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Isoelectric Focusing in Narrow pH Gradients of Kunitz and Bowman-Birk Soybean Trypsin Inhibitors

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Summary

The two distinct soybean trypsin inhibitors, namely Kunitz and Bowman-Birk inhibitors, were isolated from various commercial sources by isoelectric focusing in the narrow pH 3–6 range in sucrose gradients. The Kunitz inhibitor was also isolated by the same procedure from an aqueous soybean water extract subjected to preparative gel filtration on Sephadex G-25. The Kunitz inhibitor was focused at pH 4.5, and the Bowman-Birk inhibitor at pH 4.3. The two purified proteins were found to be immunochemically different. Disc electrofocusing on polyacrylamide gels in the pH 3–10 range was shown to be a simple and high resolution method for detection of heterogeneous preparations of the two inhibitors.

INTRODUCTION

Previous communications from this laboratory (*1–4*) have demonstrated the utility of isoelectric focusing methods (*5–7*) in the separation and isolation of biologically active proteins present in soybean seeds (*Glycine max*). These relatively new techniques allow the fractionation and determination of isoelectric points of macromolecular ampholytes by focusing into isoelectric zones in stable, natural pH gradients. Convection is avoided either by using sucrose density gradients (*5,6*) or by the formation of a polyacrylamide gel matrix (*7*). Isolation of Kunitz soybean trypsin inhibitor by electrofocusing in the broad pH 3–10 range has been reported previously (*3*). Since

remarkable resolution of bioactive soybean protein components was achieved by using narrow pH range ampholytes (*1,2,8,9*), the potential of using these techniques for improved isolation of the two distinct trypsin inhibitors—Kunitz and Bowman-Birk—present in soybeans (*10–12*) became apparent.

The present report describes the isolation of Kunitz inhibitor from soybean water extracts and various crude commercial preparations by isoelectric focusing between pH 3.0 and 6.0. Similarly, the Bowman-Birk inhibitor was isolated from a crude commercial preparation. The two inhibitors can be distinguished isoelectrically both in sucrose gradients and in polyacrylamide gel matrices. A new gel filtration procedure was also developed for the large-scale preparation of the pH 4.5 soluble proteins of soybean cotyledons which may be used as a starting material for the isolation of other biologically active soybean proteins.

MATERIALS AND METHODS

Materials

Crude Kunitz soybean trypsin inhibitor in the form of acetone powder (SIC, lot 8CA) was obtained from Worthington Biochemical Corp., Freehold, N.J. This preparation was designated as Worthington SIC. A preparation of Kunitz soybean trypsin inhibitor obtained according to the procedure of Rackis et al. (*13*) was purchased from Mann Research Laboratories, New York, N.Y. (lot T4901) and was designated as Mann SI. Crude Bowman-Birk soybean trypsin inhibitor (lot 8011) was obtained from Miles Laboratories, Elkhart, Ind. This inhibitor preparation was designated as Miles SI. Carrier ampholytes were purchased from LKB Instruments, Inc., Rockville, Md.

The pH 4.5 soluble soybean proteins were prepared from defatted soybean flakes by aqueous extraction using procedures described previously (*4*). Soybean flakes (100 g) were suspended in 1 liter of water and stirred for 1 hr at room temperature. The slurry was strained through Miracloth (Calbiochem) and adjusted to pH 4.5 with 2 *N* HCl. After centrifugation at 10 000 *g* for 15 min, the precipitate was discarded. The pH of the supernatant was brought to 8.0 with 2 *N* NaOH solution and allowed to stand at 5° overnight. After centrifugation, this yielded 750 ml of clear yellow protein solution which was used directly for the gel filtration experiments.

Preparative Gel Filtration

Large-scale preparation of the pH 4.5 soluble soybean proteins was carried out by using the specially constructed Sephadex K100/100 column (10 × 100 cm) packed with Sephadex G-25 fine (Farmacia Fine Chemicals, Inc., Piscataway, N.J.). The column was pre-equilibrated and eluted with pH 7.3 phosphate buffer (0.02 *M*). The column was developed upward at a flow rate of 200 ml/hr. The temperature was maintained at 10° by circulating water through the jacket of the column. The protein solution (750 ml), prepared as described above, was introduced into the column and elution was performed with the described buffer. The absorbance of the eluates was recorded at 254 and 280 nm with LKB Uvicord I and Uvicord II units. The first 2300 ml of eluates showed no significant absorbance and were discarded. The next 1100 ml constituted the first major peak and were retained. The protein of this fraction was precipitated by full saturation with ammonium sulfate. The precipitate was redissolved in water and dialyzed against water (at 4°) for 48 hr. The freeze-dried material weighed 1.4 g and was designated as Fraction I. Subsequent fractions gave no precipitate by ammonium sulfate saturation.

Isoelectric Focusing

The principle of this method (*5,6*) and its application to the separation of biologically active soybean proteins (*1–3,9*) have been described. A 440-ml capacity electrofocusing column (No. 8102, LKB Instruments) was used for these experiments. The carrier ampholyte (Ampholine, LKB) was selected to give a pH gradient between pH 3 and 6. The density gradient was obtained with sucrose as described by LKB Instruments (*14*). The sample was prepared by dissolving the appropriate amount of the inhibitor preparation in the solution of fractions Nos. 23 through 25 of the density gradient followed by centrifugation to remove insoluble material. The following amounts of inhibitor were used: Worthington SIC, 70 mg; Mann SI, 50 mg; Miles SI, 50 mg; mixture of Mann SI and Miles SI, 50 mg of each; and gel filtration Fraction I, 150 mg. The anode electrolyte solution was placed at the bottom of the column and the cathode solution at the top. Electrofocusing was performed for 16 hr with a final potential of 500 V (at 10°) using a constant voltage power supply. Draining of the contents of the column was performed slowly. Fractions (3.0 ml)

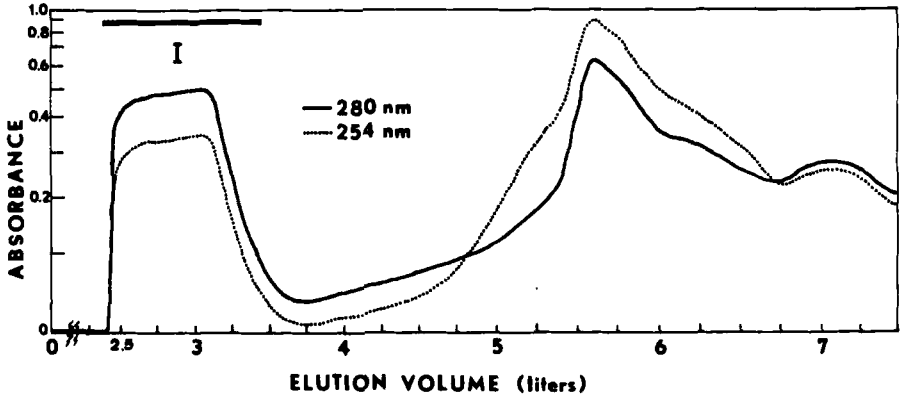

FIG. 1. Preparative gel filtration (Sephadex G-25) elution pattern of the pH 4.5 soluble soybean proteins using the Pharmacia K100/100 column: Solid line, absorbance at 280 nm; dotted line, absorbance at 254 nm. Solid bar at the upper part of the figure indicates fractions pooled.

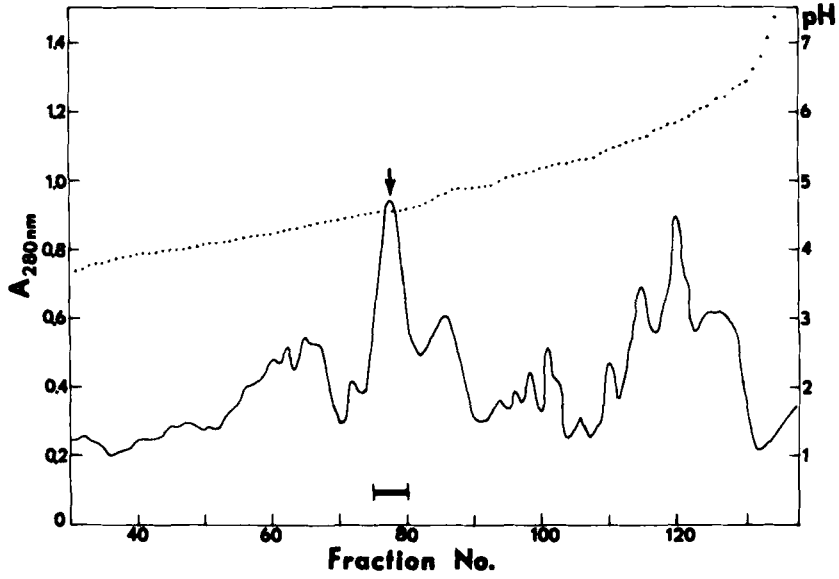

FIG. 2. Isoelectric focusing of preparative gel filtration Fraction I in the region between pH 3 and 6: solid line, absorbance at 280 nm (1-cm cell); dotted line, pH gradient (25°); solid bar, fractions pooled.

were collected and the absorbance of each fraction at 280 nm was determined using a 1-cm cell with a Gilford 2000 spectrophotometer. The pH of each fraction was measured at 25° immediately after collection. Storage of the fractions may result in deviation of the measured pH value by as much as 0.2 pH unit. Pooled fractions were dialyzed against several changes of water at 5° to remove sucrose and ampholytes and then freeze-dried. Alternatively ampholytes were removed by Sephadex G-25 (fine) gel filtration. Trypsin inhibitory activity was determined by the method of Kunitz (*10*). Disc electrofocusing in polyacrylamide gel columns was performed as described previously (*7*). The gels were photographed after standing for 1 hr in 12% trichloroacetic acid. Staining did not show additional bands.

RESULTS

Isolation of Kunitz Inhibitor from Soybean Extracts and Commercial Sources

The aqueous extract of defatted soybean flakes after precipitation of the reserve globulins at pH 4.5 exhibited the elution pattern shown

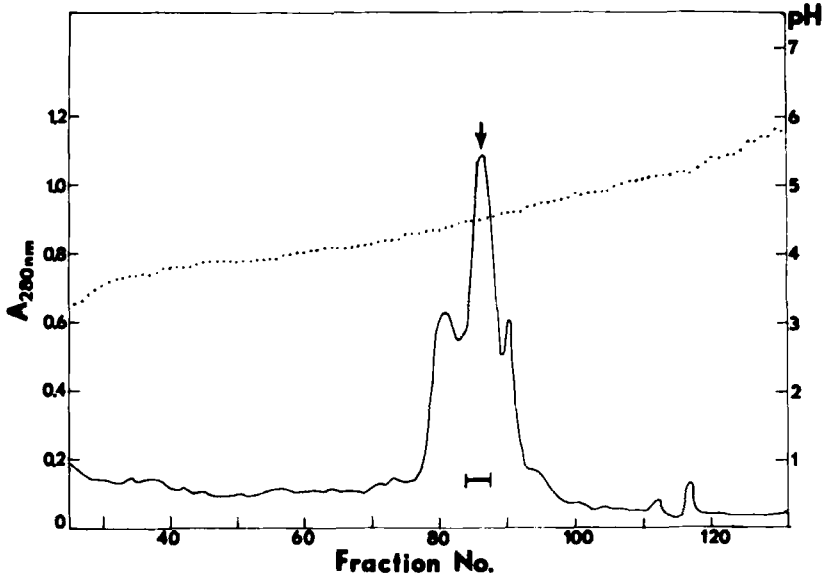

FIG. 3. Isoelectric focusing of Worthington SIC crude Kunitz inhibitor in the pH 3–6 region: solid line, absorbance at 280 nm (1-cm cell); dotted line, pH gradient (25°); solid bar, fractions pooled.

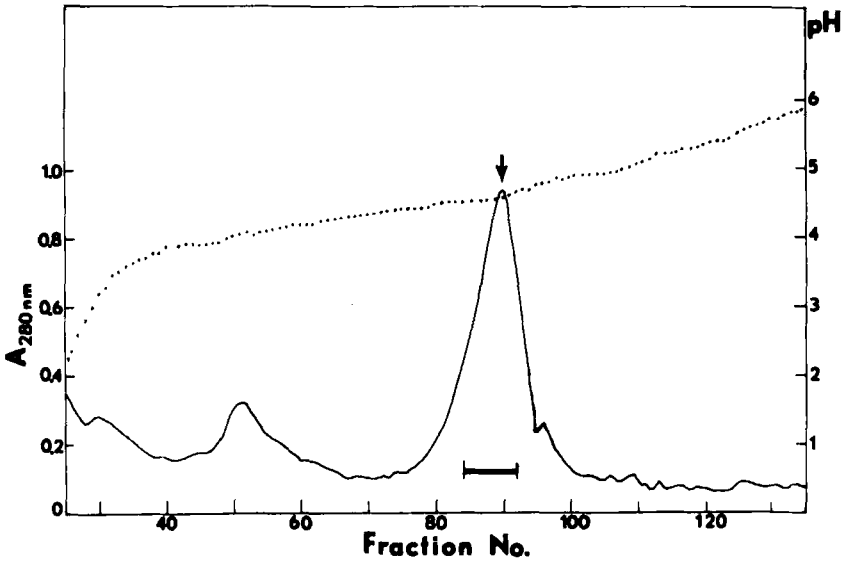

FIG. 4. Isoelectric focusing of Mann SI crude Kunitz inhibitor in the pH 3–6 region: solid line, absorbance at 280 nm (1-cm cell); dotted line, pH gradient (25°); solid bar, fractions pooled.

in Fig. 1 when subjected to preparative gel filtration through Sephadex G-25. Most of the protein was eluted in Fraction I as judged by ammonium sulfate precipitation and the higher 280/254 nm absorbance ratio of the elution curve. Isoelectric focusing of Fraction I in the pH 3–6 range showed a remarkable resolution of the protein components present. The Kunitz inhibitor was focused at the position indicated by the arrow (Fig. 2) and was identified immunochemically (*3,9,15*) and by trypsin inhibition tests (*10*). This protein constituted the major component of the gel filtration Fraction I. The mean value of its isoelectric point was found to be at pH 4.5, which is consistent with previously reported results (*3,10*).

Isoelectric focusing patterns in the pH 3–6 range of two commercial preparations of the Kunitz trypsin inhibitor, namely, Worthington SIC and Mann SI, are shown in Figs. 3 and 4. The commercial preparations were heavily contaminated with other protein components. However, the Kunitz inhibitor was isolated in homogeneous form by isoelectric focusing as judged immunochemically (*3,9*) and by the disc electrofocusing method which is discussed below.

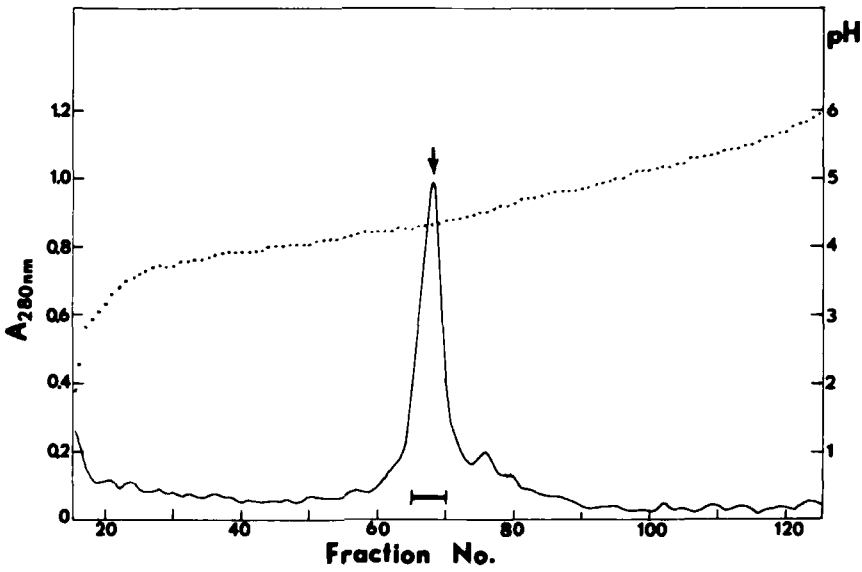

FIG. 5. Isoelectric focusing of Miles SI crude Bowman-Birk inhibitor in the pH 3–6 region: solid line, absorbance at 280 nm (1-cm cell); dotted line, pH gradient (25°); solid bar, fractions pooled.

Isolation of Bowman-Birk Inhibitor from a Commercial Source

The isoelectric focusing pattern of Miles SI preparation in the pH 3–6 range is shown in Fig. 5. The Bowman-Birk inhibitor was focused as a well-defined peak with a mean isoelectric point value at pH 4.3. The commercial preparation contained several impurities which were eliminated by electrofocusing. One of the contaminating components gave a positive immunochemical reaction with anti-Kunitz soybean trypsin inhibitor serum as judged by quantitative precipitation and double gel immunodiffusion (*9*). The purified-by-isoelectric-focusing Bowman-Birk inhibitor did not react immunochemically with anti-Kunitz inhibitor serum. This observation also indicated that the Bowman-Birk and Kunitz inhibitors are immunochemically different.

In order to test the degree of resolution obtained in the separation of the two distinct soybean trypsin inhibitors by isoelectric focusing, a mixture of Mann SI and Miles SI preparations were focused in the pH 3–6 range. The results are shown in Fig. 6. The Bowman-Birk

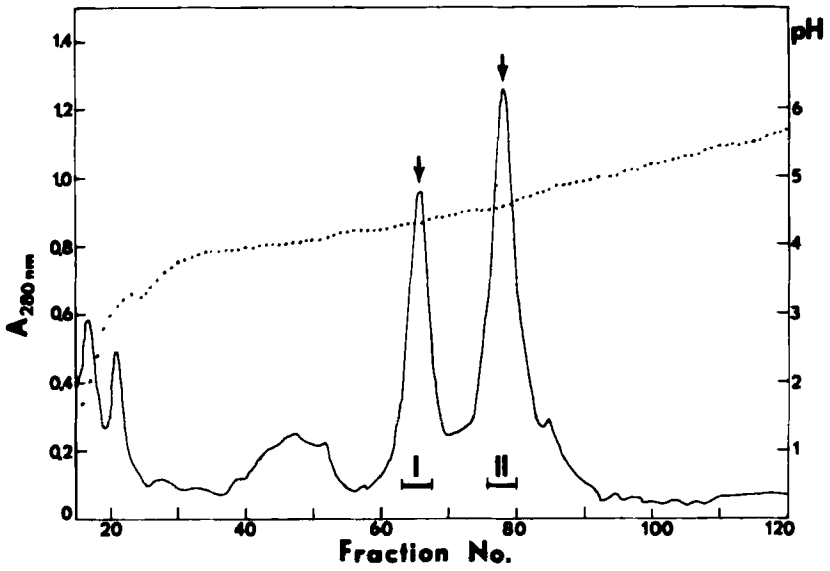

FIG. 6. Isoelectric focusing of a mixture of Mann SI and Miles SI crude inhibitor preparations in the pH 3–6 region: Solid line, absorbance at 280 nm (1-cm cell); dotted line, pH gradient (25°); solid bars, fractions pooled.

inhibitor (I) was clearly separated from the Kunitz inhibitor (II). Thus, the isoelectric focusing method appears to offer definite advantages in the separation of the two inhibitors.

Disc Electrofocusing

Disc electrofocusing patterns on polyacrylamide gels in the pH 3–10 range of commercial and purified soybean trypsin inhibitors are shown in Fig. 7. In accordance with the results obtained by isoelectric focusing in sucrose gradients, all the commercial preparations were found to be contaminated by other protein components. The position of the Kunitz and Bowman-Birk inhibitor bands are indicated by the letters K and BB, respectively. The isolated Kunitz and Bowman-Birk inhibitors formed only one band by this technique. Because of its simplicity, the disc electrofocusing method may be used as a high resolution criterion of purity of isolated inhibitors. Test of commercial samples by this method may reveal the degree of contamination since several reports have appeared in the scientific literature using these preparations. The validity of the results obtained may be seriously questioned depending on the nature of the problem.

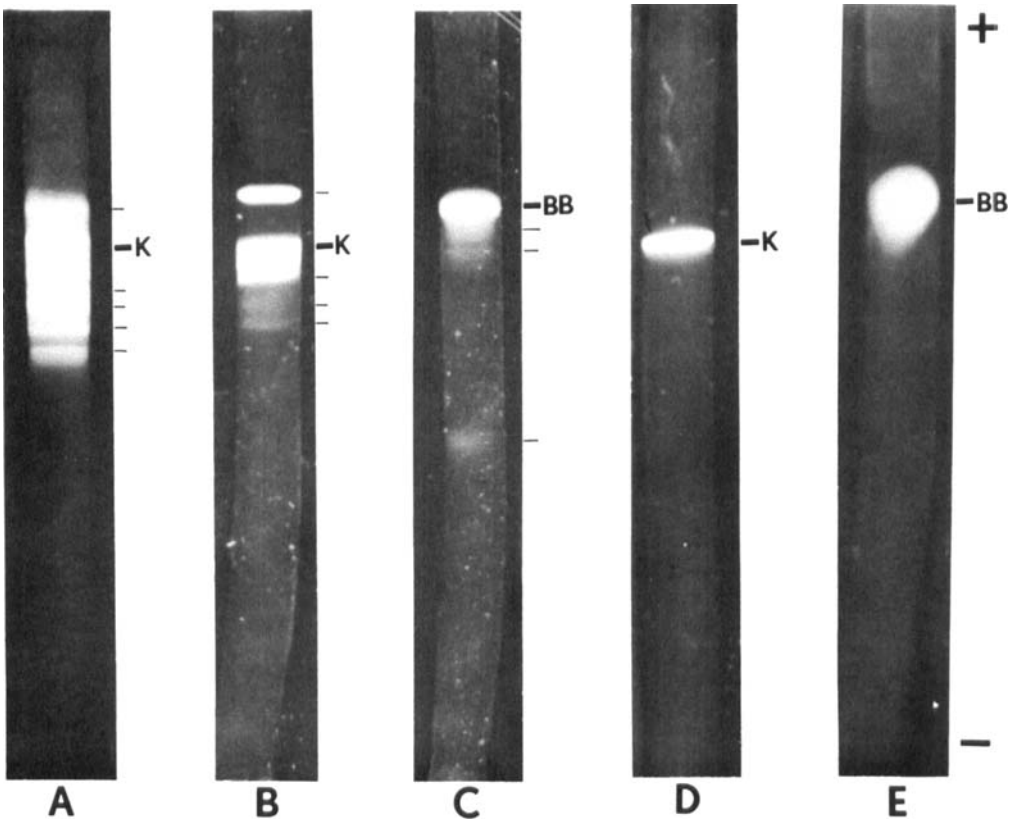

FIG. 7. Disc electrofocusing on polyacrylamide gels in the pH 3–10 region of: A, Worthington SIC inhibitor; B, Mann SI inhibitor; C, Miles SI inhibitor; D, purified by isoelectric focusing Kunitz soybean trypsin inhibitor; and E, purified by isoelectric focusing Bowman-Birk soybean trypsin inhibitor. K, Kunitz inhibitor, and BB, Bowman-Birk inhibitor.

DISCUSSION

Methods for purification of the Kunitz and Bowman-Birk soybean trypsin inhibitors involve either repeated cellulose ion-exchange chromatography using complex gradient systems (*12,16–18*) or preparative polyacrylamide gel electrophoresis (*19*). The isoelectric focusing method for the isolation of the Kunitz inhibitor was introduced recently by using broad pH range ampholytes (*3*), and was extended in these studies to include the Bowman-Birk inhibitor. The utilization of narrow pH gradients offers a definite improvement in the isolation

of the two trypsin inhibitors from various sources. In addition, the use of the analytical disc electrofocusing technique presents a sensitive tool for rapid screening of commercial or other samples for purity.

The main advantage of the isoelectric focusing method are high resolution in the separation of components, excellent reproducibility, and direct determination of the isoelectric points of isolated components at zero ionic strength. Furthermore, sizeable quantities of proteins (30 to 50 mg or higher) can be obtained in an one-step fractionation procedure. The main disadvantage of this technique is the extensive dialysis required for effective removal of sucrose and ampholytes from the isolated fractions.

The isoelectric focusing method in narrow pH gradients may be useful in the isolation of other inhibitors which reportedly are present in soybean seeds (*16,18*).

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