Isoelectric Point Differences in Commercial Soybean Trypsin Inhibitors[1]

L. C. WANG, Northern Regional Research Laboratory, Peoria, Illinois 61604

ABSTRACT

Isoelectric points of Kunitz' crystalline soybean trypsin inhibitor (STI), Bowman-Birk STI, and STI prepared by chromatography on diethylaminoethyl (DEAE)-cellulose were investigated by isoelectric focusing, gel filtration, ion-exchange resin treatment, solubility test, and titration. Results suggest that the STI of Kunitz may have an isoelectric point below pH 4.5. The isoelectric focusing pattern of the Kunitz inhibitor showed three peaks: at pH 3.5, 3.7, and 4.4. All three proteins were active inhibitors. Chromatography on anion-exchange resins separated the Kunitz STI into two fractions: one that eluted with water and focused at pH 3.5 and another that eluted with increasing concentrations of NaCl solution and focused at pH 4.0. When ion-retardation resins were used only one peak resulted, focusing at pH 3.8. Solubility tests on the Kunitz preparation before and after treatment with anion-exchange resins supported evidence for different isoelectric points. Titration of Kunitz' STI at three levels of ionic strength gave an isoionic zone lower than pH 4.5. The Kunitz inhibitor contained about 20% impurities based on gel filtration and the purified protein focused at a single isoelectric pH of 4.0. The Bowman-Birk inhibitor consistently showed an isoelectric point of pH 4.2 in focusing and solubility tests, and it had little or no impurities on gel filtration. Although STI prepared by chromatography on DEAE-cellulose contained impurities, heterogeneity of isoelectric point (pH 4.0) was less than for Kunitz' STI. The multiple isoelectric points of crystalline STI were believed caused by interaction between protein and other constituents, possibly contaminants.

Soybean trypsin inhibitor (STI) has been studied extensively. Since Kunitz (1) initially isolated and purified the inhibitor from soybeans, various chromatographic procedures have been applied to improve the isolation (2–6). At present, several purified proteins from soybeans, available commercially, are known to inhibit trypsin activity (7), although some of these preparations are believed to be impure (4,8). In 1969, Steiner and Frattali (7) summarized the purification and properties of STI.

Kunitz reported the inhibitor as a globulin with a distinct minimum solubility at its isoelectric point of pH 4.5 (9). The isoelectric pH was established by solubility test and by cataphoretic mobility. Birk et al. (3) recorded pH 4.2 as the isoelectric point of the Bowman-Birk inhibitor as determined by solubility. Apparently these inhibitors are two different proteins with different isoelectric points (7). In an isoelectric focusing pattern, the Kunitz STI showed at least three protein peaks (10). Furthermore, during focusing this crystalline preparation yielded white precipitates, often observed in handling whey proteins. Attempts to remove these white precipitates led to a shift in isoelectric focusing pattern. Solubility tests on

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crystalline preparations before and after treatment with anion-exchange resins gave two different isoelectric points.

Literature is limited on isoelectric point investigations. Isoelectric focusing of Kunitz' inhibitor isolated by focusing technique has been reported (6,11), but the changes of isoelectric point of proteins have not. This paper extends the study of isoelectric focusing on STI as follows: a) Kunitz' STI peaked at three different pH values with all proteins as active inhibitors and b) inhibitors prepared by chromatography focused below pH 4.5.

MATERIALS AND METHODS

Materials

Three STIs differing according to their method of preparation were purchased: Kunitz' inhibitor (lot No. 119B-0940) from Sigma Chemical Co., St. Louis, Mo.; Bowman-Birk inhibitor (lot No. 39-011) from Miles Laboratory, Inc., Elkhart, Ind.; and STI chromatographed with diethylaminoethyl (DEAE)-cellulose (lot No. v-1219) from Mann Research Laboratory, Inc., New York, N.Y. Sephadex G-75 (lot No. 9676, med. size) was bought from Pharmacia-Uppsala, Sweden. Analytical-grade ion-retardation resins (AG-11A8, lot No. 3690, 50 to 100 mesh) and Dowex-1 (X8, 200 to 400 mesh, chloride form) were obtained from Calbiochem, Los Angeles, Calif. Reagent-grade chemicals were used, and their solutions were prepared with distilled and deionized water.

Isoelectric Focusing

An LKB 8102 electrofocusing column of 440 ml. capacity and carrier ampholytes of pH ranges 3 to 6 were purchased from the LKB Instruments, Inc., Rockville, Md. Detailed procedures were followed as previously described (10). The pH values of the protein solution were checked before running an electrofocusing separation and were adjusted to 7.0 with 0.1N NaOH. Focusing results were obtained in duplicate after the prescribed voltage (10) was applied for 24 or 72 hr. The longer duration improved the pattern without changing the focusing pH.

Chromatography of STI Preparations

Gel Filtration. Sephadex G-75 column chromatography was used to determine purity of the STI (12). The conditions were slightly modified to fit the current study. The column (1.5 × 70 cm.), preequilibrated with 0.05M Tris-malate buffer (pH 7.0), was charged with 15 to 100 mg. STI in the same buffer (5 to 10 ml.) and eluted at a rate of 25 ml. per hr. The effluent was monitored at 280 nm., and fractions of 4.0 to 5.0 ml. were collected. The fractions in desired peak areas were pooled, and their proteins dialyzed against cold distilled water (3 liters) for at least 2 to 4 hr. before focusing.

Anionic Exchange. Dowex-1 column chromatography separated the Kunitz STI into two fractions having different isoelectric points. The column (2 × 20 cm.), filled with resin in chloride form, was loaded with 100 mg. protein in 10 ml. water and eluted with distilled water until the absorbance at 280 nm. reached nearly zero. The column was then eluted with a sodium chloride gradient from 0 to 1.0M. The gradient was formed by adding dropwise 2M NaCl solution (250 ml.) into 250 ml. water in a 500-ml. conical flask with magnetic stirring. Proteins were separated into

peaks and collected in 5-ml. fractions. The fractions in desired peak areas were pooled. The proteins eluted with water were focused after the pH (5.5) was adjusted to neutral. Proteins eluted with salt gradient were dialyzed and then focused.

Ion-Retardation. Ion-retardation resins were used to exchange inorganic ions in protein with H^+- and OH^--form ions and to eliminate any anion effect that might be present in anion-exchange resins. The detailed procedure was similar to anion-exchange chromatography with water elution. The column (2 X 22 cm.) was filled with resins AG-11A and preeluted with deionized water. The protein solution after treatment with resins had a pH about 5.0, and pH adjustment was made before focusing.

Analyses

Amino Acid Analysis. Amino acid composition of three fractions separated by focusing Kunitz' STI was determined by the method of Moore et al. (13) to check possible differences among fractions. Ampholine contained in the sample was removed by dialysis against six batches of distilled water (3 liters) at 4°C. The protein was lyophilized, weighed, and hydrolyzed for 24 hr. with 6N HCl. The number of amino acid residues per 21,500 g. of protein was calculated and compared with published results.

Trypsin Inhibition Activity. Activity in the three fractions obtained by electrofocusing of the Kunitz inhibitor was measured by the rate of hydrolysis of benzoyl-L-arginine ethyl ester hydrochloride (BAEE) according to the method described by Wu and Scheraga (14). The activity was expressed as a ratio to that of the original crystalline preparation.

Solubility Test

Isoelectric points of Kunitz' preparation and a fraction from the same preparation not retained on the Dowex-1 column were obtained by a solubility test. The detailed procedure followed was that given by Kunitz (9). Samples of 0.1 ml. of a stock suspension of 10 mg. of STI per ml. water at pH 6.0 were added to 10 ml. of 0.02M acetate buffers of varied pH. For the sample not retained on the Dowex-1 column, the protein solution was concentrated by adding a calculated amount of dry Sephadex G-25 and then centrifuging. The protein concentration was determined by absorbance measurement at 280 nm. on the basis of the factor $E_{1\,cm.}^{1\%}$ = 7.50. The pH of the solution was adjusted to neutral before use. After the solution had stood for several hours at room temperature, pH was measured with a Beckman Zeromatic meter and turbidity with a Beckman spectrophotometer at 600 nm.

pH Titration

An automatic assembly of Radiometer type TTT1c, Titrigram SBR2c, and combined glass and calomel electrodes was used to obtain titration curves of Kunitz' STI. Since the protein showed the same isoionic zone at different ionic strengths of the solution, solutions of 50 mg. of STI in 10 ml. of first 0.25, then 0.50, and finally 1.00M KCl were brought to pH 2.5 by addition of 1N HCl, and then titrated with 0.3175N KOH with automatic recording. Similar titrations were
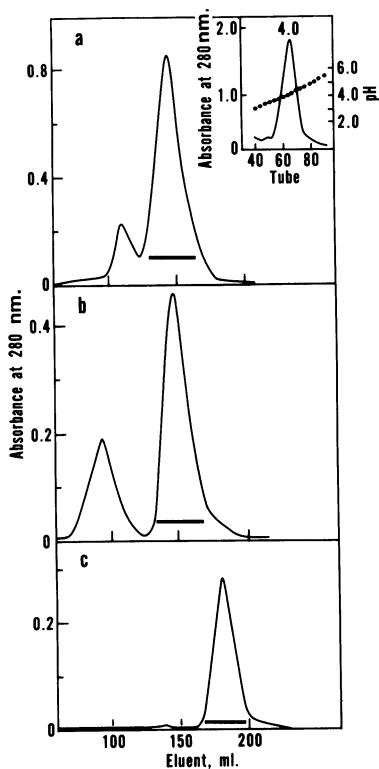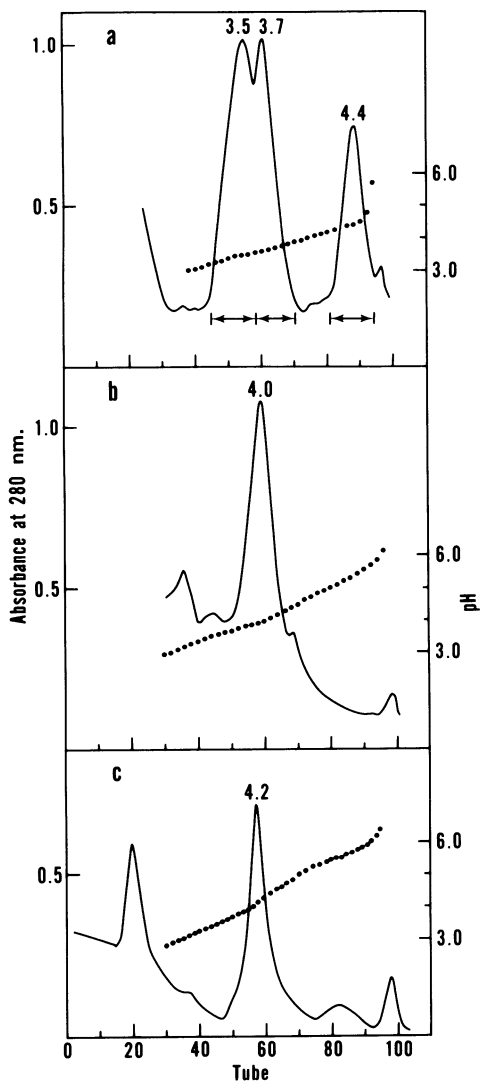
Fig. 1 (left). Isoelectric focusing of soybean trypsin inhibitors (STI). a) Crystalline preparation of Kunitz, b) STI prepared by chromatography on diethylaminoethyl (DEAE)-cellulose, and c) Bowman-Birk inhibitor. Solid line represents the protein concentration as measured by absorbance at 280 nm. Open circle lines represent the pH gradient. Solid bars show the pH range of the fractions pooled.

Fig. 2 (right). Sephadex G-75 gel filtration of STI; the bar indicates the portion of protein used in focusing as in Fig. 1. a) The Kunitz STI (insert indicates the focusing pattern), b) STI prepared by chromatography on DEAE-cellulose, and c) Bowman-Birk inhibitor.

carried out on the solvent, and the titration curve represents the difference between sample and solvent (14). Results were duplicated, with precaution being taken to adjust the initial pH. An expanding scale (0.2 pH per cm. setting on Titrigraph) was used to determine isoionic zone in a narrow pH range.

RESULTS

Isoelectric Points of STIs by Focusing

Isoelectric focusing separated Kunitz' STI into three predominate peaks in the range of pH 3 to 6 (Fig. 1, a). The pattern is compared with results for the Bowman-Birk STI and STI chromatographed on DEAE-cellusose (Fig. 1, b and c). The latter two inhibitors gave more homogeneous focusing patterns than the Kunitz inhibitor and showed no peaks at pH 4.5. The Bowman-Birk STI focused at pH 4.2, a value which agreed well with existing data (3,11). STI chromatographed on DEAE-cellulose focused at pH 4.0. Results from other tests substantiated that the isoelectric pH of Kunitz' STI might be lower than pH 4.5.

Table I tabulates an analysis for amino acids and trypsin inhibition activity of Kunitz' STI, focused and separated into three fractions as in Fig. 1, a. Practically no difference in amino acid composition and inhibitor activity existed among the fractions. Apparently all three fractions were active inhibitors with different isoelectric pH values.

TABLE I.   ANALYSES OF AMINO ACID COMPOSITION AND TRYPSIN
INHIBITOR ACTIVITY ON THREE FRACTIONS AFTER FOCUSING KUNITZ' STI[a]

Amino Acids	Fractions			Kunitz' STI[b]
	I	II	III	
	Residues/21,500 g. STI			
Lysine	11	11	11	11
Histidine	1	1	2	2
Arginine	9	9	9	9
Aspartic	27	27	29	29
Threonine	7	7	7	8
Serine	10	10	11	13
Glutamic	20	19	22	21
Proline	11	11	16	10
Glycine	16	16	17	18
Alanine	8	8	9	9
1/2 Cystine	3	2	4	4
Valine	13	13	13	12
Methionine	2	2	1	3
Isoleucine	14	14	14	14
Leucine	14	14	15	16
Tyrosine	4	4	4	4
Phenylalanine	9	8	9	9
Recovery	92.2%	99.9%	106.5%	
Relative activity[c]	0.897	0.978	0.989	

[a]STI, soybean trypsin inhibitor.

[b]Values reported by Wu and Scheraga (14).

[c]Ratio of activity of fraction to activity of original crystalline STI.

Focusing After Chromatography of the Protein

Gel Filtration . Pure STI is a single protein and should focus at one isoelectric pH. Although Kunitz' STI focused at three isoelectric pH values, the multiple focusing peaks might be caused by the presence of impurities (8,16). Gel filtration has been used to purify STI (12). Gel-filtrated protein should have an improved focusing pattern. Results in Fig. 2, a indicate that after gel filtration, the bulk of the protein in Kunitz' STI focused in a peak at pH 4.0 (Fig. 2, a, insert). The separated 20% of the protein was considered as impurities and not investigated further. Even though the focusing pattern was improved in respect to homogeneity, the peak at the pH 4.5 region was notably absent. STI chromatographed on DEAE-cellulose also contained more than 20% impurities when examined on Sephadex G-75 (Fig. 2, b). However, the focusing peak after purification was the same as Fig. 1, b. The Bowman-Birk STI was pure on the basis of gel filtration and gave the same focusing pattern before and after filtration.

Anion-Exchange Column Chromatography. The bulk of the Kunitz inhibitor was eluted from a Dowex-1 column with water. Only about 20% of the protein was eluted with a NaCl gradient. Figure 3, a indicates the separation and focusing results. The water-eluted protein focused mainly at pH 3.5, with a shoulder at 3.7 and a small peak at pH 4.6 (Fig. 3, b); the protein that eluted with NaCl focused at pH 4.0 without a peak at pH 4.5 (Fig. 3, c). Possibly the STI in the original preparation was impure, and focused at pH 4.5 as an apparent isoelectric point. Chromatographic treatment of the protein improved the purity and lowered the isoelectric pH. Although there was no single focusing peak on protein treated with Dowex-1, all predominant isoelectric pH values were lower than 4.5.

Ion-Retardation. Figure 4 shows the focusing pattern of Kunitz' STI after treatment with ion-retardation resins. The protein focused largely at pH 3.8 with a small peak at pH 5.0. The fraction at pH 5.0 contained a small amount of protein (judged by absorbance at 280 nm.) along with some white precipitate. Ion-retardation resin treatment exchanged inorganic ions for H^+ and OH^- in protein. This treatment should not alter the true isoelectric pH of the protein.

Isoelectric Point by Solubility Test

Kunitz used solubility and cataphoretic mobility to establish an isoelectric point of pH 4.5 on his crystalline preparation (9). Birk reported an isoelectric pH of 4.2 for the Bowman-Birk inhibitor based on solubility (3). Since a cataphoretic apparatus was not readily available, solubility tests were used to check Kunitz' preparation before and after treatment with ion-exchange resins (Table II). Results for STI chromatographed on DEAE-cellulose are also included in Table II. The isoelectric pH 4.5 of Kunitz' STI agreed with previous data (9). However, when the STI was chromatographed, the isoelectric pH shifted nearer to pH 4.12. STI chromatographed on DEAE-cellulose likewise showed pH 4.12 as the point of least solubility. These findings confirm that the isoelectric pH of Kunitz' STI is lower than 4.5 after treatment with ion-exchange resins, as noted earlier (Figs. 2 and 3).

Isoelectric Point by Titration

Tanford and Havenstein (15) titrated ribonuclease at three different ionic strengths and found the intersect of titration curves to be close to the isoelectric pH
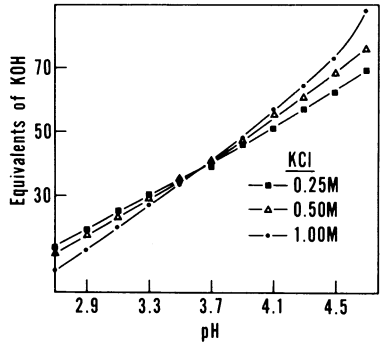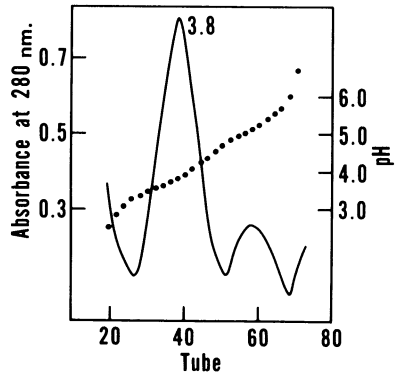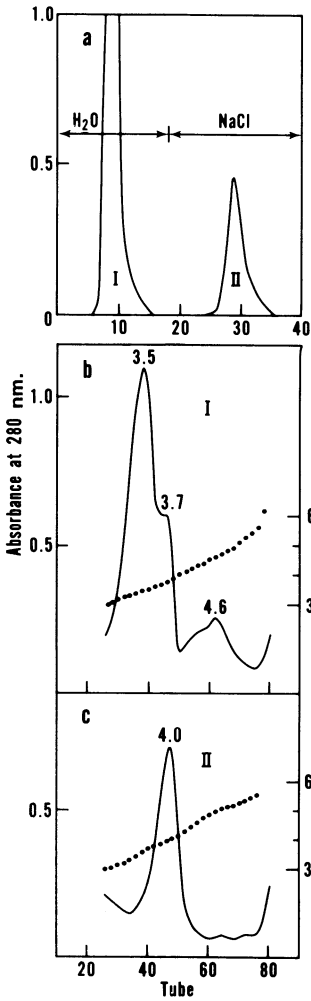
Fig. 3 (left). Elution and focusing patterns of the Kunitz STI after chromatography on Dowex-1 resin in chloride form. a) Chromatographic elution pattern, b) focusing pattern of protein eluted with water, and c) focusing pattern of protein eluted with salt gradient.

Fig. 4 (upper right). Focusing pattern of Kunitz' STI after treatment with ion-retardation resin AG-11A8.

Fig. 5 (lower right). Titration curve of Kunitz inhibitor. With 0.2 pH increments (0.2 pH per cm. setting on a Titrigraph); squares, STI in 0.25; triangles, 0.50; and circles, 1.0M KCl.

TABLE II.   ISOELECTRIC POINTS OF STIs AS MEASURED BY MINIMUM
SOLUBILITIES AT DIFFERENT pH VALUES

Inhibitor	pH	Turbidity Measured at 600 nm.
Crystalline preparation of Kunitz	3.52	0.013
	3.71	0.015
	3.92	0.060
	4.10	0.120
	4.31	0.185
	4.50	0.230
	4.70	0.220
Kunitz' crystalline preparation passed through		
Dowex-1 column	3.40	0.010
	3.58	0.012
	3.72	0.015
	3.92	0.020
	4.12	0.030
	4.30	0.015
	4.50	0.012
Preparation from chromatography on diethylaminoethyl-		
cellulose column	3.55	0.008
	3.70	0.013
	3.89	0.020
	4.12	0.040
	4.30	0.020
	4.50	0.012
	4.70	0.010

and isoionic zone of the protein. Kunitz' STI solutions in 0.3, 0.5, and 1M KCl all had the same pH, and it was assumed that there was no chloride binding (14). Because the protein should have the same isoelectric pH in different ionic strength solutions, the intersect of titration curves of Kunitz' STI will be close to its isoelectric pH.

Figure 5 presents further electrochemical aspects of Kunitz' STI. Titration of this protein in a single ionic strength (1M KCl at 25°C.) with 1N KOH gave a smooth sigmoid curve between pH 2 and 11, as reported by Wu and Scheraga (14). The curve in the whole pH range was shown to be reversible and was plotted after several runs. When a lower strength of KOH was used and ionic strength of solutions were varied, similar curves intersected near a pH of 3.7. With an expanding pH scale (0.2 pH per cm. setting on Titrigraph), the curves between pH 2.7 and 4.7 showed an isoionic zone at pH 3.7 to 3.9. Thus the true isoelectric pH of STI may be close to pH 4.0 instead of pH 4.5.

DISCUSSION

Recognizing that the isoelectric pH of STI is closer to 4.0 than to 4.5 is significant in improving the isolation of minor protein constitutents in soybeans. The bulk of water-soluble soybean protein precipitates at pH 4.5 (17) and about 6.5% protein remains soluble. Soybean whey protein is soluble at pH 4.5 and contains STI. In fact STI may be isolated and purified from soybean whey (5,6). If pH 4.0 is closer to the true isoelectric pH of STI than pH 4.5, precipitating STI at pH 4.0 may favor its removal from whey proteins. Separation of remaining whey proteins may be simplified.

In this study there are discrepancies in the value of the true isoelectric pH of STI. For instance, the Kunitz STI focused at pH 4.0 after gel filtration, 3.8 after ion-retardation resin treatment, and 3.5 and 4.0 after chromatographing with a Dowex-1 column; had a minimum solubility at pH 4.12; and on titration yielded an isoionic zone at pH 3.7 to 3.9. The fact has been established that commercial preparations of Kunitz' STI are impure (8,16). STI was also found to be one of the better proteins to complex with iron to form iron-sulfur protein complex (18). Data presented here from chromatography with ion-retardation resins support the view of STI complexing with ions. These complexes could differ and give variation in the isoelectric pH.

Soybean proteins have a common point of minimum solubility at pH 4.2 when the pH is adjusted with hydrochloric, trichloroacetic, oxalic, sulfuric, and phosphoric acids (19). In the presence of salt the ability of acids to precipitate soybean proteins decreases. The minimum solubility point broadens out as the salt concentration is increased, and practically disappears in 0.5N calcium chloride. Thus salts affect the solubility of soybean proteins. Salt or impurities in STI, or both, might also affect its minimum solubility. The different titration curves of STI in various salt concentrations exemplify this effect (Fig. 5, a). Kunitz dissolved STI in a salt solution, and the isoelectric point of pH 4.5 for his crystalline STI may be an apparent value because of impurities. More investigations are under way, particularly in the area of impurities that may occur in STI.

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