

Soybean Trypsin Inhibitor: A Reference Protein for Gel Electrophoretic Studies of Soybean Proteins¹

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ABSTRACT

Earlier studies showed commercial samples of crystalline soybean trypsin inhibitor (SBTI) to be heterogeneous. An automated diethylaminoethyl (DEAE) cellulose chromatographic system was used to purify several commercial samples of SBTI. The SBTI's purified by column chromatography were analyzed by seven different gel electrophoretic procedures. The protein migrated as a characteristic, fast-moving band in most gel systems studied. SBTI can be used as a reference protein to calculate the relative mobilities of other protein bands in electrophoretic studies of soybean proteins.

Gel electrophoresis is generally carried out with starch (1) or polyacrylamide (2,3) as the supporting medium. Both starch and polyacrylamide gels have been used for electrophoretic analysis of soybean protein fractions (4 to 12). Since electrophoretic mobilities vary with conductivity, temperature, and other factors, it is difficult to obtain reproducible results between different analyses. A helpful adjunct to any electrophoretic study would be to include in the analysis a well-defined, readily available, homogeneous, fast-migrating protein as a reference. We have examined the properties of crystalline soybean trypsin inhibitor (SBTI) and conclude that it is a good reference protein for electrophoretic examinations of soybean protein.

MATERIALS AND METHODS

Samples

Several grades of crystalline SBTI were purchased from Sigma Chemical Corp., St. Louis, Mo.; California Corp. for Biochemical Research, Los Angeles, Calif.; Worthington Biochemical Corp., Freehold, N.J.; Mann Research Laboratories, New York, N.Y.; and Nutritional Biochemical Corp., Cleveland, Ohio.

Purification of SBTI

Initial column chromatographic fractionation of SBTI was performed by the methods of Rackis et al. (13) and Frattali and Steiner (12) on diethylaminoethyl (DEAE) cellulose columns; gradients used were as described by these authors. In the Rackis purification the starting buffer was 0.01M potassium phosphate, pH 7.6, the limit buffer being 0.01M potassium phosphate, pH 7.6, containing 0.3M NaCl. The gradient was from 0M NaCl to 0.3M NaCl in 1 liter. The Frattali and Steiner starting buffer was 0.05M NH₄OAc, pH 6.5. A 1-liter gradient was used with a limit buffer of 0.5M NH₄OAc, pH 5.0. Conductivity measurements of the column effluents showed that the gradients were essentially linear for each method.

After the gradients had been established, routine purifications were performed on a Beckman Spectrochrom Model 130 with a linear program cam and a Beckman

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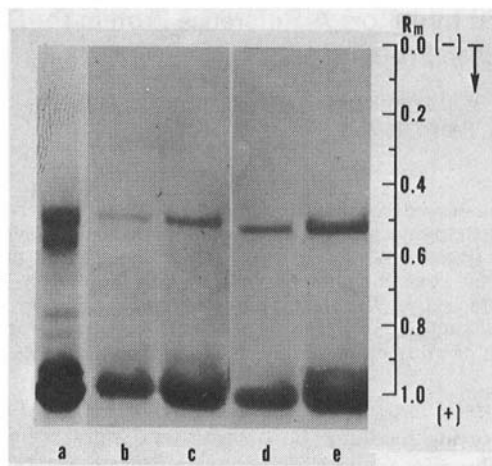


Fig. 1. Polyacrylamide gel electrophoretic patterns with 0.025M glycine, pH 9.2, and 8M urea (5,6). Sample a, commercial soybean trypsin inhibitor (SBTI); b and c, SBTI purified by Frattali and Steiner's procedure (12), 200 and 400 γ , respectively; d and e, SBTI purified according to Rackis et al. (13), 200 and 400 γ , respectively.

gradient pump Model 131. These routine procedures were identical with those described above except that a 3-liter gradient cam was used for the Frattali and Steiner procedure.

For purification of SBTI, columns (1.9 \times 24.0 cm.) were filled with buffer-equilibrated DEAE (Cellex D Bio-Rad Laboratories, Richmond, Calif.). Usually 200 mg. of SBTI was applied to the column in \sim 5 ml. of starting buffer. The protein sample was washed into the column with \sim 10 ml. of starting buffer; then the gradient was started.

After chromatography of SBTI, the protein in the center of the major peak from each elution was dialyzed against water for 1 week at 2°C., then freeze-dried. Purity of the freeze-dried proteins was determined by the polyacrylamide gel electrophoresis that we developed earlier (5,6).

Electrophoresis

Purified SBTI was evaluated as a reference protein in seven different electrophoretic procedures (5,7,9,10,11,12,14). In all tests, each procedure was reproduced as closely as possible; i.e., the same buffers and gels were used but not necessarily the same equipment. In all experiments we used a horizontal or vertical flat bed for the respective gels. Although all gels were stained with Amido Black 10B, solvent for the stain was as specified in each reference.

RESULTS

Gel electrophoretic patterns for commercial, crystalline SBTI before and after purification by chromatography are shown in Fig. 1. Typically, the protein applied to each column was heterogeneous (Fig. 1, a); after purification by either the Rackis (13) or the Frattali system (12), the protein appears to be fairly homogeneous according to patterns in Fig. 1, b and c and Fig. 1, d and e, respectively. The purified protein migrates as a major band which is arbitrarily

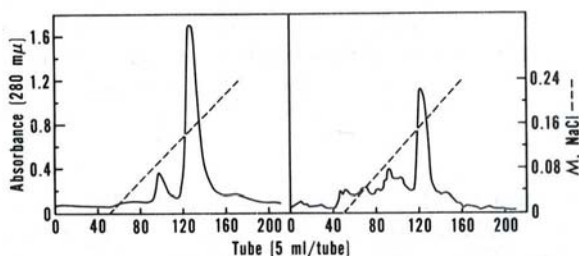


Fig. 2. Diagram of results from column chromatography of different lots of SBTI: Curve I, Sigma Lot 47B-0330; II, Nutritional Biochemical Lot 3097. A 1.9 X 24 cm. column and the buffers of Rackis et al. (13) were used.

assigned relative mobility (R_m) of 1.00. Minor bands also occur at R_m 0.48 to 0.52; the possible origin of these bands is discussed later.

Figure 2 contains elution curves obtained when crystalline SBTI from two sources was chromatographed with the buffers of Rackis et al. (13). The major peak in each sample eluted when the salt concentration reached ~ 0.16 M NaCl. Similar elution curves were obtained when crystalline SBTI from other sources was chromatographed. The major component in each elution diagram corresponded to the electrophoretic band assigned an R_m of 1.00 (Fig. 1). Presumably this protein is identical with Kunitz's trypsin inhibitor (15), Frattali and Steiner's inhibitor F_2 (12), and to Rackis's inhibitor SBTI-A₂ (13).

Homogeneity and mobility of the purified SBTI were examined by seven electrophoretic systems. In four of these systems polyacrylamide gel was the support; in three, starch was used. Figure 3 shows the results when SBTI was examined by the seven procedures.

The purified SBTI appears to be relatively homogeneous in each electrophoretic system studied except Fig. 3, a and b. As is apparent in Fig. 3, a, and also in Fig. 1,

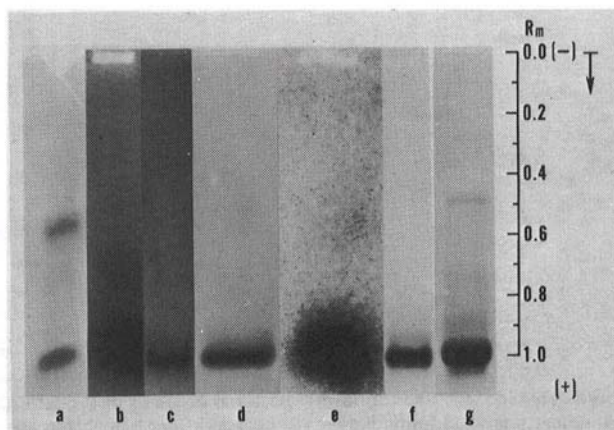


Fig. 3. Gel electrophoretic patterns of purified SBTI: a and b, Alkaline- and acid-buffered starch gels, respectively, of Puski and Melnychyn (11); c, polyacrylamide gel of Tombs (10); d, polyacrylamide gel of Catsimpoilas et al. (9); e, starch gel according to Shibasaki and Okubo (7); f and g, polyacrylamide gels according to Frattali and Steiner (12) and Eldridge et al. (5).

components at $R_m \sim 0.5$ are observed in alkaline gels that contain urea. The origin of these slow-moving components has been investigated. If SBTI is dissolved in freshly prepared 0.025M glycine, pH 9.2, containing 8M urea and immediately analyzed, the bands at $R_m \sim 0.5$ are minimal (Fig. 4, a). If the protein is allowed to stand in the buffer containing urea for 2 to 3 days at room temperature, the band at $R_m 1.00$ diminishes in intensity and the bands at $R_m 0.48$ to 0.52 (Fig. 4, b) become more prominent. After standing 1 week under the same conditions, nearly all the SBTI that originally traveled at $R_m 1.00$ has been modified and has a mobility of 0.48 to 0.52 (Fig. 4, c).

The decrease in R_m of SBTI on standing in 8M urea is similar to changes noted when the disulfide bonds in the protein are reduced or reduced-alkylated (6). Since cleavage of the disulfide bonds in SBTI also causes an increase in the intrinsic viscosity of the protein (6), we measured viscosities of SBTI after various storage times in 8M urea. Freshly prepared SBTI in buffer containing urea had an intrinsic viscosity of ~ 0.06 ; however, after it had stood 1 week we observed an intrinsic viscosity of 0.22. This high value suggests that the SBTI molecule has lost its tertiary structure because of some modification. It is noteworthy that the value of 0.22 is similar to that found earlier for either the reduced or the reduced-alkylated SBTI (6) when the protein was dissolved in buffer containing urea.

Apparently the modification of SBTI which is occurring cannot proceed in the absence of urea, since a control sample without urea showed no change in mobility after standing 1 week.

Figure 5 shows the use of purified SBTI as a marker or reference protein. For this analysis, samples were applied to the gel as isolated; no SBTI was added to the samples. It is apparent that the water-extractable, the acid-precipitated, and the whey proteins contain SBTI which can serve as an internal reference. It has been demonstrated that SBTI migrates as a well-defined, fast-moving band in most gel

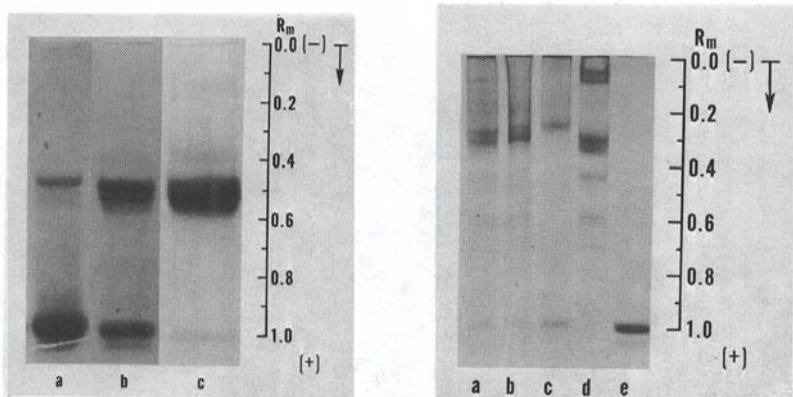


Fig. 4 (left). Polyacrylamide gel electrophoretic patterns of SBTI in 0.025M glycine, pH 9.2, with 8M urea. Sample a analyzed immediately after preparation; b analyzed after 3 days; and c analyzed after 1 week.

Fig. 5 (right). Polyacrylamide gel electrophoresis of soybean proteins according to the procedure of Eldridge et al. (5). Sample a, water-extractable proteins; b, acid-precipitated proteins; c, whey proteins; d, cold-insoluble fraction (CIF); e, purified SBTI.

electrophoresis systems; thus if a value of 1.00 is assigned to SBTI any protein band in a mixture can be designated relative to SBTI by its R_m value. This technique compensates for variations in temperature, conductivity, and other variables in different analyses. For example, the 0.28 band in soybean whey protein or the 0.60 and 0.62 bands in the cold-insoluble fraction (CIF) are easily designated. Bands with slight differences in mobility can be differentiated as seen in the water-extractable protein sample.

DISCUSSION

We have examined the properties of SBTI, which is available commercially, and found it well suited as a reference protein for gel electrophoresis. As purchased, most lots of SBTI are heterogeneous. To eliminate uncertainty about identity of the R_m 1.00 band, we prefer to purify commercial samples by anion-exchange chromatography. Purification of commercial SBTI is relatively easy by either the procedure of Frattali and Steiner (12) or the method of Rackis et al. (13). The purity of SBTI prepared by either chromatographic procedure does not differ greatly (Fig. 1).

In the electrophoretic patterns given in Fig. 3, SBTI migrates rapidly and forms a characteristic band in most of the gel and buffer systems. Therefore, SBTI should serve as a good protein marker. The proposed marker also appears fairly homogeneous in each electrophoretic protein pattern. Rather diffuse bands were observed when SBTI was analyzed on starch gels (Fig. 3, a, b, and e), possibly because of our limited experience with this gel medium. Other workers (7,11), however, have used starch gels and obtained good separations of a variety of soybean protein fractions such as the water-extractable and the acid-precipitated proteins which also contain SBTI.

In alkaline buffer systems containing 8M urea, bands at $R_m \sim 0.5$ are present. Since the concentration of the R_m 0.48 to 0.52 bands increases with time, all SBTI samples should be analyzed immediately after they are dissolved in alkaline buffers containing urea. The exact nature of the modification occurring is not known. Since urea is necessary for modification of SBTI, there is a possibility that cyanate may have formed from the urea and reacted with the protein (16). If amino groups in the protein reacted with cyanate, the modified form should be less positively charged and should migrate faster in alkaline gels than the unmodified protein (17). The urea-modified SBTI, however, migrates slower than the native protein; hence, carbamylation of amino groups by reaction with cyanate seems unlikely. It should be noted, however, that relative mobility and intrinsic viscosity of the modified SBTI and reduced-alkylated SBTI agree closely. The similarity of the modified protein to SBTI in which the disulfides are cleaved suggests that alkaline hydrolysis of the cystine cross-linkages occurs at pH 9.2 in 8M urea. Swelling of the protein molecule by urea may make the disulfide bonds more accessible to hydroxyl ions than in the absence of urea.

If a mobility of 1.00 is assigned to SBTI as we proposed earlier (5), then all other bands present in soybean proteins can be designated relative to this value. This system is similar to that proposed by Wake and Baldwin (18) for milk proteins. The use of SBTI as a marker protein is exemplified in Fig. 5. Any band present can be easily located in relation to SBTI. Figure 5 also shows that several crude soybean protein fractions contain SBTI. Such fractions can be used as a source of reference

protein in routine analyses after the crude samples are compared with purified SBTI.

It should be emphasized that relative mobilities may change with variations in experimental conditions such as pH, buffer, and the concentrations of urea and gel medium. The conditions of analysis must therefore be held constant when this reference system is used. However, we do not want to imply that our conditions for gel electrophoresis are ideal. Figure 5 shows that streaking and overlapping of bands is still a serious problem with several fractions of soybean proteins. Further work is needed to find conditions which give better separations, but use of SBTI as a marker protein should be helpful in describing results obtained with any gel system.

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