated from the ascending boundaries.

Sedimentation experiments were carried out at room temperature, using a Spinco ultracentrifuge, Model E, at 59,790 r.p.m. The protein fractions were dialyzed against the same buffer used for electrophoresis. Sedimentation constants were calculated according to the method of Svedberg and Pedersen.[16] The method of Pickels[17] was used to calculate areas, assuming a specific refractive index value of 0.00186.

Results

Chromatographic Behavior of Whey Proteins Using Gradient Elution.—In Fig. 1 is shown the elution diagram of soybean whey proteins, using a

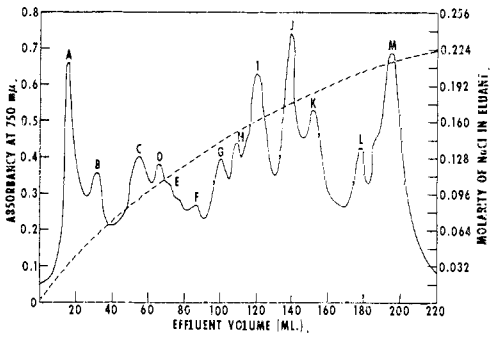

Fig. 1.—Gradient elution diagram of soybean whey: 5 mg. dialyzed and lyophilized protein in 5 ml. applied to 2.8 g. of adsorbent; effluent collected in 1-ml. fractions. Gradient limit 0 to 0.3 *M* sodium chloride in 0.01 *M* potassium phosphate buffer, *p*H 7.6. Absorbancy is the optical density at 750 mμ of 1 ml. of effluent with Folin reagent.[12] Broken line and scale at right represent salt concentration of eluent. Mixing chamber volume was 160 ml.

gradient limit of 0 → 0.3 *M* sodium chloride in 0.01 *M* potassium phosphate buffer, *p*H 7.6 and a mixing chamber volume of 160 ml. About 90–95% of the protein applied had been eluted when the salt concentration in the eluent (input) reached 0.219 *M* sodium chloride (tube 220). The method of Alm, *et al.*,[18] was used to calculate the salt concentration of the eluent. At least 13 distinct peaks designated A to M in the order of their elution were resolved. By using the same gradient limit (0.3 *M* sodium chloride with a mixing chamber volume of 240 ml.) better resolution of some of the components was obtained. However, extended elution ranges in the region of components D to I tended to obscure the rather good resolution of other components.

For a given elution schedule, in both analytical and preparative columns, the reproducibility of the position and height of the chromatographic peaks were very good. Identical elution patterns were obtained for fresh or lyophilized whey.

There was a marked decrease in the resolution of whey proteins when the amount of protein applied to the analytical column was 50 mg. or more. This decrease in resolution was due primarily to a decrease in the affinity of components G through M for the adsorbent. It was determined that component M which normally is eluted when the salt concentration of the eluent reached 0.219 *M* now peaks at a concentration of 0.15 *M* sodium chloride. This behavior is typical of substances having strongly curved adsorption isotherms; other factors, *e.g.*, strong displacement effects and changes in buffer capacity because of more protein can be implicated. The same degree of resolution was obtained at *p*H 7.0 with the same buffer and gradient limit as in Fig. 1. At lower *p*H values varying amounts of whey protein will precipitate. Where buffers at *p*H 8.0 or above are used, the decrease in the anion-binding capacity of the ad orbent results in poor resolution of the whey proteins. Decreased resolution was obtained, by using 0.005 *M* phosphate buffer, *p*H 7.6, as the starting buffer and progressively decreasing *p*H and increasing the salt concentration. The large number of components in soybean whey, their narrow *p*H elution region and the excessive trailing of some place a limit on the degree of resolution that can be obtained with gradient elution chromatograms. Regardless, significant conclusions on homogeneity and the distribution of enzymatic activity in purified preparations can be obtained.

Differentiation between SBTI A_1 and A_2 isolated by chromatographic methods (Table I) and the homogeneity of SBTI (5X)[19] is illustrated in Fig. 2. Trypsin inhibitor A_2 and the 5X crystallized inhibitor have identical elution characteristics, which peak at tube 126 when the salt concentration of the eluent reaches 0.219 *M* sodium chloride and corresponds to component M in Fig. 1. The material which is eluted before the major peak (tube 126) in the commercial preparation is devoid of any antitryptic activity and cannot be separated from the active component by conventional boundary electrophoresis. SBTI A_2 is chromatographically pure. SBTI A_1 peaks at tube 90 (0.20 *M* sodium chloride in eluent) and corresponds to component L in Fig. 1. The small peak at tube 80 has no activity.

If Kunitz[14] procedures for denaturation by heat and alkali are used, the undenatured and denatured components of both SBTI (5X) and A_2 are eluted from DEAE-cellulose columns with 0.219 and 0.35 *M* sodium chloride, respectively.

A crude soybean phosphatase preparation,[20] having a specific activity value of 2400, after chro-

(14) M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).

(15) I. E. Liener, *Arch. Biochem. Biophys.*, **54**, 223 (1955).

(16) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, New York, N. Y., 1940.

(17) E. G. Pickels, *Methods in Medical Research*, **5**, 107 (1952).

(18) R. S. Alm, R. J. P. Williams and A. Tiselius, *Acta Chem. Scand.*, **6**, 826 (1952).

(19) Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

(20) The phosphatase preparation was supplied by Dr. F. M. Mayer of this Laboratory, who is presently engaged in a more definitive study of the properties of soybean acid phosphatases.

TABLE I

ANALYSIS OF ISOLATED PROTEINS FROM SOYBEAN WHEY

Fraction	Electrophoretic data: Mobility[b] × 10^{-5}	Electrophoretic data: Protein concn., %	Ultracentrifuge data: s_{20w} values × 10^{-13}	Ultracentrifuge data: Total area, %	Ultracentrifuge data: Protein concn., %	Biological assay specific activity values[a]: Phosphatase	β-Amylase	SBH	SBTI
I	A 0.08	0.98	0.99	84	0.6	1.2	0	868	0
	B 0.55		5.85	16					
	C 1.13								
II	D 2.70	1.28	2.85	21	.6	24	0	9123	0
			6.14	79					
III	E 3.56	0.90	1.58	36	.6	43	3.6	1595	0
	F 3.89		5.66	64					
	G 4.37								
IV	H 5.0	0.91	1.73	73	.6	14	2.4	66	0.45
	I 5.45		5.64	27					
	J 5.99								
V	K 6.55	1.08	1.75	87	.6	2	1.7	100	1.28[c]
	L 7.17		5.62	13					
VI	M 7.86	1.10	2.29	100	.6	0	0	23	1.0[d]
Whole whey	1 3.11	1.10	1.75	41					
(VII)	2 4.72		6.13	59	.6	20	2.9	1480	0.387
	3 6.30								
	4 7.56								
	5 9.96								

[a] Values calculated on the basis of 1 mg. of protein (N × 6.14). [b] Mobilities as measured on the ascending boundaries. [c,d] (Referred to in the text as) SBTI A_1 and A_2, respectively.

matography exhibited two maxima having phosphatase activity; one corresponds to component G of Fig. 1 (specific activity 6400) and one to component H having a specific activity of 1700.

Discontinuous Elution of Whey Proteins.—Because of the multiplicity of peaks obtained in the gradient elution diagrams, further work was deferred until development of a preparative procedure for the isolation of specific components. A typical stepwise elution diagram is shown in Fig. 3. The stepwise increases of sodium chloride concentration in 0.01 *M* potassium phosphate buffer, *p*H 7.6, are shown in the legend.

Six fractions designated I, II, III, IV, V and VI in the order of their elution, were obtained. These fractions were dialyzed against distilled water, lyophilized and subjected to various physical and biological tests.

Characterization of the Proteins Fractionated by Discontinuous Elution. A. Rechromatography.—Since single proteins with markedly curved isotherms may have extended elution ranges, it is possible that several chromatographic peaks may be obtained with each stepwise increase in sodium chloride concentration of the eluent.[21] On dialysis and rechromatography, each of the six fractions was eluted as a single peak at the same salt concentration as in the first experiment (broken lines Fig. 3). The behavior of these fractions upon rechromatography indicates that a true fractionation took place.

As a further test for determining whether the chromatographic method had fractionated whey proteins into distinctly different components, lyophilized fractions of Fig. 3 were rechromatographed on the analytical columns using gradient elution. In each case the salt concentration of the eluent required to elute each fraction completely from the column was the same as the stepwise increases employed in Fig. 3. Again, the chromatographic behavior corresponded to what would be expected if a real separation had taken place. Fractions III and IV were eluted with considerable tailing, a behavior typical of substances with highly curved adsorption isotherms. For this reason, the resolu-

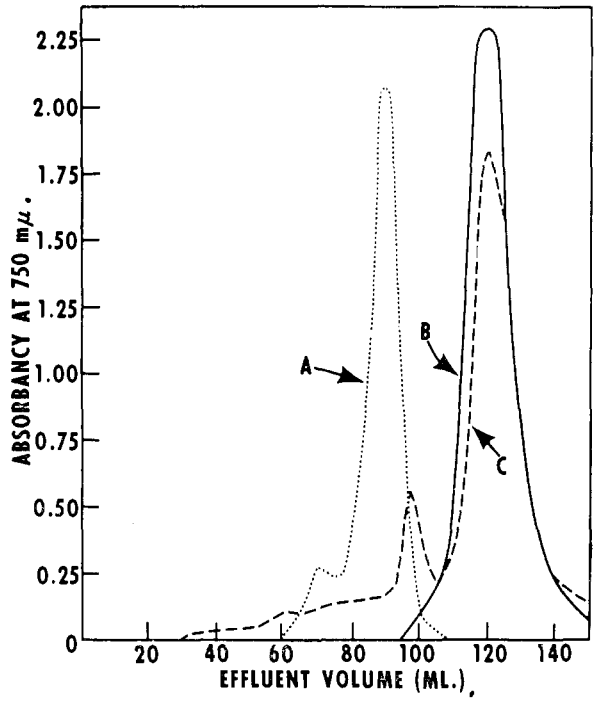

Fig. 2.—Gradient elution diagrams of soybean trypsin inhibitors; A, trypsin inhibitor A_1; B, trypsin inhibitor A_2; C, commercial 5X crystallized inhibitor. See Table I for data. Mixing chamber volume was 80 ml; other conditions same as Fig. 1.

(21) A. Tiselius, S. Hjerten and O. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).

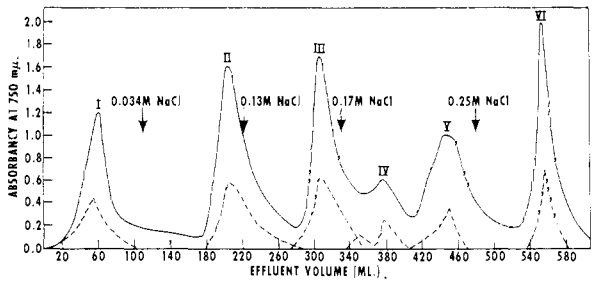

Fig. 3.—Stepwise elution diagram of soybean whey: 600–700 mg. dialyzed and lyophilized protein in 50 ml. applied to 11 g. of adsorbent; effluent fraction was 10 ml.; absorbancy is the optical density at 750 mμ of a 0.1-ml. aliquot of effluent with Folin reagent.[12] Numbers on the peaks correspond to those given in Table I. Vertical arrows indicate the point of change of the sodium chloride concentration in 0.01 *M* potassium phosphate buffer, *p*H 7.6. Broken lines indicate the rechromatographic behavior of each fraction; protein concentration, g. of adsorbent and effluent volumes were reduced by one-third. Absorbancy value was determined on 0.1-ml. aliquots.

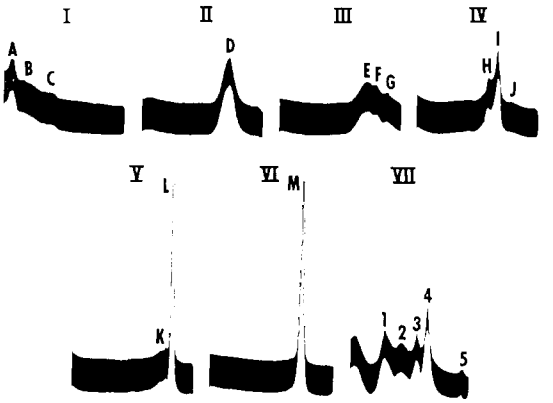

Fig. 4.—Electrophoresis patterns of chromatographic fractions from Fig. 3 and of the original whey. The symbols used for the pattern number and the components correspond to those given in Table I. Field strengths were adjusted to allow the migration of all fractions, except I, to continue the length of the cell in approximately 120 min. Only ascending patterns are shown. Direction of migration is to the right.

tion of components of whole whey by gradient elution is never as good as by stepwise elution. Probably it is more correct to assume that tailing in stepwise as well as gradient elutions is a consequence of an incomplete resolution of several proteins of similar physical properties such as in fractions III and IV. The establishment of differently shaped gradients (Varigrad device) on these fractions may greatly increase the degree of resolution.[22]

B. Electrophoresis Analysis.—The electrophoretic mobilities of the components in unfractionated whey, as well as those of the fractions obtained from Fig. 3, are given in Table I.

The data show that the 6 chromatographic fractions contain a total of 13 electrophoretic components having distinctly different mobilities in contrast to only 5 components in unfractionated whey. The electrophoretic patterns for these proteins are shown in Fig. 4. There are 3 components each in fractions I, III and IV, whereas fraction II appears to be essentially one component; however, from the ultracentrifuge data it appears that component D contains two proteins having widely different S_{20w} values. Except for the presence of a small component K in fraction V, both fraction V and VI exhibit a high degree of electrophoretic homogeneity. Even after 3 hours of back-compensation, components L and M remained symmetrical and no additional peaks were formed. The pattern for the whole whey (pattern VII) shows the presence of 4 major and 1 minor components. Rechromatography of fractions V and VI has no effect on their electrophoretic behavior or mobility values.

For a direct comparison between the mobilities of the components present in the 6 chromatographic fractions and those of the whole whey, the electrophoretic patterns were traced and projected along the mobility axis. By following the solid line, it can be seen that the 3 components in fraction I increase in mobility, but do not overlap with the component found in fraction II. The same is true for all of the other chromatographic fractions. This direct correlation between the increase in mobility values and their order of elution from the column indicates that ion exchange had the dominant role in this chromatographic fractionation. Under the conditions used for the column and electrophoresis, all of the proteins have a net negative charge. One would expect, therefore, that the component having the greatest anionic character would require a higher salt concentration for elution.

By following the dotted line which represents the pattern of whole whey, the first peak appears to have a mobility value which is intermediate to mobilities of the proteins found in fractions II and III. The same effect occurs with the other peaks as well. Electrophoresis of a mixture containing equal amounts of fraction V and VI gave only one highly symmetrical peak having a mobility value of 7.6 which is identical to the value for component 4 of the whole whey (Fig. 4) and which is the same component shown between fractions V and VI of Fig. 5. Yet, this mixture of fraction V and VI can be completely resolved upon rechromatography with either the stepwise or gradient elution procedure. With the addition of a calculated amount of fraction II to a solution of whole whey, it was found that the area of the pattern in the region of component 1 of pattern VII (Fig. 3) was increased after electrophoresis of this mixture. Additions of other fractions to whole whey solutions gave comparable results.

It appears that the presence of only 5 electrophoretic components in unfractionated whey is due to the interaction of protein components to form single peaks having intermediate mobility values.

C. Ultracentrifugal Analysis.—Results given in Table I show that the chromatographic components have s_{20w} values in the range of 0.99 to 6.14 *S* in contrast to only two resolvable peaks in the unfractionated whey corresponding to s_{20w} values of 1.75 and 6.13 *S*. The narrow distribution of sedi-

(22) E. A. Peterson and H. A. Sober, *Anal. Chem.*, **31**, 857 (1959).

mentation values accounts for the minimum number of sedimenting species in whole whey. The relative areas of resolvable peaks for each fraction also are given in Table I. Only fraction VI was homogeneous in both electrophoresis and ultracentrifugation.

D. Biochemical Characterization.—The distribution of various biologically active proteins in the effluent of Fig. 3 was examined (Table I). Acid phosphatase activity was distributed over a fairly wide area, having a twofold concentration of activity at its maximum in fraction III. The distribution of β-amylase activity was more narrow and its maximum activity also was in fraction III. Since fractions III and IV have an especially highly curved adsorption isotherm, the proteins present in these fractions would then have an extended elution range and their enzymatic activity would be widely distributed.

Even though some hemagglutinating activity was distributed in all of the fractions, 79 and 13% of the total activity applied to the column was recovered in fractions II and III, respectively. The observed activities were corrected with respect to an SBH standard, having an assumed activity of 7500 hemagglutinin units/mg. protein as defined by Liener.[15] The specific activity of fraction II is greater than the standard even though ultracentrifuge data indicate that the SBH did not exceed 79%. The s_{20w} value for SBH given in Table I agrees with the value of 6.15 for chromatographically pure SBH.[23] An electrophoretic mobility value of 2.66 was obtained for a highly purified SBH, which also agrees with the value obtained for fraction II. Using the data of Table I and a specific activity value of 9100, we calculated that SBH should account for nearly 19% of the protein found in fraction III. Electrophoretic mobility data and rechromatographic behavior of fraction III indicate that no SBH was present. The reason for this discrepancy was not further investigated, but the data presented would seem to indicate that there are two different components in soybean whey having hemagglutinin activity.

The elution of trypsin inhibitor activity occurred over a very narrow range. There were two maxima: one at fraction V with a specific activity of 1.28 and one at fraction VI with an activity of 1.00. About 99% of the activity applied to the column was recovered. All activity measurements were made against a SBTI (5X) standard.[19]

The gradient elution diagrams of SBTI A_1, A_2 and (5X) are shown in Fig. 2. In moving boundary electrophoresis, trypsin inhibitors A_2 and (5X) have identical mobilities in both the presence and absence of each other. Because a complex is formed which has an intermediate mobility value, SBTI A_1 and A_2 cannot be resolved electrophoretically. A mixture containing equal amounts of SBTI A_1 and A_2 can be resolved chromatographically to give elution diagrams identical to those shown in Fig. 2. These results indicate that two trypsin inhibitors of high purity were isolated directly from the whey and simultaneously from each other.

(23) S. Wada, M. J. Pallansch and I. E. Liener, *J. Biol. Chem.*, **233**, 395 (1958).

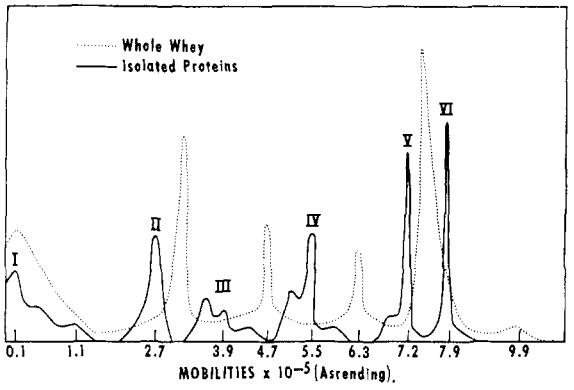

Fig. 5.—Direct comparison of the mobilities of the components found in the whole whey and the chromatographic fractions.

Discussion

DEAE-cellulose may be superior in many respects to free electrophoresis and ultracentrifugation as an analytical method for characterization of the complex protein system present in soybean whey. Chromatographic fractions with distinctly different mobilities may appear as single electrophoretic peaks when combined (Fig. 5).

The multiplicity of chromatographic components in relation to the apparent homogeneity of certain proteins in electrophoresis does not appear to be caused by artifacts arising from the chromatographic procedure. Since the advent of protein chromatography, evidence for the multiple nature of albumins, γ-globulins and other proteins has been accumulating.[10,21,24] One must conclude from the rechromatographic behavior of these proteins that a true separation has taken place and that these chromatographic methods have marked specificity for proteins.

The poor separation of phosphatase and β-amylase activities is in marked contrast to the good separation of an SBH and two trypsin inhibitors. Carboxymethyl cellulose appears useful for further purification of some of the proteins, particularly the phosphatase and components D to H (Fig. 1). It also was found from these preliminary experiments that there was very little correlation between electrophoretic and chromatographic behavior of the whey fractions of Fig. 3 when chromatographed on carboxymethyl cellulose. The order of elution was fractions III, V, IV, VI, II and I.

Usually, concentrated whey solutions are deeply colored solutions, which produce a series of colored bands on elution ranging from yellow-red to brown-black. Most of the brown-black color remains at the starting position even after the elution of fraction VI. The significance of these colored bands has not been investigated further.

Results to date have indicated that chromatography of whey proteins on cellulosic ion-exchange adsorbents can become the basis for the separation of this protein system into its individual components and that it is a valuable tool both for analytical and preparative purposes. Apparently

(24) M. B. Rhodes, P. R. Azari and R. E. Feeney, *ibid.*, **230**, 399 (1958).

no artifacts are formed since the rechromatographic behavior corresponds to what is expected if a real separation occurred. The electrophoretic mobility of fraction VI also remains unchanged after rechromatography on DEAE-cellulose.

Bowman[25,26] and Ham and Sandstedt[27] claim that soybeans contain at least three trypsin inhibitors on the basis of their solubility in alcohol, ammonium sulfate and trichloroacetic acid. Except for the isolation of a crystalline trypsin inhibitor by Kunitz,[28] all of these solutions containing antitryptic activity were very crude preparations. A more extensive chemical and enzymatic characterization of the whey fractions is being developed, with particular emphasis on the elucidation of the apparent presence of two trypsin inhibitors in soybean whey.

(25) D. E. Bowman, *Proc. Soc. Exptl. Biol. Med.*, **63**, 547 (1946).
(26) D. E. Bowman, *Arch. Biochem.*, **16**, 109 (1948).
(27) W. E. Ham and R. M. Sandstedt, *J. Biol. Chem.*, **154**, 505 (1944).
(28) M. Kunitz, *J. Gen. Physiol.*, **29**, 149 (1946).

Acknowledgments.—The authors are indebted to Dr. F. M. Mayer for the phosphatase and β-amylase determinations, Mr. A. M. Nash for the trypsin inhibitor assays, Mr. A. C. Eldridge for performing the hemagglutinin analyses, Mr. G. E. Babcock for carrying out the ultracentrifugal experiments and to Dr. I. E. Liener for a sample of SBH.

PEORIA, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, SCHOOL OF MEDICINE, YALE UNIVERSITY]

2-Selenobarbiturates. Studies of Some Analogous Oxygen, Sulfur and Selenium Compounds[1-3]

BY HENRY G. MAUTNER AND EDWIN M. CLAYTON[4]

RECEIVED JUNE 4, 1959

2-Selenobarbituric acid and several of its derivatives were prepared by the condensation of selenourea with appropriately substituted diethyl malonates. The changes in acid dissociation constants, ultraviolet spectra and water–lipid partitions when oxygen in the 2-position of barbiturates was replaced with sulfur and selenium were determined and compared with the changes seen when sulfur and selenium were substituted for oxygen in members of the uracil and hypoxanthine series.

One of the major problems in medicinal chemistry is the frequent lack of lipid solubility of otherwise useful agents, preventing them from penetrating the blood–brain barrier.[5] The usual method of increasing fat solubility in such cases is the addition of alkyl chains. Although the introduction of such groups usually results in the desired solubility in lipids, it also modifies the steric configuration of the molecule considerably and may interfere with the ability of the modified drug to attach itself to the receptor sites on which its action is exerted.

Another method of increasing lipid solubility is the replacement of oxygen by sulfur, the classical example of such fat solubilization being the replacement of oxygen in the 2-position of pentobarbital (a short-acting hypnotic) by sulfur to yield thiopental (an ultra-fast acting anesthetic).[6-8] Here, profound changes in pharmacological action are achieved with only negligible changes in the size and configuration of the molecule.

Since selenium is related to sulfur in the same way in which sulfur is related to oxygen, it was of interest to measure water-lipid partitions in several series of isologous oxygen, sulfur and selenium compounds in order to determine whether the effect of replacement of oxygen by sulfur on solubility in lipids is a more-or-less general one, and to learn what effect the replacement of sulfur by selenium would have. 2-Selenobarbituric acid and several of its derivatives were synthesized as part of this work.

2-Selenobarbituric acid was prepared by the condensation of selenourea with diethyl malonate in the presence of sodium ethoxide, similarly to the method used previously for the synthesis of 2-selenouracil.[9] 5-Phenyl-, 5-benzyl-, and 5-pentyl-2-selenobarbituric acid could be obtained by the condensation of selenourea and the appropriately substituted diethyl malonate in the presence of sodium methoxide.

All attempts to condense selenourea with diethyl diethyl malonate or with diethyl ethyl (2-methylbutyl)-malonate to obtain the selenium analogs of barbital and pentobarbital, respectively, have been unsuccessful; furthermore, these compounds could not be obtained by substituting magnesium ethoxide for sodium ethoxide. The only disubstituted selenobarbiturate which could be made is 5-phenyl-5-ethyl-2-selenobarbituric acid, the selenium analog of phenobarbital. It seems likely that the other disubstituted selenobarbiturates were too unstable to be isolated. Also, among the monosubstituted selenobarbiturates only the phenyl compound appeared to be stable, presumably due to resonance stabilization, while the pentyl compound decomposed to form a red sludge within two weeks, even when protected from light and stored in a vacuum desiccator.

(1) Presented before the Medicinal Chemistry Section at the American Chemical Society Meeting, Boston, Mass., April, 1959, p. 25-N.
(2) The material presented here is based on a thesis submitted by Edwin M. Clayton to the Yale University School of Medicine in partial fulfillment of the requirements for the M.D. degree.
(3) This work was supported, in part, by a grant (CY-3937) from the National Institutes of Health, Public Health Service.
(4) Research Fellow, summers of 1957 and 1958, Yale University Medical Student Research Program, supported by Public Health Service Training Grant, CRTY-5012.
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