Purification and Partial Characterization of Four Trypsin/Chymotrypsin Inhibitors from Red Kidney Beans (*Phaseolus vulgaris*, var. Linden)

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Four protease inhibitors were purified to homogeneity from red kidney bean (*Phaseolus vulgaris* var. Linden) by extraction, followed by affinity chromatography with trypsin–Sepharose, ion-exchange chromatography with DEAE-Sephacel and CM-cellulose, and hydrophobic chromatography with phenyl-Sepharose. Amino acid analysis indicated all four inhibitors are high in $^1/_2$ Cys (12–14 mol/mol of protein), Ser (11–12 mol/mol of protein), Asp (12 mol/mol of protein), and Pro (7–10 mol/mol of protein) and low in Gly, Ala, and aromatic amino acids. The four inhibitors have similar molecular weights. Molecular weights determined under native condition indicate the inhibitors exist primarily as dimers. Isoelectric points of the four inhibitors range from 4.46 to 5.09. Inhibitors R(A), R(B2), and R(C) bind to one trypsin and one chymotrypsin; R(B1) binds to only one trypsin. Inhibition constants of the inhibitors to trypsin and chymotrypsin were measured.

INTRODUCTION

Protease inhibitors are found in most plants and several of them have been purified and extensively investigated. Extensive studies have been carried out on protease inhibitors purified from soybean (*Glycine max*; Kunitz, 1946; Birk, 1961; Yamamoto and Ikenaka, 1967), lima bean (*Phaseolus lunatus*; Jones et al., 1963; Haynes and Feeney, 1967), navy bean (*Phaseolus vulgaris*; Wagner and Riehm, 1967; Whitley and Bowman, 1975), broad bean (*Vicia faba*; Warsy et al., 1974), runner bean (*Phaseolus coccineus*; Weder et al., 1975), garden bean (*P. vulgaris*; Wilson and Laskowski, 1975), mung bean (*Phaseolus aureus Roxb.*; Baumgartner and Chrispeels, 1976), bush bean (*P. vulgaris* var. nanus; Gerstenberg et al., 1980), Brazilian pink bean (*P. vulgaris Rosinha G2*; Whitaker and Sgarbieri, 1981; Sgarbieri and Whitaker, 1981), and lentils (*Lens culinaris Medik.*; Weder et al., 1983).

Pusztai (1966) reported the isolation of a trypsin inhibitor from kidney bean (*P. vulgaris*, var. haricot). Jacob and Pattabiraman (1986) reported the isolation and some properties of an apparently different trypsin/chymotrypsin inhibitor from kidney bean (var. red bean). Neither of them reported multiple isoinhibitors in their studies. Purification and characterization of the physical and chemical properties of the inhibitors from red kidney beans have not been done before. Our preliminary study indicated that there were at least four major trypsin isoinhibitors in red kidney beans. They were purified to homogeneity to study their physical, chemical, and biological properties. This paper describes the purification of all four major trypsin isoinhibitors and presents some of their molecular properties.

MATERIALS AND METHODS

Materials and Reagents. Red kidney beans (*P. vulgaris* var. Linden) were obtained from Dr. Carl Tucker, Department of Agronomy and Range Science, University of California, Davis. Bovine pancreas trypsin (type III, 2× crystallized), soybean trypsin inhibitor (Kunitz), soybean Bowman–Birk inhibitor, N^{α}-benzoyl-DL-arginine *p*-nitroanilide, *N*-glutaryl-L-phenylalanine *p*-nitroanilide, phenyl-Sepharose, and CM-cellulose were purchased from Sigma Chemical Co. Bovine pancreas α-chymotrypsin (code: CDI, 3× crystallized) was purchased from Worthington Biochemical Corp. DEAE-Sephacel and Sepharose-4B were purchased from Pharmacia. Lima bean trypsin inhibitors were obtained from Professor R. E. Feeney, Department of Food Science and Technology, University of California, Davis. All chemicals were of analytical grade. Deionized water was used in all studies.

Purification of Isoinhibitors. *Extraction.* Extraction of inhibitors was carried out by stirring a suspension of bean flour containing 1% added poly(vinylpyrrolidone) in 0.5 M NaCl solution (1:6 w/v) adjusted to pH 2.5 with HCl for 2 h at room temperature (25 °C). Insoluble materials were removed by centrifugation at 10000*g* for 40 min, and the supernatant (acid extract), after the pH was adjusted to 7.0, was centrifuged at 10000*g* for 40 min. The supernatant (neutral bean extract) was used for further purification by chromatographic methods.

Affinity Chromatography. Bovine pancreas trypsin was coupled to Sepharose 4B beads by mixing trypsin solution in 0.1 M sodium bicarbonate buffer, pH 8.6, with activated Sepharose 4B and stirring for 16 h at 4 °C. Sepharose 4B was activated with cyanogen bromide by the cyano-transfer method of Kohn and Wilchek (1982). The affinity beads (50 mL packed) were mixed with 200 mL of neutral bean extract adjusted to 0.02 M phosphate (pH 7.6) and allowed to stand for 30 min at 4 °C with occasional stirring. Unbound proteins were removed by filtering the mixture on a sintered glass funnel and washing the Sepharose with 4 × 400 mL of 0.02 M phosphate buffer (pH 7.6) containing 0.4 M NaCl followed by 2 × 200 mL of deionized water. The Sepharose beads were packed into a column, and the inhibitors were eluted with 0.02 N HCl at 4 °C. Fractions containing the inhibitors were pooled, neutralized, dialyzed against deionized water, and lyophilized.

Ion-Exchange Chromatography. Ion-exchange chromatography and hydrophobic chromatography were carried out at room temperature (25 °C). Lyophilized inhibitors (200 mg) were dissolved in 40 mL of 0.02 M ammonium acetate buffer, pH 5.90, and loaded onto a DEAE-Sephacel column (1.5 × 30 cm) equilibrated with the same buffer. After the column was washed with 150 mL of buffer, elution of the inhibitors was carried out with a linear gradient of 0–0.3 M NaCl in 0.02 M ammonium acetate buffer (pH 5.90). Flow rate was 0.25 mL/min, and 500 mL total volume was collected. Three major peaks, all with inhibitory activity, were obtained and were named peaks A–C according to their elution order.

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Peak A was rechromatographed on the DEAE-Sephacel column. Pooled peak A was dialyzed against deionized water and lyophilized. A sample (10 mg) was weighed, dissolved in 10 mL of 0.02 M ammonium acetate buffer, pH 5.90, and loaded onto the column. A linear gradient of 0–0.25 M NaCl in the 0.02 M ammonium acetate buffer was started after the column was washed with 100 mL of buffer. A total volume of 520 mL of elution buffer was used. Inhibitor collected from the major peak was designated R(A).

The pooled fractions collected for peak C of DEAE-Sephacel chromatography were dialyzed against 0.02 M acetate buffer, pH 4.20, and loaded onto a CM-cellulose column (1.5 × 16 cm) equilibrated with the same buffer. After the column was washed with 40 mL of the buffer, a linear gradient of 0–0.25 M NaCl in the same buffer was used to elute the inhibitor. The flow rate was 0.3 mL/min, and a total volume of 300 mL of elution buffer was collected. The inhibitor in the major peak was designated R(C).

Hydrophobic Chromatography. A sample (20 mg) of dialyzed and lyophilized inhibitors collected from peak B of the DEAE-Sephacel column was dissolved in 5 mL of 30% saturated ammonium sulfate in 0.02 M phosphate buffer, pH 6.20, and the solution was loaded onto a phenyl-Sepharose 4B column (1.5 × 8 cm) equilibrated with the same buffer. After the column was washed with 40 mL of the same buffer, the inhibitors were eluted with a linear gradient of 0–50% ethylene glycol and 30–0% saturated ammonium sulfate in 0.02 M phosphate buffer, pH 6.20, with a flow rate of 0.3 mL/min; the total volume of the gradient was 260 mL. Fractions of the two major peaks were pooled separately, dialyzed against deionized water, lyophilized, and designated R(B1) and R(B2).

Characterization. *Amino Acid Analysis.* Lyophilized inhibitors were weighed on a Cahn gram electrobalance and hydrolyzed as described by Moore and Stein (1963). Amounts of $^1/_2$ Cys were determined from cysteic acid by performic acid oxidation before acid hydrolysis. Amino acid determination was performed on a Dionex D-500 amino acid analyzer.

SDS–PAGE and Isoelectric Focusing. The molecular weights of the inhibitors were determined by SDS–PAGE on 15% separating gel following Laemmli's (1970) procedure. Bovine serum albumin (M_r 66 000), egg white albumin (M_r 43 000), bovine pancreas carboxypeptidase A (M_r 34 000), Kunitz soybean trypsin inhibitor (M_r 23 000), and horse heart myoglobin (M_r 17 000) were used as molecular weight markers. Isoelectric focusing of the inhibitors was carried out in 7.5% polyacrylamide gel to determine the isoelectric points. Focusing range was pH 4.0–6.5, using ampholine from Pharmacia.

Gel Electrophoresis. Gel electrophoresis under native condition was carried out to evaluate the purity of the inhibitors at different stages of purification by using the procedure described by Davis (1964). Acrylamide and bis(acrylamide) concentrations in the separation gel were 12.5% and 0.4%, respectively. Native molecular weights of the inhibitors were estimated by the method of Hedrick and Smith (1968) using anodic PAGE with a continuous buffer system (Tris/glycine, pH 9.3); total gel concentrations were 8%, 10%, 12%, 14%, and 16%.

Inhibitory Activity Staining. Staining of inhibitory activity followed the procedure of Uriel and Berges (1968) with some modification. After electrophoresis, a 0.75 mm thick gel was immersed in trypsin or chymotrypsin solution (5 mg/100 mL) in 0.05 M phosphate buffer, pH 7.60, for 20 min at room temperature. The gel was then rinsed with water and stained in a solution containing acetyl-DL-phenylalanine β-naphthyl ester (0.7 mM) and tetrazotized *o*-dianisidine (1 mg/mL) in 0.05 M phosphate buffer, pH 7.60.

Gel Filtration Chromatography. The inhibitors or enzyme–inhibitor complexes were chromatographed by gel filtration on a FPLC system using a Superose 12 HR10/30 column. The column was equilibrated with 0.02 mM Tris buffer containing 0.25 M NaCl, pH 8.10, to determine the molecular weights of native inhibitors and enzyme–inhibitor complexes. Gel filtration under denaturing condition was also carried out by using the same column. A 0.05 M phosphate buffer containing 0.15 M NaCl and 6 M urea was used to equilibrate the column and as the elution buffer. All inhibitors and cystine-containing M_r standards were treated with NTSB solution containing 7.5 mM 2-nitro-5-sulfothiobenzoate, 0.4 M sodium sulfite, and 6 M guanidine hydrochloride in 50 mM glycine buffer, pH 9.0. The preparation of NTSB was according to the method of Thannhauser et al. (1987). Such treatment ensured complete denaturation of proteins of different stabilities and greatly eliminated deviation of many proteins on a plot of V_e/V_0 vs log (M_r).

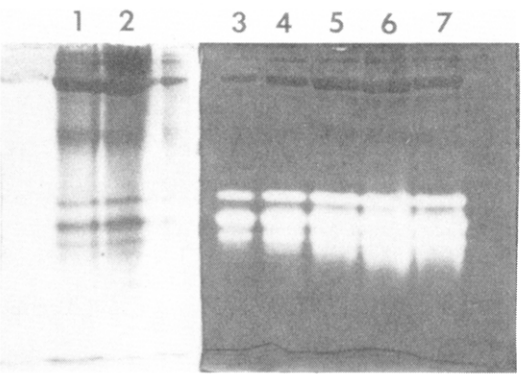

Figure 1. Gel electrophoresis patterns of crude extract of RKB stained for protein and inhibitory activity. Lanes 1 (100 μg) and 2 (400 μg) were stained for protein; protein bands were blue in clear background. Lanes 3 (20 μg), 4 (40 μg), 5 (80 μg), 6 (200 μg), and 7 (80 μg) were stained for trypsin inhibitory activity, which was shown by clear bands on dark pink background.

Binding Stoichiometry and Inhibition Constant of Inhibitors to Trypsin and Chymotrypsin. The binding stoichiometry of the inhibitors to different enzymes was determined by activity titration. The inhibitor concentrations were determined by weighing inhibitors on an electromicrobalance and making to a fixed volume with 0.05 M Tris buffer, pH 8.0. The active trypsin concentration was determined by titration with *p*-nitrophenyl *p*-guanidinobenzoate, following the method of Chase and Shaw (1970). The active chymotrypsin concentration was determined by titration with *p*-nitrophenyl acetate according to the method of Bender et al. (1966). N^{α}-Benzoyl-DL-arginine *p*-nitroanilide (BAPA) and *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) were used as specific substrates of trypsin and chymotrypsin, respectively. The dissociation constants of enzyme–inhibitor complexes (K_i) were determined according to the method of Bieth (1974).

RESULTS

A. Purification. The inhibitors were effectively extracted by the described procedure. The extraction of inhibitors from RKB at low pH not only gave extracts of higher specific activity but also prevented enzymatic browning. The resulting supernatant was very light in color. Storage of this solution at 4 °C for a few days did not result in loss of inhibitor activity or change in color. Electrophoresis of the crude extract and subsequent staining of the inhibitory activity indicated there are four trypsin inhibitors in red kidney bean extract (Figure 1). This activity staining technique is very sensitive. It could easily detect less than 100 ng (10 pmol) of inhibitor in a band. Neutralization of the supernatant precipitated some proteins; removal of the precipitate resulted in about 10% increase in total activity. The neutralized extract slowly became darker in color even when stored in the cold, presumably due to oxidation of polyphenol compounds.

The result of the purification at different steps is summarized in Table I. The affinity chromatography gave satisfactory purification. After the affinity column, the inhibitors were purified to more than 30-fold with a recovery of 51.8%. At this stage, 1 μg of protein inhibited 1.82 μg of trypsin. The relatively low recovery simply reflected the intentional overloading of the affinity beads to keep them saturated with inhibitors to prevent proteolysis. The electrophoresis pattern showed that the

Table I. Purification of the Inhibitors from Red Kidney Bean

purification steps	volume, mL	protein, mg/mL	act., μg of Tr[a]/mL	sp act., μg of Tr[a]/mg	yield, %
acid extract	420	18.6	1110	59.8	100
neutral extract	431	16.0	1200	75.5	111
affinity column	227	0.580	1070	1840	51.8
DEAE-Sephacel column (100 mg RAP[b])					
peak A	26	0.549	1120	2040	15.8
peak B	41	0.893	1430	1600	31.8
peak C	31	0.316	507	1610	8.6
rechromatography of peak A (10 mg)					
R(A)	15	0.584	1070	1830	87.0
CM-cellulose column (15 mg peak C)					
R(C)	25	0.352	630	1790	65.5
phenyl-Sepharose column (20 mg peak B)					
R(B1)	20	0.105	121	1150	7.6
R(B2)	35	0.313	592	1890	64.8

[a] Micrograms of trypsin inhibited. [b] RAP, preparation after affinity column.

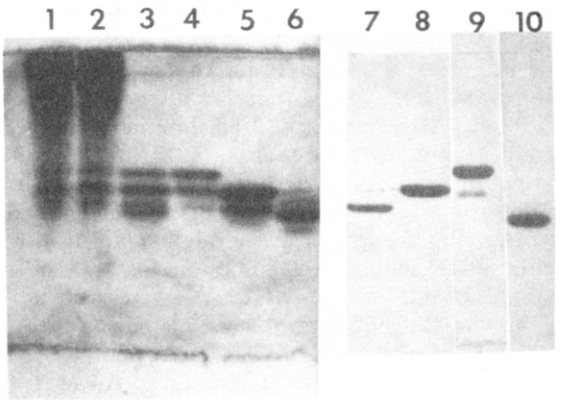

Figure 2. Gel electrophoresis patterns of preparations at different purification steps. (Lane 1) Acid extract; (lane 2) neutralized extract; (lane 3) inhibitors eluted from affinity column; (lanes 4, 5, and 6) peaks A, B, and C from DEAE-Sephacel column, respectively; (lane 7) R(B1); (lane 8) R(B2); (lane 9) R(A); (lane 10) R(C).

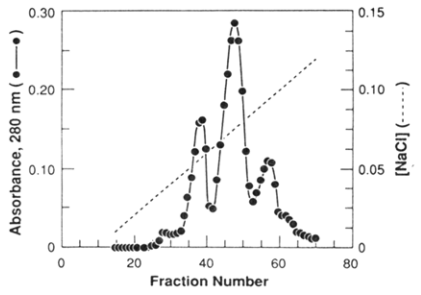

Figure 3. Chromatogram from DEAE-Sephacel column (1.5 × 30 cm). Sample was dialyzed, lyophilized preparation after affinity chromatography (200 mg). Fraction size, 5 mL; flow rate, 0.25 mL/min.

preparation at this stage was mainly a mixture of the four isoinhibitors (Figure 2, lane 3).

Separation of the isoinhibitors through ion-exchange chromatography and hydrophobic chromatography was followed by the protein peaks and by the electrophoresis patterns of the peaks (Figure 2). Three major peaks were obtained from the DEAE-Sephacel column (Figure 3; Figure 2, lanes 4–6). Peak A, after rechromatography on the same column, gave purified R(A) (Figure 4; Figure 2, lane 9). Peak B from the DEAE-Sephacel column, after hydrophobic chromatography on the phenyl-Sepharose column, gave two peaks corresponding to pure R(B1) and R(B2) (Figure 5; Figure 2, lanes 7 and 8). Peak C from the DEAE-Sephacel column was chromatographed on CM-cellulose column and gave purified R(C) (Figure 6; Figure 2, lane 10). The four major inhibitors were purified to

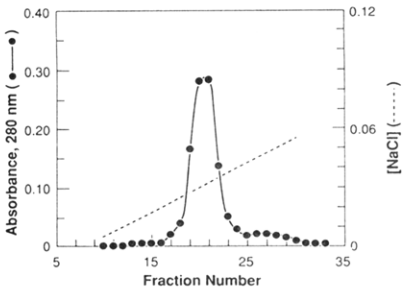

Figure 4. Chromatogram from DEAE-Sephacel column (1.5 × 30 cm). Sample was pooled peak A (10 mg) from Figure 3, after dialysis and lyophilization. Fraction size, 5 mL; flow rate, 0.25 mL/min.

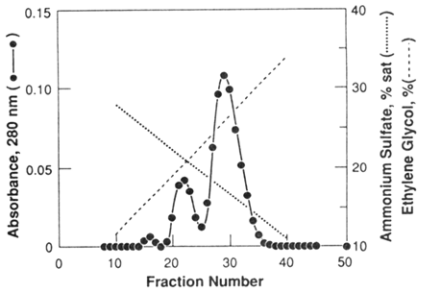

Figure 5. Hydrophobic chromatography on phenyl-Sepharose 4B column (1.5 × 8 cm). Sample was pooled peak B (20 mg) from Figure 3, after dialysis and lyophilization. Fraction size, 5 mL; flow rate, 0.3 mL/min.

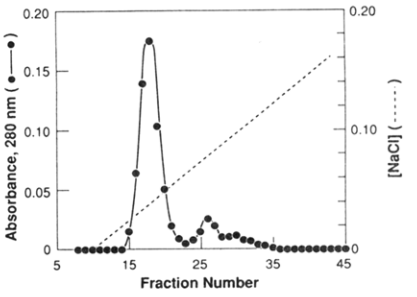

Figure 6. Chromatogram on CM-cellulose column (1.5 × 16 cm). Sample was pooled peak C from Figure 3, after dialysis against 0.02 M acetate buffer, pH 4.20. Fraction size, 5 mL; flow rate, 0.3 mL/min.

homogeneity as judged by their electrophoresis patterns (Figure 2) and isoelectric focusing patterns (Figure 7). The minor band in R(A) (Figure 2, lane 9) was a modified form of R(A) because it did not appear in the isoelectric focusing pattern of R(A).

B. Characterization. Results of amino acid analyses of the purified inhibitors are shown in Table II. The amino

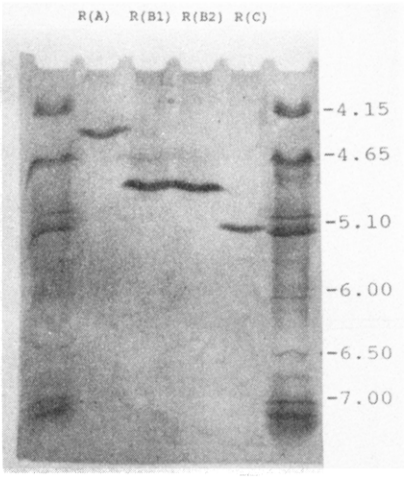

Figure 7. Isoelectric focusing pattern of the purified red kidney bean inhibitors. Focusing range was pH 4.0–6.5. The following proteins were used as p*I* standards: glucose oxidase (4.15), phycocyanin (4.65), β-lactoglobulin B (5.10), bovine carbonic anhydrase (6.00), and human carbonic anhydrase (6.50). Equine myoglobin (7.00), whale myoglobin (8.05), α-chymotrypsin (8.80), and cytochrome *c* were also included in the standard mixture, but these proteins stayed at the bottom of the gel or migrated off the gel.

Table II. Amino Acid Composition of the Four Red Kidney Bean Trypsin Inhibitors

	relative amino acid composition, mol %				AA residues/mol[c]			
	R(A)	R(B1)	R(B2)	R(C)	R(A)	R(B1)	R(B2)	R(C)
$^1/_2$Cys[a]	13.9	15.0	16.5	15.0	12	12	14	12
Asp	14.0	13.5	13.8	14.8	12	12	12	12
Thr	5.9	5.7	5.6	6.2	5	5	5	5
Ser	14.4	13.7	13.5	14.0	12	12	11	11
Glu	8.8	8.6	8.7	9.4	7	7	7	7
Pro	10.4	11.4	10.2	9.3	9	10	8	7
Gly	2.5	2.5	2.4	2.0	2	2	2	2
Ala	3.7	3.6	3.8	4.0	3	3	3	3
Val	0.0	1.1	0.0	0.0	0	1	0	0
Met	0.0	0.0	0.0	0.0	0	0	0	0
Ile	4.5	3.9	4.7	4.9	4	3	4	4
Leu	2.9	3.8	2.6	2.8	2	3	2	2
Tyr	1.2	1.2	1.2	1.3	1	1	1	1
Phe	2.2	1.4	2.5	2.5	2	1	2	2
Trp[b]	0.0	0.0	0.0	0.0	0	0	0	0
His	6.5	6.9	5.8	5.4	5	6	5	4
Lys	6.2	5.0	4.9	5.3	5	4	4	4
Arg	3.7	2.9	3.9	3.1	3	2	3	2
M_r					9210	9120	9110	8510

[a] Determined as cysteic acid. [b] Determined by spectrometric method (Blackburn, 1968). [c] Amino acid composition data relative to 1.0 tyrosine/mol and rounded to whole integer.

acid compositions of all four inhibitors are similar: they are all high in $^1/_2$ Cys and Ser, Asp, and Pro and low in Gly, Ala, and aromatic amino acids. There are no Met, no Trp, and, except in one inhibitor [R(B1)], no Val. The numbers of Asp, Glu, Thr, Gly, Ala, and Tyr residues are very similar for all the inhibitors, but there are obvious differences in their contents of His and Arg residues. There are also differences in $^1/_2$ Cys, Ser, Pro, Val, Ile, Phe, and Lys contents among the inhibitors. The molecular weights calculated from amino acid composition (taking the number of tyrosines in each inhibitor as 1) were as follows: R(A), 9210; R(B1), 9120; R(B2), 9110; R(C), 8510 (Table II).

Different isoinhibitors have distinct isoelectric points as shown in isoelectric focusing gel (Figure 7). The p*I*s of the inhibitors were measured by comparing the isoelectric position of each inhibitor with the p*I* markers. The p*I*s decrease in the order R(A), R(B1), R(B2), and R(C) (Table III), in the order eluted from the DEAE-Sephacel column, as one would predict. The nearly identical p*I* of R(B1) and R(B2) made the separation of the two inhibitors by ion-exchange chromatography impossible. Use of hydrophobic interaction chromatography effectively separated the two inhibitors, indicating differences in their surface hydrophobicity.

Figure 8 shows the SDS–PAGE patterns of the purified inhibitors. All the inhibitors have similar molecular weights. Figure 9 shows the determination of native M_r of inhibitors by Ferguson plot. The native molecular weights of the inhibitors estimated from native PAGE ranged from 17 100 to 19 400, and the molecular weights determined by SDS–PAGE ranged from 18 800 to 20 400 (Table III). Table III also lists molecular weights of the inhibitors determined by gel filtration chromatography under different conditions.

Under native conditions, the molecular weights determined by both polyacrylamide gel electrophoresis (17 100–19 400) and gel filtration chromatography (16 500–17 600) were nearly twice as high as the minimum molecular weights calculated from the amino acid compositions. The molecular weights determined by gel filtration in 6 M urea after complete denaturation of the inhibitors (11 200–11 900) and by gel filtration of trypsin–inhibitor complexes (11 100–12 800) were reasonably close to the values from amino acid analysis. Although the molecular weights of inhibitors R(A) and R(B1) in chymotrypsin complexes were similar to those in trypsin complexes, inhibitors R(B2) and R(C) appeared to be twice as large in chymotrypsin complexes as in trypsin complexes. In the course of molecular weight determination, parallel experiments were carried out on soybean Bowman–Birk inhibitor (M_r 8000; Yamamoto and Ikenaka, 1967) and lima bean inhibitor IV (M_r 9400; Jones et al., 1963) for comparison. The molecular weights of red kidney bean inhibitors as determined by SDS–PAGE and gel filtration under native condition and in the presence of 6 M urea were all similar to that of the lima bean inhibitor but higher than that of the soybean Bowman–Birk inhibitor (Table III). Therefore, the molecular weights calculated from amino acid composition are believed to be close to accurate assessments.

Three of the inhibitors, R(A), R(B2), and R(C), inhibited both trypsin and chymotrypsin simultaneously. By use of specific substrates, the inhibition of trypsin could be measured in the presence of excess chymotrypsin and vice versa. In this way, binding of the three inhibitors to trypsin and chymotrypsin was shown to be independent. The results on stoichiometry determination indicated that, at a total enzyme concentration of 0.1–0.2 μM, R(A), R(B2), and R(C) all bind to one trypsin and one chymotrypsin molecule independently, whereas R(B1) binds only to one trypsin molecule (Table IV). In the presence of trypsin, R(B1) bound about 30% less chymotrypsin (0.15 CT/inh in the absence of trypsin to 0.11 CT/inh in the presence of trypsin). Possible explanations are nonspecific binding of chymotrypsin to the trypsin binding site or the site that bound to chymotrypsin also had affinity to trypsin. Measurement of inhibition constants showed that binding of the inhibitors to trypsin and chymotrypsin was very tight (Table IV). Except inhibitor R(B2), the binding to trypsin was tighter than to chymotrypsin. The dissociation constants (K_d) of trypsin–inhibitor complexes ranged from 0.34 to 0.84 nM; K_d of chymotrypsin–inhibitor complexes ranged from 0.55 to 4.0 nM.

Table III. Molecular Properties of the Four RKB Inhibitors

	R(A)	R(B1)	R(B2)	R(C)	BBI[a]	LBI[a]
isoelectric point	5.09	4.84	4.82	4.46		
molecular weight by						
SDS–PAGE	19 500	20 400	20 100	18 800	16 500	19 000
native PAGE	17 100	18 900	19 400	17 400		
gel filtration						
native inhibitor	16 500	17 600	16 900	17 400	11 300	17 800
in trypsin complex[b]	12 500	12 800	12 500	11 100	7 500	12 300
in chymotrypsin complex[c]	11 700	13 100	20 600	21 900	18 500	21 500
in 6 M urea[d]	11 300	11 900	11 700	11 200	10 700	11 800
amino acid composition	9 210	9 120	9 110	8 510	8 000[e]	9 400[f]

[a] Results on M_r of soybean Bowman–Birk inhibitor (BBI) and lima bean trypsin inhibitor (LBI) in parallel experiments are included for a comparison. [b] Estimated by subtracting MW of trypsin from apparent M_r of inhibitor–trypsin complexes. The inhibitors and trypsin were mixed in elution buffer in molar ratios of 2:1 (I:Tr) and held at room temperature for 10 min before chromatography. [c] Estimated by subtracting M_r of chymotrypsin from apparent M_r of inhibitor–chymotrypsin complexes. Similar procedure as in footnote *b* was carried out. [d] Inhibitors were denatured by NTSB reagent (see text). [e] From Yamamoto and Ikenaka (1967). [f] From Jones et al. (1963).

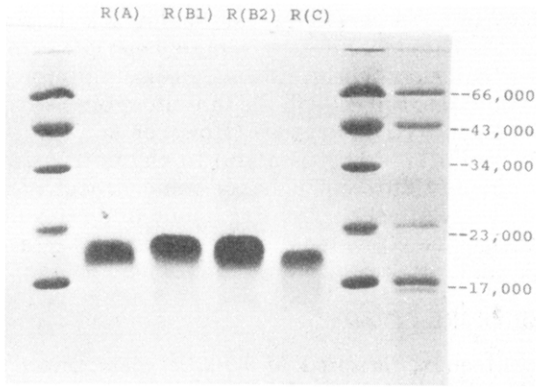

Figure 8. SDS–PAGE patterns of the four red kidney bean inhibitors. Separating gel contained 15% acrylamide and 0.9% bis(acrylamide). M_r standards are bovine serum albumin (66 000), egg white albumin (43 000), bovine pancreas carboxypeptidase A (34 000), Kunitz soybean trypsin inhibitor (23 000), and horse heart myoglobin (17 000).

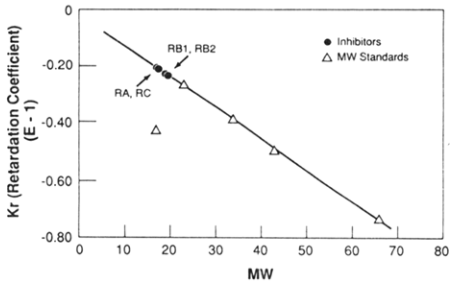

Figure 9. Determination of native M_r by Ferguson plot. Proteins were run on five gels of different concentration (8–16% total gel concentration). K_r's of different proteins were obtained by plotting R_f vs gel concentration and determining the slope. The M_r standards were the same as in SDS–PAGE except they were not denatured. Note: The K_r of myoglobin was omitted in calculation due to its deviation from the expected behavior.

DISCUSSION

One concern previously expressed about extracting inhibitors from beans at low pH was that the acid condition may cause modification of the inhibitors. Chu et al. (1964) reported that inhibitor A from mung bean was derived from inhibitor B after hydrolysis of some amide bonds in the protein during treatment with trichloroacetic acid. Since all the inhibitors isolated in this work were stable at low pH for several days without detectable change in electrophoretic mobility, it is not likely that such modification occurred under the conditions used. The inhibitors extracted at neutral pH also gave four major isoinhibitors that were electrophoretically indistinguishable from the acid-extracted inhibitors. This further indicates that the four isoinhibitors are components in beans, not products formed during extraction.

Table IV. Binding Stoichiometry and K_d of the RKB Inhibitors to Trypsin and Chymotrypsin[a]

	no. of enzyme bound/inh		K_d, E-10 M	
inhibitor	Tr	CT	to Tr	to CT
R(A)	1 (1.09)[b]	1 (0.91)	3.4	40
R(B1)	1 (0.71)	0 (0.13)	4.0	
R(B2)	1 (0.81)	1 (0.61)	8.4	5.5
R(C)	1 (1.02)	1 (0.87)	4.8	19.3

[a] BANA and GPNA were used in binding stoichiometry measurement and K_d measurement as substrates for trypsin and chymotrypsin, respectively. Total trypsin concentration was 0.1 μM, and total chymotrypsin concentration was 0.2 μM in binding stoichiometry measurements. [b] Figures in parentheses are experimental values.

Because of the relatively low contents of these protease inhibitors in beans (each inhibitor accounting for less than 0.5% of total acid extractable protein) and the existence of multiple isoinhibitors, the purification was quite difficult. The affinity column as the first chromatographic method was very effective in isolating the isoinhibitors as a group. It has been a concern that the trypsin inhibitors purified by immobilized trypsin might be proteolytically modified at the reactive sites (Fritz et al., 1971), and the use of anhydrotrypsin as affinity material has been suggested (Feinstein and Feeney, 1966; Xavier-Filho and Campos, 1983). To prevent any enzymatic modification in purification, we kept all the trypsin saturated with inhibitors by overloading the affinity beads and eluted the inhibitors quickly with 0.02 N HCl after the beads were washed rapidly with water. It has been shown that rapid dissociation of the enzyme–inhibitor complexes gives unmodified inhibitor (Laskowski, 1970). In our study, the purified R(A) contained a minute amount of modified form which was believed to be enzymatically cleaved R(A). Other than that, we did not observe formation of any new components after affinity chromatography by comparing the protein bands (Figure 2, lanes 1–3) and inhibitor bands (Figure 1) in gel electrophoresis patterns. This method is applicable to the purification of protease inhibitors from other beans, as long as the inhibitors are stable at low pH and rapid elution is carried out in chromatography.

Purification of the four isoinhibitors, which has not been reported previously, allowed studies of their molecular properties. The amino acid compositions of the purified inhibitors are similar to those of the low molecular weight

cystine-rich Bowman–Birk-type inhibitors such as trypsin isoinhibitors from garden beans (Wilson and Laskowski, 1973), Kintoki beans (Tsukamoto et al., 1983), lima beans (Jones et al., 1963), navy beans (Whitley and Bowman, 1975), and pinto beans (Wang, 1975), although the percentage of cystine content of the RKB inhibitors is generally a little lower than these Bowman–Birk-type inhibitors. The amino acid compositions are completely different from that of the high molecular weight Kunitz-type inhibitors (Wu and Scheraga, 1962). The trypsin inhibitor purified from kidney beans by Pusztai (1966) had somewhat similar amino acid composition as the RKB inhibitors, but there are obvious and significant differences. The compositions of the RKB inhibitors are very similar to the Brazilian pink bean inhibitors (Whitaker and Sgarbieri, 1981), indicating that they may be closely related.

The methods for the molecular weight determination deserve some discussion. The molecular weights of the inhibitors determined by amino acid composition (around 9000) and by gel filtration in 6 M urea (around 11 000) are within the molecular weight range of 8000–13 000 for most of the trypsin inhibitors isolated from *Phaseolus* species (Jones et al., 1963; Pusztai, 1968; Belitz et al., 1972; Wilson and Laskowski, 1973; Wang, 1975; Whitley and Bowman, 1975; Tsukamoto et al., 1983). However, M_rs determined by SDS–PAGE, gel filtration chromatography, and gel electrophoresis under native conditions were much higher. It has been shown that some of the Bowman–Birk-type inhibitors, such as those from soybean (Birk, 1985), kidney bean (Pusztai, 1968), black-eyed pea (Gennis and Cantor, 1976a), and lima bean (Haynes and Feeney, 1967), have a tendency to self-associate, forming dimers or tetramers. The apparent higher molecular weights observed for the red kidney bean inhibitors under nondenaturing conditions are likely due to such self-association. The tendency of self-association for the red kidney bean inhibitors must be quite strong since, apparently, the inhibitors existed primarily in dimer form even at a concentration as low as 2 μM. Inhibitors R(B2) and R(C) appeared to exist as dimers even in the complexes with chymotrypsin.

The dimer formation may sterically block the chymotrypsin binding site on one monomer, which may be the reason why the numbers of chymotrypsin bound to R(B2) and R(C) determined by activity titration are less than unity (Table IV). Gennis and Cantor (1976b) reported that black-eyed pea proteinase inhibitors exist in dimer form in complexes with trypsin and chymotrypsin when the inhibitors were in excess. Our observation suggests that the existence of inhibitor dimer in an enzyme–inhibitor complex in excess of inhibitor may be general to self-associating proteinase inhibitors.

The reason SDS–PAGE gives higher molecular weights for the proteinase inhibitors is not clear. Dimerization of inhibitors after disulfide bond cleavage and SDS treatment is extremely unlikely. Oxidation of cysteine during the course of electrophoresis is probably the reason for broad bands in gel but not the reason for slower migrating bands (with apparent higher molecular weights) because inhibitors with all the disulfide bonds cleaved and thiol groups blocked by treatment with NTSB reagent gave sharper protein bands but with similar higher molecular weights (results not included). Whether these inhibitors inherently bind less SDS and hence migrate slower needs to be investigated. Meanwhile, one should be cautious in applying this technique for the molecular weight determination of cystine-rich, low molecular weight proteinase inhibitors.

Three of the inhibitors inhibit both trypsin and chymotrypsin. Inhibition of the two enzymes is independent, indicating separate reactive sites for the two enzymes. Bowman–Birk-type inhibitors, besides being small in M_r and high in cystine content as contrasted to Kunitz-type inhibitors, are also characterized as having two independent reactive sites in two domains. Kunitz inhibitors have been shown to have only one reactive site; they bind to trypsin but not chymotrypsin, although recently Boesterling and Quast (1981) showed that there is a chymotrypsin binding site on soybean Kunitz inhibitor which binds much less tightly ($K_d = 10^{-6}$ M) than the trypsin binding site ($K_d = 10^{-11}$ M). The four inhibitors from red kidney bean have similar amino acid compositions and molecular properties as Bowman–Birk-type inhibitors, and at least three of the inhibitors are double-headed; therefore, they appear to belong to the family of Bowman–Birk inhibitors.

The K_d values of the inhibitor–enzyme complexes indicated that binding of the inhibitor to both trypsin and chymotrypsin was very tight. The binding is less tight than that of the Kunitz inhibitors from soybean (10^{-11} M), but tighter than the soybean Bowman–Birk inhibitor (10^{-7}–10^{-8} M). Except R(B2), all the inhibitors bind to trypsin tighter than to chymotrypsin. However, since K_d values of the inhibitors to trypsin and to chymotrypsin were determined at different pHs and ionic strengths, which was shown to affect the binding in our study (results not included), the K_d values to the two enzymes are not comparable.

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Received for review August 10, 1989. Revised manuscript received January 30, 1990. Accepted March 5, 1990.