Screening of Heat-Stable Trypsin Inhibitors in Dry Beans and Their Partial Purification from Great Northern Beans (*Phaseolus vulgaris*) Using Anhydrotrypsin-Sepharose Affinity Chromatography

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Nine dry bean cultivars (*Phaseolus vulgaris*) showed evidence of heat-stable trypsin inhibitory activity (TIA). TIA exhibited minimum heat stability in the whole intact bean matrix, medium heat stability in the dry bean flour, and maximum heat stability in the extracted bean albumin. The boiling of soaked, whole intact beans for 30 min resulted in 2.5–5% retention of TIA when compared to that of nonboiled, soaked, whole intact beans. Heating extracted bean albumin in a boiling water bath for 30 min resulted in 97–100% retention of TIA when compared to that of the unheated albumin. There was no effect of storage (4 °C for 20 h) on the level of TIA in the extract of heated bean samples. The purification of heat-stable trypsin inhibitors from Great Northern beans was performed using solubility in salt/water, DE-52 cellulose, phenyl-Sepharose, and affinity chromatography on anhydrotrypsin-Sepharose. The apparent molecular weight of the pool of inhibitors was approximately 16 000 as estimated by gel filtration and approximately 18 000 as estimated by SDS-PAGE. Eleven isolated trypsin isoinhibitors with pI values of approximately pH 4–5 were observed by trypsin inhibitor staining of an isoelectric focusing gel. Some of the 11 isoinhibitors that exhibited trypsin inhibitory activity also exhibited chymotrypsin inhibitory activity. Isoelectric focusing of albumin extracts from nine dry bean cultivars revealed differences between the varieties in the amounts of individual trypsin inhibitors present.

INTRODUCTION

Numerous studies of dry beans (*Phaseolus vulgaris*) have noted the presence of heat-stable trypsin inhibitors (TIs). Questions have been raised about their possible adverse effects with regard to protein digestibility and nutritive value of dry beans (Friedman and Gumbmann, 1986; Deshpande and Nielsen, 1987b). In studies of heat-stable TIs in dry bean cultivars, it has been reported that 50–80% of the original trypsin inhibitor activity (TIA) is retained after various heat treatments (Wagner and Riehm, 1967; Pusztai, 1968; Elias et al., 1976; Miyoshi et al., 1978; Jacob and Pattabiraman, 1986; Deshpande and Nielsen, 1987b). These studies suggest that dry been cultivars differ in their level of heat-stable TIA, but no study has made comparisons under identical conditions.

The main concern with residual protease inhibitor activity in dry beans is their affinity for human anionic trypsin. Lima bean trypsin inhibitor (Bowman–Birk family) severely inhibits both human and bovine trypsin (Feeney et al., 1969). The inhibition of human anionic trypsin by lima bean trypsin inhibitor is reportedly somewhat stronger than that of human cationic trypsin (Mallory and Travis, 1975). However, it was later reported that human trypsin appears less sensitive than rat trypsin to soybean trypsin and chymotrypsin inhibitors (Temler et al., 1983). Temler et al. (1983) reported that the Bowman–Birk inhibitor in soybean forms a very weak interaction with human cationic trypsin.

The molecular weight (MV) of the dry bean TIs is in the range 8000–23 000 depending on the cultivar investigated and the method of molecular weight estimation (Pusztai, 1968; Wagner and Riehm, 1967; Belitz et al., 1972; Wilson and Laskowski, 1973; Mosolov and Fedurkina, 1974, 1978; Wang, 1975; Whitley and Bowman, 1975; Wilson and Laskowski, 1975; Mosolov et al., 1976, 1977; Miyoshi et al., 1978; Gomes et al., 1979; Sgarbieri and Whitaker, 1981; Whitaker and Sgarbieri, 1981; Jacob and Pattabiraman, 1986; Wu and Whitaker, 1990). The reported number of trypsin isoinhibitors has ranged from four to six in *Phaseolus lunatus* (lima beans) (Jones et al., 1963; Haynes and Feeney, 1967), four in *Vicia faba* (broad beans) (Warsy et al., 1974), and from two to six in *P. vulgaris* (Bowman, 1971; Belitz et al., 1972; Wilson and Laskowski, 1973; Wang, 1975; Whitley and Bowman, 1975; Gerstenberg et al., 1980; Whitaker and Sgarbieri, 1981; Jacob and Pattabiraman, 1986; Wu and Whitaker, 1990). The high Cys content of TIs is important due to the low content of sulfur-containing amino acids in the storage proteins (Pusztai, 1968; Harms-Ringdahl et al., 1979; Chang and Satterlee, 1981). Moreover, the large number of disulfide bonds reported in trypsin inhibitors from *P. vulgaris* (i.e., no free SH groups) and the high amount of half-cystine (14 residues) (Wilson and Laskowski, 1973; Odani and Ikenaka, 1977) contribute to the heat stability of TIs (Birk, 1976), as demonstrated by Friedman and Gumbmann (1986).

Many techniques have been used to isolate TIs from dry beans, including trypsin affinity chromatography. While very specific, this technique has the potential to modify the trypsin inhibitors by proteolysis. This problem is essentially eliminated using anhydrotrypsin, a derivative of trypsin in which the active site serine has been converted to dehydroalanine. Similar to anhydrochymotrypsin, anhydrotrypsin lacks catalytic activity but retains its ability to bind stoichiometrically to trypsin inhibitors (Weiner et al., 1966; Ako et al., 1972). Anhydrotrypsin-Sepharose affinity chromatography has recently been employed to isolate trypsin inhibitors from soybeans (Pusztai et al., 1988, 1991). It is of interest to compare the results of dry

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bean trypsin inhibitors isolated with anhydrotrypsin–Sepharose to reports in the literature using trypsin–Sepharose (Wu and Whitaker, 1990) and trypsin–agarose (Gomes et al., 1979).

The first objective of this study was to screen the heat stability of TIA in the whole intact bean matrix, in the ground bean flour, and in the albumin extract of nine cultivars of dry beans from the United States. The second objective was to purify the heat-stable trypsin isoinhibitors from Great Northern beans for further studies, using a variety of chromatographic techniques including anhydrotrypsin–Sepharose affinity chromatography.

MATERIALS AND METHODS

Materials and Reagents. The U.S. dry bean (*P. vulgaris*) cultivars were a gift from Roger Brothers Seed Co., Boise, ID: Viva pink, pinto, dark red kidney, Black Beauty, light red kidney, Sanilac navy, small white, small red, and Great Northern.

DE-52 (diethylaminoethyl)cellulose was purchased from Whatman, Ltd., Hillsboro, OR. Trypsin (bovine pancreas, TPCK treated), α-chymotrypsin (from bovine pancreas), *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), DNP–glycine, aprotinin, cytochrome *c*, carbonic anhydrase, bovine serum albumin, blue dextran, Coomassie Blue (G-250 and R-250), *N*-acetyl-D,L-phenylalanine β-naphthyl ester, and bicinchoninic acid (BCA) were purchased from Sigma Chemical Co., St. Louis, MO. Phenyl-Sepharose CL-4B, Sephadex G-50, and ampholines were purchased from Pharmacia LKB, Piscataway, NJ. Fast blue B salt was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. All other chemicals used were of reagent grade.

Isolation of Crude Albumins. Beans were ground in a Udy Mill (Tecator, Model 1000-2016) using a 0.4-mm mesh screen. Albumin and globulin proteins were extracted using a 1:10 ratio (w/v) of flour:2% NaCl solution (Deshpande and Nielsen, 1987a,b). Samples were stirred on a magnetic stir plate for 4 h in a cold room (4 °C) and clarified by centrifugation at 5000*g* for 30 min at 4 °C. The pellet was reextracted at a 1:5 ratio (w:v) of flour:2% NaCl stirred for 1 h at 4 °C and centrifuged as above. Supernatants were pooled, filtered through glass wool, and dialyzed (MW cutoff 3500) extensively against deionized distilled water (DDW). Insoluble globulin proteins were removed by centrifugation at 5000*g* for 30 min at 4 °C. The supernatant containing albumin proteins was freeze-dried and stored at −20 °C until analyzed.

Screening for Heat-Stable Trypsin Inhibitory Activity (TIA). Nine cultivars of dry beans were screened for heat-stable TIA in the whole intact bean matrix, ground bean flour, and bean albumin extract.

Preparation of Boiled Intact Beans for TIA Assay. Dry beans (5 g) were added in triplicate to both 500-mL flat-bottom round flasks and 250-mL glass beakers. One hundred milliliters of DDW was then added to each container. The flasks and beakers were sealed and held overnight (16 h) at 4 °C. The contents of flasks containing the soaked beans in the original soaking water were boiled for 30 min (timed from the start of a rolling boil) on hot plates. The come up time to reach a rolling boil was approximately 10 min. Cold water condensers (55 cm long) were attached to the flasks (ground glass fittings) to prevent loss of water during heating. Immediately following boiling, the flasks and contents were cooled in an ice water bath. The contents of the flasks were transferred to 250-mL glass beakers in preparation for homogenization.

Homogenization of both the unheated (raw) and heated (boiled) soaked beans (both equilibrated to room temperature) was accomplished using a high-shear Polytron (Brinkman Instruments Co., Westbury, NY), Model PT 10/35, using a small probe (PTA 105) at medium speed. Before homogenization, the beans were cut into smaller pieces with a metal spatula to facilitate homogenization. Raw and boiled beans were homogenized in their original soak water for 3 min. Any unhomogenized beans remaining were further cut into smaller pieces, and the entire mixture was homogenized for an additional 1 min.

The homogenized bean solutions were centrifuged at 15000*g*, 20 °C, for 30 min. The resulting pellets were discarded, and the supernatants were used to assay for TIA as given below. After the boiled bean samples were assayed for TIA, these samples were stored at 4 °C overnight (20 h) and reassayed for TIA. The protein content of all supernatants was determined using the BCA method of Smith et al. (1985) using bovine serum albumin as the standard.

Preparation of Boiled Ground Bean Flour for TIA Assay. To assay for TIA in the raw flour, ground bean flour (0.5 g) and 20 mL of DDW were added to screw-cap test tubes (20 × 150 mm) in triplicate. The flour was dispersed in the water, and then the samples were homogenized with a high-shear Brinkman Polytron using a small probe (PTA 105) at medium speed for 1 min. The homogenized samples were centrifuged at 10000*g* for 30 min. The pellets were discarded, and the supernatants were assayed for TIA.

Heat stability of the TIA in bean flour was measured by placing 0.5 g of ground dry bean flour plus 10 mL of DDW into screw-cap test tubes (25 × 150 mm) in triplicate. The flour was dispersed in the water, and the tubes were tightly capped and placed into a boiling water bath for 30 min. Immediately following boiling, the tubes were cooled in an ice water bath to room temperature. Ten milliliters of DDW was then added to each tube, bringing the total volume of water in the tubes to 20 mL. The samples were then homogenized and centrifuged, and the supernatants were assayed for TIA, all as described above. Following TIA analysis of the boiled samples, these same samples were stored at 4 °C overnight (20 h) and then reassayed for TIA. The protein content of all supernatants was determined using the BCA method of Smith et al. (1985) using bovine serum albumin as the standard.

Preparation of Dry Roasted Ground Bean Flour for TIA Assay. Ground bean flour (0.5 g) was weighed (in triplicate) into disposable aluminum weigh pans (6 cm diameter × 1.5 cm high) and spread to a thickness of approximately 0.2 cm in the center of each pan. The weigh pans containing samples were centered on an aluminum tray (26.5 × 38.5 × 2 cm) in a randomized complete block distribution and subjected to dry roasting at 200 °C for 5 min in a forced air convection oven (Model DK-42, American Scientific Products, McGraw Park, IL). Following heating, the samples were cooled to room temperature, quantitatively transferred to scintillation vials, and stored at −20 °C until analyzed for residual TIA.

Both heated and raw bean flour were analyzed for TIA by placing 0.5 g of flour and 20 mL of DDW into large screw-cap test tubes (25 × 150 mm). The flour was dispersed in the water, and then the samples were homogenized with a high-shear Brinkman Polytron using a small probe (PTA 105) at medium speed for 30 s. The homogenized samples were centrifuged at 10000*g* for 30 min. The pellets were discarded, and the supernatants were assayed for TIA.

Preparation of Bean Albumin Extracts for TIA Assay. To screen for heat-stable TIA in the albumin extracts of nine bean cultivars, 50 mg of each albumin sample was dissolved in 50 mL of DDW in triplicate; 4-mL aliquots of the albumin solutions were then transferred to screw-cap test tubes, and the tubes were capped tightly and placed in a boiling water bath for 30 min. Following heat treatment, the tubes were cooled immediately to room temperature in an ice water bath and then centrifuged at 10000*g* for 30 min at 20 °C. Both the unheated and boiled albumin samples were assayed for TIA. The heat-treated albumin samples were stored at 4 °C for 20 h and then reassayed for TIA.

Trypsin Inhibitor Activity (TIA) Assay. TIA was determined with a spectrophotometric assay, using the trypsin substrate BAPNA according to a modification of the method by Kakade et al. (1974). The total sample volume and the reagent volumes used in the assay were decreased proportionally to yield a final volume of 2 mL instead of a final volume of 10 mL. Seed, flour, and albumin sample aliquots were chosen to result in a 40–60% inhibition of trypsin activity compared to a control containing no sample, as recommended by Kakade et al. (1974). Following the addition of trypsin to all tubes, the tubes were incubated for 10 min at 37 °C before addition of the BAPNA solution. The addition of the reagents to the tubes during the assay was timed at 10-s intervals to ensure that all of the tubes were incubated for the same time period.

One unit of trypsin activity (TA) was defined as an increase of 0.001 absorbance unit at 410 nm of the reaction mixture under assay conditions.

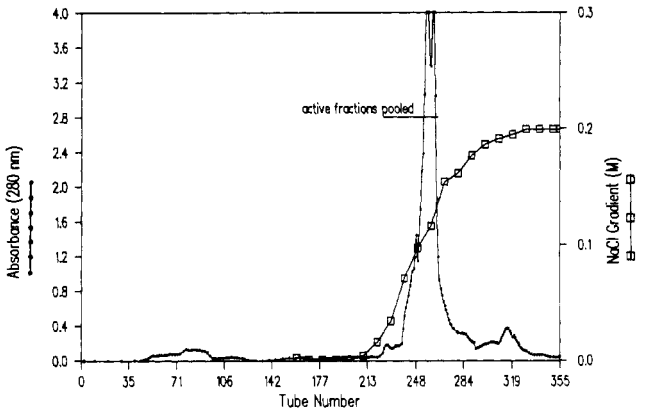

Figure 1. Anion-exchange chromatography of heated (75 °C for 15 min) albumin extract of Great Northern beans. A DE-52 cellulose column (73 × 3.5 cm) was equilibrated with 20 mM Tris buffer, pH 8.1, containing 20 mM $CaCl_2$, and eluted with a linear gradient (total Volume 1 L, 0–0.3 M NaCl) in the same buffer. Fraction size, 4.5 mL; flow rate, 40 mL/h.

One unit of trypsin inhibitor activity (TIA) was defined as a 10% decrease in the number of units of TA under assay conditions. By this definition, a 50% decrease in units of TA (i.e., 50% inhibition of TA) would equal 5 units of TIA.

Statistical Analysis. Statistical analysis was performed using SAS and the general linear model (GLM) procedure with a paired-comparison *t*-test for boiled vs boiled and stored samples (SAS Institute Inc., Cary, NC).

Chromatography Purification Steps. Chromatographic separations were carried out at 4 °C. Dialysis tubing (Spectrum Medical Industries, Inc., Houston, TX) used on samples had a MW cutoff of 3500.

Anion Exchange. The sample for application to the anion-exchange column was prepared by dissolving the Great Northern bean albumin extract in 20 mM Tris buffer, pH 8.1, at a 1:50 ratio (w/v). The sample solution was then heated in a water bath at 75 °C for 15 min and cooled immediately to room temperature in an ice water bath. Separation of precipitated protein was done by centrifugation at 20000*g* for 1 h at 4 °C, followed by filtration through Whatman No. 1 filter paper (Whatman). The supernatant was loaded onto a DE-52 cellulose column, 73 × 3.5 cm. Unbound material was eluted with 20 mM Tris and 20 mM $CaCl_2$, pH 8.1 (buffer A). Bound proteins were eluted with a linear gradient (1 L total), 0–0.3 M NaCl in buffer A. Fractions of 4.5 mL/tube were collected using a flow rate of 40 mL/h. The salt gradient was monitored by conductivity using a Model 31 YSI conductivity bridge (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). Tubes with TIA were pooled as indicated in Figure 1.

Hydrophobic Interaction. The TIA pool from DE-52 cellulose was brought to 25% ammonium sulfate (w/v) and 20 mM sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$), and adjusted to pH 7.0 (buffer B). The sample was chromatographed on a phenyl-Sepharose CL-4B column, 17 × 35 cm. Unbound proteins were eluted with buffer B. Bound proteins were eluted with a linear gradient (1 L total) of 30% ethylene glycol in buffer B to 60% ethylene glycol in DDW. Fractions of 2.7 mL/tube were collected using a flow rate of 40 mL/h. Tubes with TIA were pooled, as indicated in Figure 2, dialyzed (MW cutoff 3500) extensively against DDW or concentrated to about 15 mL using an Amicon YM-5 membrane (Amicon Division, W. R. Grace and Co., Danvers, MA), and freeze-dried.

Affinity Chromatography. The methods of Ishii et al. (1983) and Ako et al. (1972) were used to prepare anhydrotrypsin–Sepharose affinity resin. A freeze-dried TI sample pooled from phenyl-Sepharose was dissolved in 50 mM Tris, 500 mM NaCl, and 20 mM $CaCl_2$, pH 7.8 (buffer C), and loaded onto the 4.5 × 3.5 cm anhydrotrypsin–Sepharose column. Unbound proteins were eluted with buffer C. Trypsin inhibitors bound to the column were eluted with 10 mM HCl containing 500 mM NaCl and 20 mM $CaCl_2$. Fractions of 2.3 mL/tube were collected using a 10 mL/h flow rate. Fractions with TIA were pooled as shown in Figure 3, dialyzed (MW cutoff 3500), and freeze-dried.

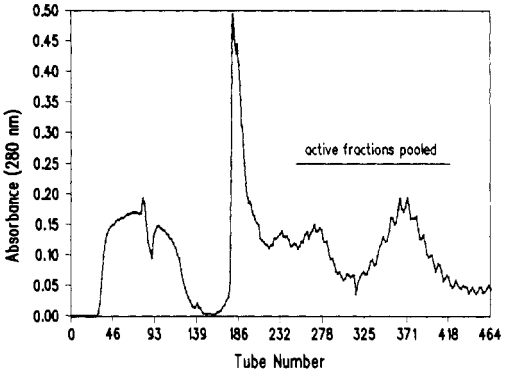

Figure 2. Hydrophobic interaction chromatography of pooled fractions from DE-52 cellulose column (Figure 1). Phenyl-Sepharose CL-4B column (17 × 3.5 cm) was equilibrated with 20 mM sodium phosphate buffer, pH 7, containing 25% ammonium sulfate, and eluted with linear gradient of ethylene glycol 30–60% and 25–0% ammonium sulfate in the same buffer (1 L total volume). Fraction size, 2.7 mL/tube; flow rate, 40 mL/h.

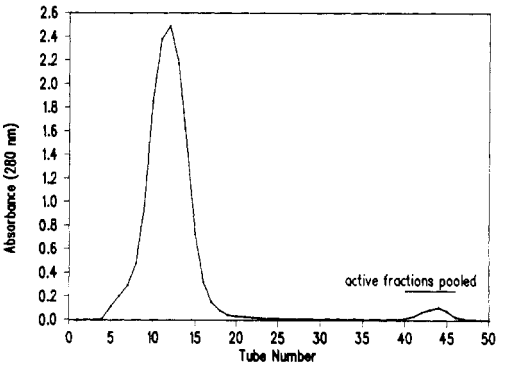

Figure 3. Anhydrotrypsin–Sepharose chromatography of pooled fractions from phenyl-Sepharose CL-4B column (Figure 2). The column (4.5 × 3.5 cm) was equilibrated with 50 mM Tris buffer, pH 7.8, containing 500 mM NaCl and 20 mM $CaCl_2$. TIA eluted with 20 mM HCl containing 500 mM NaCl and 20 mM $CaCl_2$. Fraction size, 2.3 mL/tube; flow rate, 10 mL/h.

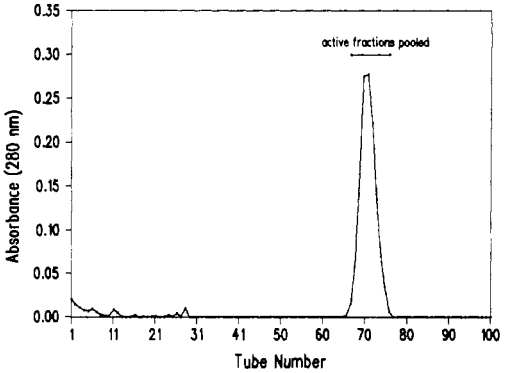

Figure 4. Gel filtration chromatography of pooled fractions from anhydrotrypsin–Sepharose column (Figure 3), dialyzed and lyophilized. Sephadex G-75 column (90 × 1.0 cm) was eluted with 10 mM Tris buffer, pH 7, containing 500 mM NaCl and 20 mM $CaCl_2$. Fraction size, 1.0 mL/tube; flow rate, 20 mL/h.

Gel Filtration. A freeze-dried TI sample pooled from the affinity column was dissolved in 3 mL of 10 mM Tris, pH 7, containing 500 mM NaCl and 20 mM $CaCl_2$, and applied to a Sephadex G-75 column, 90 × 1.0 cm, to estimate apparent molecular weight (MW). Fractions of 1 mL/tube were collected using a flow rate of 20 mL/h. Fractions with TIA were pooled as indicated in Figure 4, dialyzed against DDW, and freeze-dried. The gel filtration column was calibrated using DNP-glycine (259), aprotinin (6500), cytochrome *c* (12 400), carbonic anhydrase (29 000), bovine serum albumin (66 000), and blue dextran (2 000 000).

Table I. Effect of Boiling and Refrigerated Storage on Trypsin Inhibitor Activity[a] in Soaked, Raw Intact Beans (*P. vulgaris*)

	units of TIA[b] of whole bean			% original TIA retained	
sample	raw[c]	boiled[d]	boiled and stored[e]	boiled	boiled and stored
Black Beauty	14810 ± 480a	492 ± 18b	459 ± 14c	3.32	3.10
pinto	10687 ± 302a	389 ± 43b	363 ± 75b	3.64	3.40
Viva pink	8337 ± 129a	402 ± 16b	400 ± 17b	4.82	4.80
Great Northern	12365 ± 862a	450 ± 23b	496 ± 31c	3.64	4.01
small white	17558 ± 83a	505 ± 55b	521 ± 61c	2.88	2.97
dark red kidney	15329 ± 817a	387 ± 85b	456 ± 119b	2.52	2.97
small red	6462 ± 58a	326 ± 10b	297 ± 15b	5.04	4.60
Sanilac navy	11881 ± 497a	547 ± 12b	597 ± 25c	4.60	5.02
light red kidney	11668 ± 397a	288 ± 60b	269 ± 98b	2.47	2.31

[a] Mean ± standard deviation of three replicates, expressed on a wet weight basis. Means within each variety in the same horizontal line followed by a different letter are significantly different ($P < 0.05$). [b] TIA activity in bean extracts; one unit of trypsin activity (TA) = an increase of 0.001 absorbance unit at 410 nm of the reaction mixture. One unit of trypsin inhibitor activity (TIA) = a 10% decrease in the number of units of TA. [c] Nonheated, soaked whole dry beans. [d] Boiled for 30 min in flat-bottom round flasks with attached cold water condensers and then extracted for TI. [e] Boiled for 30 min and extracted for TI; extract stored at 4 °C for 20 h and then reassayed for TIA.

Table II. Effect of Boiling and Refrigerated Storage on Trypsin Inhibitor Activity[a] in Soaked, Raw Intact Beans (*P. vulgaris*)

	units of TIA[b]/mg of protein extracted			% of original TIA retained	
sample	raw[c]	boiled[d]	boiled and stored[e]	boiled	boiled and stored
Black Beauty	138.6 ± 3.8a	12.3 ± 0.2b	11.5 ± 0.1c	8.87	8.30
pinto	132.0 ± 6.6a	12.7 ± 0.8b	11.8 ± 1.8b	9.62	8.94
Viva pink	80.8 ± 0.9a	14.2 ± 1.7b	14.1 ± 1.7b	17.57	17.45
Great Northern	119.9 ± 6.7a	22.8 ± 1.3b	25.1 ± 1.8c	19.02	20.93
small white	161.4 ± 4.5a	28.9 ± 1.6b	29.8 ± 1.8c	17.91	18.46
dark red kidney	123.1 ± 6.4a	12.2 ± 2.9b	14.5 ± 4.0b	9.91	11.78
small red	62.3 ± 2.4a	9.4 ± 0.8b	8.5 ± 0.8b	15.09	13.64
Sanilac navy	104.0 ± 2.8a	23.6 ± 1.8b	25.8 ± 2.5c	22.69	24.80
light red kidney	88.3 ± 3.3a	4.9 ± 0.3b	4.6 ± 1.3b	5.55	5.21

[a] Mean ± standard deviation of three replicates, expressed on a wet weight basis. Means within each variety in the same horizontal line followed by a different letter are significantly different ($P < 0.05$). [b] TIA activity in bean extracts; one unit of trypsin activity (TA) = an increase of 0.001 absorbance unit at 410 nm of the reaction mixture. One unit of trypsin inhibitor activity (TIA) = a 10% decrease in the number of units of TA. [c] Nonheated, soaked whole dry beans. [d] Boiled for 30 min in flat-bottom round flasks with attached cold water condensers and then extracted for TI. [e] Boiled for 30 min and extracted for TI, extract stored at 4 °C for 20 h and then reassayed for TIA.

Protein Analysis. Protein concentration during the TI purification steps was determined using the BCA method with 30 min of incubation at 37 °C for color development (Smith et al., 1985). Bovine serum albumin was used as the standard protein. Crude protein content of the dry beans and of the albumin fractions was determined according to the micro-Kjeldahl method, using the conversion factor of 6.25 × N.

Electrophoresis. *Native Gels.* Native (nondissociating), discontinuous polyacrylamide gel electrophoresis was performed with an electrode buffer at pH 8.3 (Hames, 1990). A resolving slab gel of 12.5–25% acrylamide gradient with a 4.5% stacking gel was used for protein staining (180 × 160 × 0.75 mm slab, Protean 16 cm electrophoresis unit, Bio-Rad Laboratories, Richmond, CA).

Native gels were stained for protein using 0.1% Coomassie Blue R-250, in a 40% methanol and 10% trichloroacetic acid solution, and destained in the same solution without the dye. Gels were also double-stained in 0.25% Coomassie Blue G-250 followed by a silver nitrate stain as described by Moreno et al. (1985). A negative staining procedure used to visualize trypsin and chymotrypsin inhibitory activity bands employed *N*-acetyl-DL-phenylalanine β-naphthyl ester and fast blue B salt as described by Hejgaard (1981). A trypsin solution of 0.2 mg/mL or chymotrypsin solution of 0.3 mg/mL was used in the incubation step.

SDS–PAGE. Discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using an electrode buffer at pH 8.3 (Hames, 1990). A resolving slab gel of 7.5–24% acrylamide gradient with a 3.75% acrylamide stacking gel was employed (180 × 160 × 1.5 mm slab, Protean 16 cm electrophoresis unit, Bio-Rad Laboratories). The SDS–PAGE gels were stained with 0.1% Coomassie Blue R-250 in water/methanol/glacial acetic acid (5:5:2 by volume) (Hames, 1990) and destained with a 5% methanol/7.5% acetic acid solution. Standard proteins used for molecular weight estimation were from an electrophoresis calibration kit for molecular weight determination of low molecular weight proteins (Pharmacia LKB) and consisted of phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400). Molecular weight was estimated by relative mobility with respect to bromophenol blue tracking dye.

Isoelectric Focusing (IEF). Albumin fractions and purified inhibitors were separated using 7.5% polyacrylamide gels containing 1.92% ampholine, pH 3.5–5, and 5.68% ampholine, pH 4–6 (LKB 2117 Multiphor electrophoresis unit, Pharmacia LKB, 125 × 260 × 0.5 mm plate). The anode electrode strip was soaked in 1 M phosphoric acid, and the cathode electrode strip was soaked in 2% ampholine, pH 5–7. Negative stains of trypsin and chymotrypsin inhibitors and protein double stain (Coomassie Blue R-250 and silver stain) were carried out as described earlier.

RESULTS AND DISCUSSION

Screening for Heat-Stable Trypsin Inhibitory Activity (TIA). Results of studies to screen intact beans, bean flours, and bean albumin extracts for heat-stable TIA are found in Tables I–V. The TIA of raw (unheated) dry beans varied significantly between varieties, from a low of approximately 6500 units of TIA/g of bean (30 units of TIA/mg of total bean protein or 62 units of TIA/mg of protein extracted) for small red beans to a high of approximately 17 600 units of TIA/g of bean (83 units of TIA/mg of total bean protein or 161 units of TIA/mg of protein extracted) for small white beans (Table I). Crude protein (Kjeldahl N × 6.25) values of the dry beans varied from approximately 22% for small red beans to approximately 25% for Sanilac navy beans on a wet weight basis (data not shown). These protein values are in agreement with literature reports (Koehler et al., 1987). However, it

Table III. Effect of Boiling and Refrigerated Storage on Trypsin Inhibitor Activity[a] of Ground Bean Flour (*P. vulgaris*)

sample	units of TIA[b]/g of bean flour			% of original TIA retained	
	rw[c]	boiled[d]	boiled and stored[e]	boiled	boiled and stored
Black Beauty	14681 ± 87a	5970 ± 85b	6551 ± 87c	40.67	44.62
pinto	9322 ± 119a	5879 ± 250b	5473 ± 229c	63.07	58.71
Viva pink	6882 ± 55a	3402 ± 108b	3603 ± 214b	49.43	52.35
Great Northern	10748 ± 154a	3401 ± 107b	3213 ± 277b	31.64	29.88
small white	16548 ± 312a	7644 ± 145b	7362 ± 153b	46.19	44.49
dark red kidney	13718 ± 223a	6830 ± 272b	6879 ± 409b	49.79	50.15
small red	6314 ± 97a	2955 ± 166b	3182 ± 167c	46.80	50.40
Sanilac navy	12987 ± 55a	3943 ± 49b	3677 ± 28c	30.36	28.31
light red kidney	10304 ± 100a	5377 ± 133b	5602 ± 9b	52.18	54.37

[a] Mean ± standard deviation of three replicates, expressed on a wet weight basis. Means within each variety in the same horizontal line followed by a different letter are significantly different ($P < 0.05$). [b] TIA activity in bean extracts; one unit of trypsin activity (TA) = an increase of 0.001 absorbance unit at 410 nm of the reaction mixture. One unit of trypsin inhibitor activity (TIA) = a 10% decrease in the number of units of TA. [c] Nonheated, ground bean flour. [d] Suspended in water, placed in screw-cap test tubes, and heated in a boiling water bath for 30 min and then extracted for TI. [e] Boiled for 30 min and extracted for TI; extract stored at 4 °C for 20 h and then reassayed for TIA.

Table IV. Effect of Dry Roasting on Trypsin Inhibitor Activity[a] of Ground Dry Bean Flour (*P. vulgaris*)

sample	units of TIA[b]/g of bean flour		% of original TIA retained (dry roasted)
	raw[c]	dry roasted[d]	
Black Beauty	14958 ± 26a	1445 ± 367b	9.67
pinto	10519 ± 121a	603 ± 94b	5.73
Viva pink	7526 ± 65a	900 ± 317b	11.96
Great Northern	12284 ± 141a	966 ± 310b	7.86
small white	16869 ± 137a	1227 ± 493b	7.27
dark red kidney	14324 ± 154a	1831 ± 969b	12.78
small red	7003 ± 48a	555 ± 132b	7.93
Sanilac navy	12988 ± 105a	1155 ± 71b	8.89
light red kidney	11618 ± 38a	875 ± 132b	7.53

[a] Mean ± standard deviation of three replicates, expressed on a wet weight basis. Means within each variety in the same horizontal line followed by a different letter are significantly different ($P < 0.05$). [b] One unit of trypsin activity (TA) = an increase of 0.001 absorbance unit at 410 nm of the reaction mixture. One unit of trypsin inhibitor activity (TIA) = a 10% decrease in the number of units of TA. [c] Nonheated, ground bean flour. [d] Dry roasted at 200 °C for 5 min.

Table V. Effect of Heating and Refrigerated Storage of the Heated Albumin Samples on Trypsin Inhibitor Activity[a] of Albumin Extracts of Dry Beans (*P. vulgaris*)

sample	units of TIA[b]/mg of bean albumin			% of original TIA retained	
	native[c]	boiled[d]	boiled and stored[e]	boiled	boiled and stored
Black Beauty	170.2 ± 2.8a	168.5 ± 1.2a	167.0 ± 1.2a	99.0	98.12
pinto	156.4 ± 1.8a	149.2 ± 4.6a	157.1 ± 6.3b	95.40	100.45
Viva pink	102.2 ± 2.7a	108.8 ± 7.2a	102.1 ± 1.1a	106.45	99.90
Great Northern	140.8 ± 4.7a	142.9 ± 7.2a	144.3 ± 3.2a	101.49	102.49
small white	169.0 ± 4.2a	167.9 ± 2.5a	171.0 ± 5.7a	99.35	101.18
dark red kidney	157.4 ± 0.5a	152.2 ± 1.3b	153.1 ± 0.5b	96.70	97.27
small red	87.8 ± 0.3a	87.5 ± 1.9a	86.0 ± 0.7a	99.66	97.95
Sanilac navy	137.2 ± 2.6a	129.7 ± 3.0a	135.3 ± 4.8a	94.53	98.62
light red kidney	135.1 ± 1.0a	132.0 ± 1.8a	127.8 ± 1.3a	97.71	94.60

[a] Mean ± standard deviation of three replicates, expressed on a wet weight basis. Means within each variety in the same horizontal line followed by a different letter are significantly different ($P < 0.05$). [b] One unit of trypsin activity (TA) = an increase of 0.001 absorbance unit at 410 nm of the reaction mixture. One unit of trypsin inhibitor activity (TIA) = a 10% decrease in the number of units of TA. [c] Freeze-dried bean albumin extract, not heated. [d] Boiled in solution (1 mg/mL in distilled water) in a boiling water bath for 30 min. [e] Boiled for 30 min, stored at 4 °C for 20 h, and then reassayed for TIA.

should be noted that total nitrogen slightly overestimates the true protein content of dry beans (Sgarbieri et al., 1979; Antunes and Sgarbieri, 1980; Deshpande and Nielsen, 1987a).

Heat stability of trypsin inhibitors in dry beans varied depending on the nature of the dry bean being subjected to the heat treatment. TIA exhibited minimum heat stability in the intact bean matrix, medium heat stability in dry bean flour, and maximum heat stability in the extracted bean albumins. Boiling soaked, whole, intact dry beans for 30 min resulted in a substantial reduction in TIA as compared to raw (unheated) beans (Table I). Approximately 2.5 (light red kidney)–5.0% (small red) of the original TIA was retained in the intact dry beans following heat treatment when expressed as units of TIA per gram of whole bean. This is in agreement with several authors who reported that boiling (30 min) whole intact dry beans resulted in 4.5% residual TIA for Rosinha G2 (*P. vulgaris*) beans (Antunes and Sgarbieri, 1980), 7.8% residual TIA for Biala Wyborowa (*P. vulgaris*) beans (Borowska and Kozlowska, 1981), and 2.9% and 2.8% residual TIA for navy and kidney beans, respectively (Dhurandhar and Chang, 1990). Wang and Chang (1988) reported that heating navy beans in steam (100 °C) for 10 min resulted in 14% residual TIA. Literature reports on the effect of autoclaving on TIA of whole intact beans are somewhat similar. Heating navy and pinto beans at 120–121 °C for 14–15 min resulted in 2.5–5% residual TIA (Wang and Chang, 1988; Wang et al., 1988; Dhurandhar and Chang, 1990), while heating Rosinha G2 beans at 121 °C for 7.5 min (Antunes and Sgarbieri, 1980) or Biala Wyborowa beans at 120 °C for 15 min (Borowska and Kozlowska, 1981) resulted in 0% and 45% residual TIA, respectively.

The significance of low levels of dietary trypsin inhibitors is in question. Short-term feeding trials (28 days) elicit

no pancreatic hypertrophy in rats fed soy flour in which 55–69% of the trypsin inhibitory activity has been destroyed (Rackis et al., 1975). However, a major trypsin inhibitor chronic feeding study showed that long-term feeding of low levels of trypsin inhibitors does indeed cause adverse physiological effects in rats, and the investigators found no evidence of a trypsin inhibitor concentration threshold that elicited the adverse physiological effects (Gumbmann et al., 1985; Liener et al., 1985; Rackis et al., 1985; Spangler et al., 1985). There are no studies in the literature to date that investigate the long-term effects of low dietary exposure to trypsin inhibitors in humans. Short-term exposure to Bowman–Birk soybean trypsin inhibitor, however, causes increased pancreatic secretion in humans (Liener et al., 1988). More work is warranted to determine the effect to humans of long-term dietary exposure to low levels of trypsin inhibitors.

Near identical results for residual TIA in boiled whole intact beans were obtained when the data were expressed on a units of TIA per milligram of total bean protein basis (data not shown) compared to on a units of TIA per gram of whole bean basis, but a substantially higher TIA heat stability was obtained when the data were expressed on a units of TIA per milligram of protein extracted basis (Table II). On a units of TIA per milligram of protein extracted basis, the percent of original TIA retained after boiling ranged from approximately 5.6% (light red kidney) to approximately 22.7% (Sanilac navy). The apparent differences in heat stability of TIA between the data expressed on a per gram of dry bean basis (or on a per milligram of total bean protein basis) and on a per milligram of protein extracted basis is due to the differences in protein extractability between the raw (unheated) and boiled beans. More protein was extracted from the raw beans than from the heat-treated beans. This effect of lower protein solubility of soaked, boiled whole beans is consistent with a report by Antunes and Sgarbieri (1980) of a decrease in protein solubility upon boiling of Rosinha G2 beans. The lower protein solubility effect was also evident after boiling and dry roasting ground bean flour (data not shown).

The effect of storage (20 h, 4 °C) of the extract of boiled intact beans on TIA was investigated (Tables I and II). Of the nine cultivars of beans examined, only three cultivars exhibited a significant ($P < 0.05$) increase in TIA after refrigerated storage. This indicates that little to no renaturation of the inactivated TIA occurred following refrigerated storage.

Boiling (30 min) ground bean flour that had been dispersed in water resulted in a substantial increase in TIA heat stability (Table III) as compared to boiling whole intact beans. Approximately 30 (Sanilac navy)–63% (pinto) of the original TIA was retained following heat treatment when expressed as units of TIA per gram of ground bean flour. This is in agreement with Borowska and Kozlowska (1981), who reported 20% residual TIA in Biala Wyborowa bean flour dispersed in water (1:25 bean: water ratio, w/v) and heated at 100 °C for 60 min as opposed to 7.8% TIA retained in boiled (100 °C, 30 min) whole intact beans.

Nearly identical results were obtained for percent of original TIA retained following heat treatment of the ground bean flour when the data were expressed on either a units of TIA per milligram of total bean protein basis or on a units of TIA per milligram of protein extracted basis (data not shown). Refrigerated storage (20 h, 4 °C) of the extracts of the nine cultivars of boiled dry bean flour resulted in a significant ($P < 0.05$) increase in TIA for only two cultivars. Again, this would indicate that little to no renaturation of the inactivated TIA occurred following refrigerated storage.

Dry roasting (200 °C, 5 min) ground bean flour resulted in levels of heat-stable TIA that were intermediate to that of boiling whole intact beans and boiling dry bean flour (Table IV). Approximately 6 (pinto)–13% (dark red kidney) of the original TIA was retained in the ground bean flour following dry roasting, on a units of TIA per gram of bean flour basis. Nearly identical results were obtained for dry roasting bean flour when expressed on a units of TIA per milligram of total bean protein basis (data not shown). However, when residual TIA was expressed on a units of TIA per milligram protein extracted basis, values for percent of original TIA retained were substantially higher (34% for pinto to 79% for dark red kidney) (data not shown). The apparent higher heat stability of TIA in dry roasted bean flour when expressed on a units of TIA per milligram of protein extracted basis is due again to the difference in protein extractability between the raw (unheated) and the heat-treated beans.

Preliminary studies of dry heating ground bean flour at 100 °C for 30 min resulted in no loss of TIA (data not shown), indicating high heat stability of the TIA to dry heat treatment as opposed to moist (boiling) heat treatment of the bean flour. This is consistent with a report by Pusztai (1968) that isolated TIs from kidney beans were stable to dry heating at 90 °C for 90 min. There are papers in the literature on the effect of dry roasting on TIA of whole intact dry beans. Navy beans dry roasted in a bed of salt at 196–204 °C for 20 to 25 s resulted in 24.9% residual TIA in the bean (Yadav and Liener, 1977), while dry roasting whole intact beans at 190 °C for 30 s or at 220 °C for 10 s resulted in 28% and 18% residual TIA, respectively (Carvalho et al., 1977). Dry roasting of whole intact navy beans in a particle-to-particle dry roaster at 230 °C for 1 min or at 275 °C for 2 min resulted in 92% and 22% residual TIA, respectively (Aguilera et al., 1982).

Albumin extracts of nine cultivars of dry beans (*P. vulgaris*) were screened for heat-stable TIA (Table V). TIA in the raw (unheated) albumin fractions ranged from a low of approximately 88 units of TIA/mg of bean albumin (149 units of TIA/mg of albumin protein) for small red bean albumin to a high of approximately 170 units of TIA/mg of bean albumin (268 units of TIA/mg of albumin protein) for Black Beauty beans. The TIA in dry bean albumins was extremely heat stable. Albumin extracts of all but one (dark red kidney) of the nine bean cultivars studied showed no significant decrease in TIA after boiling for 30 min. Dark red kidney albumin retained nearly 97% of its original TIA following boiling for 30 min. There is evidence in the literature which indicates that the TIA of crude extracts of dry beans or purified trypsin inhibitors from dry beans are resistant to inactivation by heat treatment (Bowman, 1944, 1971; Wagner and Riehm, 1967; Pusztai, 1968; Mosolov and Fedurkina, 1974; Mosolov et al., 1977; Miyoshi et al., 1978; Jacob and Pattabiraman, 1986).

Only one of the nine cultivars exhibited a significant ($P < 0.05$) increase in TIA after refrigerated storage (20 h, 4 °C) of the heated albumins, again indicating that little to no renaturation of the inactivated TIA occurred over time. The albumin fractions from the nine cultivars of dry beans investigated contained approximately 50–65% crude protein (Kjeldahl N × 6.25) on a wet weight basis (data not shown). This is similar to the crude protein level of 70.3% for the albumin fraction from Rosinha G2 beans reported by Antunes and Sgarbieri (1980).

Table VI. Purification of Trypsin Inhibitors from Great Northern Beans (*P. vulgaris*)

purifn step	total protein[a], mg	total UTIA[b]	sp act., UTIA/mg of protein	recovery, %	purifn factor
albumin extract	804.0	191 565	238.3	100	1
heated albumin[c]	712.8	179 269	251.5	93.6	1.1
DE-52 cellulose	253.5	167 856	662.2	87.6	2.8
phenyl-Sepharose	58.1	110 870	1908.3	57.9	8.0
anhydrotrypsin–Sepharose	13.6	44 850	3297.8	23.4	13.8

[a] Based on bicinchoninic acid and copper sulfate assay. [b] Units of trypsin inhibitor activity. One unit of trypsin activity (TA) = an increase of 0.001 absorbance units at 410 nm of the reaction mixture. One unit of trypsin inhibitor activity (TIA) = a 10% decrease in the number of units of TA. [c] Albumin extract heated at 75 °C for 15 min, centrifuged, and filtered.

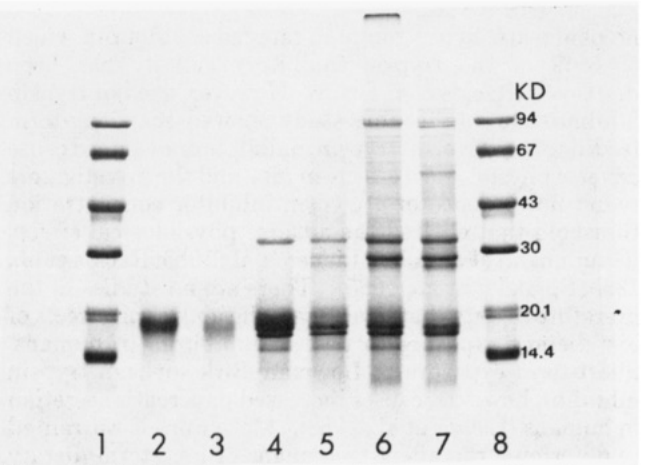

Figure 5. SDS–polyacrylamide gel electrophoresis pattern of samples from purification steps of Great Northern bean trypsin inhibitors. Stained with 0.1% Coomassie Blue R-250. (Lane 1) Standard (10 μg of each component); (lanes 2 and 3) anhydrotrypsin–Sepharose step (20 and 10 μg of protein, respectively); (lane 4) hydrophobic interaction phenyl-Sepharose step (42 μg of protein); (lane 5) anion-exchange DE-52 step (56 μg of protein); (lane 6) heated albumin extract (70 μg of protein); (lane 7) crude albumin extract (70 μg of protein); (lane 8) standard (10 μg of each component). Molecular weights of standards are given at the right.

Previous studies on the heat stability of TIA are found in the literature, but differences in conditions of analysis, heat treatments, and expression of results make a direct comparison difficult. This is, to our knowledge, the first time that a systematic screening for the heat stability of TIA in *P. vulgaris* whole intact bean matrix, ground bean flour, and albumin extracts is reported. This study revealed that different cultivars of raw (unheated) dry beans vary in their levels of TIA. TIA exhibited minimum heat stability in the whole intact bean matrix, medium heat stability in dry bean flour, and maximum heat stability in the extracted bean albumin. This is in agreement with literature reports that isolated trypsin inhibitors are stable against heat treatment but not heat stable in the presence of high molecular weight substances such as RNA, DNA, bovine serum albumin, high molecular weight proteins, cytochrome *c*, gelatin, and starch (Ellenrieder et al., 1980; Tsukamoto et al., 1983). A study that confirmed the greater heat stability of the Kunitz type vs the Bowman–Birk type soybean trypsin inhibitor when contained within a soybean flour matrix also showed that purified soybean inhibitors were more heat stable than the inhibitors contained within a soybean flour matrix (DiPietro and Liener, 1989).

Partial Purification and Characterization of the Isoinhibitors. Chromatograms from the DE-52 cellulose, phenyl-Sepharose, anhydrotrypsin–Sepharose, and Sephadex G-75 columns are shown in Figures 1, 2, 3, and 4, respectively.

A summary of the purification steps for heat-stable TIs from Great Northern beans is presented in Table VI. Purification of the TIs through the five purification steps, concluding with anhydrotrypsin–Sepharose affinity chromatography, resulted in a 13.8-fold purification with a recovery of 23.4%. Trypsin inhibitors accounted for 1.69% of the albumin protein (or 0.91% of the total albumin fraction) and approximately 0.08% of the total bean. Values of the amount of TIs present in dry beans, percent recovery, and purification factors vary greatly among papers in the literature. Gomes et al. (1979) reported that TIs make up 2.7% of the protein in the albumin fraction of navy beans, while Whitaker and Sgarbieri (1981) reported that TIs make up 0.245% of the total water-soluble proteins of Rosinha G2 beans. Trypsin inhibitors constitute 1.69% of the protein from an acidic salt extract of red kidney beans (Wu and Whitaker, 1990).

Three literature papers give differing recovery and purification factors when trypsin affinity chromatography was employed in the purification of TIs from dry beans. Whitaker and Sgarbieri (1981) reported 61.4% recovery with 60.1-fold purification of the TIs from Rosinha G2 beans following trypsin–Sepharose chromatography and 42.2% recovery with 92.0-fold purification when the eluted TI active fractions were reapplied to the same column. Jacob and Pattabiraman (1986) reported 53% recovery with 45-fold purification of the TIs from kidney beans following trypsin–Affi-Gel chromatography, while Wu and Whitaker (1990) reported 52.1% recovery with a 30.8-fold purification of the TIs from kidney beans following trypsin–Sepharose chromatography.

Three additional research groups report differing recovery and purification factors for TIs in dry beans using purification procedures that did not include affinity chromatography. Wagner and Riehm (1967) reported 15% recovery with a 40-fold purification of TIs from navy bean, Wilson and Laskowski (1973) reported 54.7% recovery with a 96-fold purification of TIs from Great Northern beans, and Miyoshi et al. (1978) reported 21% recovery with a 200-fold purification of TIs from Kitoki beans. The variability in the values of the amount of TIs present in dry beans, percent recovery, and purification factors could be attributed to varietal differences and to differences in isolation and assay procedures.

The pooled TIs from the anhydrotrypsin–Sepharose column were judged by SDS–PAGE gel electrophoresis to be homogeneous (Figure 5). Reports of trypsin inhibitor broad bands on gels in the literature are abundant. The oxidation of cysteine during electrophoresis may cause the broadening of trypsin inhibitor bands (Wu and Whitaker, 1990). When the purified pool of TIs was electrophoresed in native (no SDS) polyacrylamide gels, a double-protein stain (Coomassie Blue and silver stain) resolved three broad bands (Figure 6) which stained differentially. Differences in protein fixing and staining for one of the four inhibitors from mung beans have been reported (Wilson and Chen, 1983). Two protein bands with low mobility and high molecular weight were also evident in trace amounts. These bands were most probably the result of either trace amounts of contaminating proteins or associated trypsin inhibitors. The three broad bands in Figure 6 indicate that in nondenaturing conditions Great Northern bean trypsin isoinhibitors can be separated into three groups, as reported earlier by Wilson and Laskowski (1973).

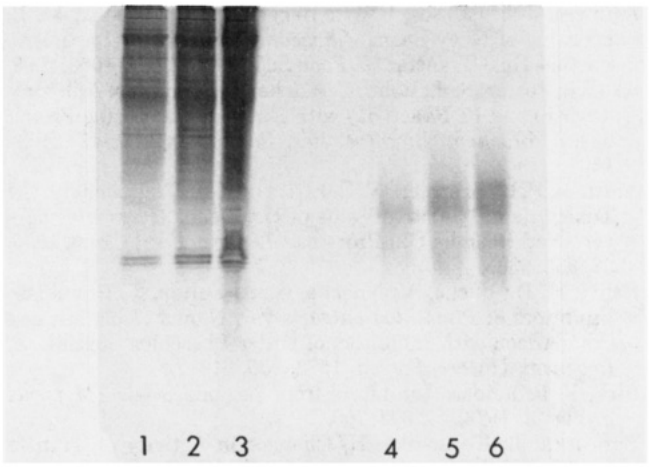

Figure 6. Native polyacrylamide gel electrophoresis pattern of albumin extract and purified trypsin inhibitors from Great Northern beans. Double protein stained with Coomassie and silver stain (see Materials and Methods). (Lanes 1, 2, 3) Crude albumin extract (100, 200, 400 μg of protein, respectively); (lanes 3, 4, 5) purified trypsin inhibitors (9, 18, 27 μg of protein, respectively).

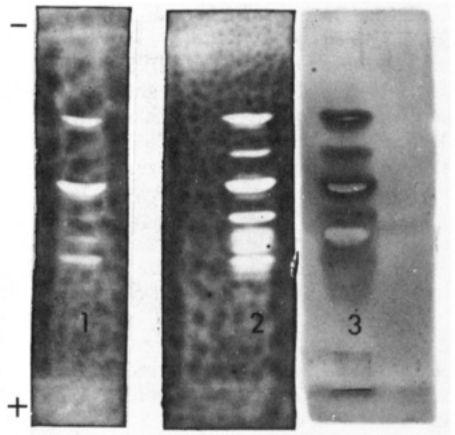

Figure 7. Isoelectric focusing pattern of purified trypsin inhibitors from Great Northern beans. Gels were stained for chymotrypsin (lane 1) or trypsin (lane 2) activity or protein double stained (lane 3) (see Materials and Methods). Purified trypsin inhibitors were loaded onto gel at levels of 60, 15, and 75 μg of protein for lanes 1, 2, and 3, respectively.

Isoelectrofocusing of the purified TIs followed by trypsin inhibitor staining revealed the presence of at least 11 trypsin isoinhibitor bands (Figure 7, lane 2, and Figure 8, lane 2). The occurrence of several isoinhibitors in the genus *Phaseolus* has been widely documented, i.e., kidney beans with six isoinhibitors (Jacob and Pattabiraman, 1986) and lima beans with at least six closely related isoinhibitors (Jones et al., 1963; Haynes and Feeney, 1967; Wilson and Laskowski, 1973). Wilson and Laskowski (1973) reported that one of the three trypsin inhibitors found in Great Northern beans, inhibitor II, had weaker inhibition of bovine trypsin, with a dissociation constant 2–3 orders of magnitude greater than that of inhibitors I and III at pH above 4.5. The isoinhibitors from Great Northern beans had p*I*s of approximately pH 4–5. Some of the bands that exhibited trypsin inhibitor activity also exhibited chymotrypsin inhibitory activity (Figure 7, lanes 1 and 2). One of the methods used to identify the Bowman–Birk type inhibitors is their strong inhibition of both trypsin and chymotrypsin (DiPietro and Liener, 1989). Thus, on the basis of this dual inactivation of trypsin and chymotrypsin, it appears that at least some of the isoinhibitors purified from Great Northern beans could be of the Bowman–Birk type. However, further studies are needed to confirm their classification.

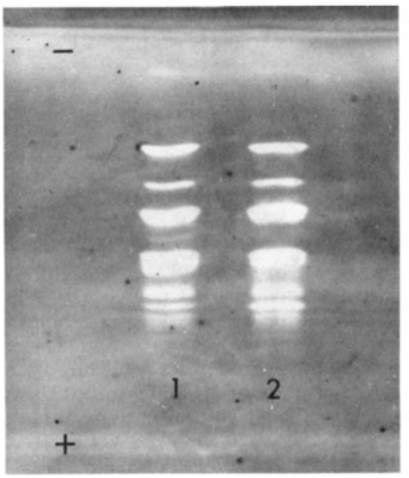

Figure 8. Isoelectric focusing pattern of native albumin extract (lane 1) and purified trypsin inhibitor (lane 2) from Great Northern beans. Gels were negatively stained for trypsin inhibitory activity (see Materials and Methods). Lanes 1 and 2 were loaded with 215 and 15 μg of protein, respectively.

An IEF gel stained for protein with Coomassie Blue followed by silver stain showed fewer protein bands (Figure 7, lane 3) compared to the 11 bands with trypsin inhibitory activity stain (Figure 7, lane 2, and Figure 8, lane 2). This was due to the highly sensitive inhibitory activity stain and the difficulty in staining individual bands of isoinhibitors present in very small quantities. The low content of basic amino acids in the trypsin isoinhibitors might be the cause of incomplete protein staining since the poorest staining was obtained when Coomassie Blue was used alone. Groups that react with silver stain are free amines and sulfur groups, while groups that react with Coomassie Blue are arginine and lysine residues (Merril, 1990). Thus, further work is needed to optimize the protein staining procedure of trypsin inhibitors in polyacrylamide gels.

Concerning the relationship of the multiple forms of the TIs, some authors have reported evidence of the production of new species of isoinhibitors during germination. The new isoinhibitors are produced after hydrolysis of terminal segments of amino acids from a major TI (Lorensen et al., 1981). Lorensen et al. (1981) noted that the cleavage of aspartyl and glutamyl residues from amino- and carboxyl-terminal sequences common in Bowman–Birk inhibitors could produce a decrease in charge and size. To ensure that the original inhibitors were not modified during the isolation procedure to generate new isoinhibitors, the isoelectrofocusing patterns of the native albumin extract and purified inhibitors were compared. Figure 8 shows the same number of isoinhibitors in the albumin extract as in the purified pool of isoinhibitors when a negative stain of the TIs was performed in IEF gels. Thus, the 11 trypsin isoinhibitors were present originally in the albumin fraction and not produced during the isolation.

Albumin extracts of nine dry bean cultivars (*P. vulgaris*) were subjected to IEF followed by trypsin inhibitor staining of the IEF gel (Figure 9). Differences exist between the varieties in the amounts of individual trypsin inhibitors present. Consequently, care should be taken when TI characterization results of one cultivar are compared to those of another.

The apparent molecular weight (MW) of the pool of purified isoinhibitors was estimated to be approximately 16 000 by gel filtration chromatography and approximately

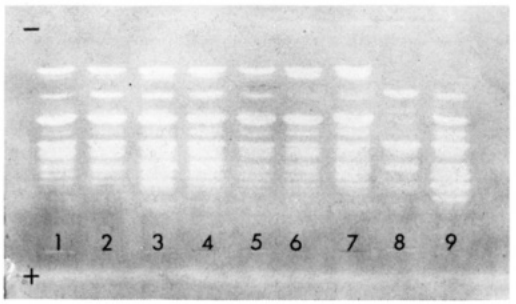

Figure 9. Isoelectric focusing pattern of albumin extracts of nine cultivars of dry beans (*P. vulgaris*). (Lane 1) Great Northern (99 μg of protein); (lane 2) Sanilac navy (129 μg of protein); (lane 3) small white (135 μg of protein); (lane 4) pinto (117 μg of protein); (lane 5) Viva pink (132 μg of protein); (lane 6) light red kidney (110 μg of protein); (lane 7) dark red kidney (130 μg of protein); (lane 8) small red (133 μg of protein); (lane 9) Black Beauty (133 μg of protein). Gels were negatively stained for trypsin inhibitory activity (see Materials and Methods).

18 000 by SDS–PAGE. This value is greater than the MW of approximately 8000–10 000 of the Bowman–Birk type inhibitor of soybean and garden bean (*P. vulgaris*) (Wilson and Laskowski, 1973) and the 8510–9210 reported for kidney beans by Wu and Whitaker (1990) as calculated from amino acid analysis. Other literature papers include navy beans with MW values of 23 000, as calculated by ultracentrifugation data and amino acid analysis, and 11 500, by stoichiometry of reaction between the inhibitor and trypsin (Wagner and Reihm, 1967). Kidney bean MW estimated by gel filtration is 14 000 (Pusztai, 1968).

Studies by Whitley and Bowman (1975) of navy bean trypsin inhibitors showed the presence of two inhibitors (MW 7900) and observed self-association of the inhibitors on Sephadex G-75 and G-100. Observations of dimer associations have also been reported in soybean Bowman–Birk inhibitors (Millar et al., 1969), lima bean trypsin inhibitors (Haynes and Feeney, 1967), and black-eyed peas (Gennis and Cantor, 1976b). Gennis and Cantor (1976b) found that the trypsin and chymotrypsin inhibitors (black-eyed peas) dimeric bonds were most stable in 2 M HCOOH (6 h), followed in stability by 3 M KSCN (6 h) and 6 M guanidine hydrochloride in 0.1 M HCOOH (24 h), and were destabilized with 60% saturation Na_2SO_4 in HCOOH (6 h). The surface of the inhibitor apparently is composed largely of disulfide loops with stretches of exposed peptide backbone (Gennis and Cantor, 1976b). Reduced carbamidomethylated trypsin and chymotrypsin inhibitors from black-eyed peas with MWs of 18 000 and 17 000, respectively, have dissociated into single bands of 10 000 and 9000, respectively. This dissociation was achieved by denaturation in 1% SDS for 10 min at 60 °C, followed by standing overnight (Gennis and Cantor, 1976a). Thus, our estimated MW for TIs in Great Northern beans is most probably a dimer form that was not disrupted by a 3-min boiling treatment in 2% SDS and 1% mercaptoethanol before electrophoresis. Further characterization of the Great Northern bean trypsin/chymotrypsin isoinhibitors is underway in our laboratory.

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