Homology among Trypsin/Chymotrypsin Inhibitors from Red Kidney Bean, Brazilian Pink Bean, Lima Bean, and Soybean

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Three trypsin/chymotrypsin inhibitors [R(A), R(B2), R(C)] from red kidney bean (RKB; *Phaseolus vulgaris* var. Linden) and three [B(A), B(B), B(C)] from Brazilian pink bean (BPB; *P. vulgaris* var. Rosinha G2) inhibited one trypsin and one chymotrypsin/mol simultaneously. One RKB inhibitor [R(B1)] inhibited one trypsin/mol. Dissociation constants of trypsin- and chymotrypsin–inhibitor complexes ranged from 0.48 to 5.37 nM. High homology was shown among the seven inhibitors by tryptic and chymotryptic peptide maps by using anion-exchange and reverse-phase chromatographies. The inhibitors also showed significant homology with lima bean and soybean Bowman–Birk inhibitors but not with soybean Kunitz inhibitor. The N-terminal 16 amino acid sequence of inhibitor B(B) showed high homology with several Bowman–Birk inhibitors. A convenient peptide mapping procedure especially suitable for cysteine-rich proteinase inhibitors and analysis of peptide mapping data are described. The procedure and data analysis method provide an approach to assess homology among proteins without sequence data.

INTRODUCTION

Proteinase inhibitors isolated from legumes have been generally classified into two families on the basis of their molecular weights and cystine contents (Odani and Ikenaka, 1982). The best studied inhibitors in the two families are the Kunitz and Bowman–Birk inhibitors from soybean. The complete amino acid sequences of the two inhibitors were reported in 1972 and 1973 (Odani and Ikenaka, 1972; Koide and Ikenaka, 1973). Complete amino acid sequences of a number of inhibitors in each family are also known. Examples of those in the Kunitz inhibitor family are the trypsin inhibitors from winged bean (*Psophocarpus tetragonolobus*; Yamamoto et al., 1983) and from *Erythrina latissima* (Joubert et al., 1985). Examples in the Bowman–Birk inhibitor family are inhibitors from lima bean (Stevens et al., 1974), garden bean (*Phaseolus vulgaris*; Wilson and Laskowski, 1975), azuki bean (Yoshikawa et al., 1979; Kiyohara et al., 1981), and mung bean (Wilson and Chen, 1983). High homology among inhibitors in the same family (especially inhibitors from the Bowman–Birk inhibitor family) was observed.

Trypsin/chymotrypsin inhibitors from Brazilian pink beans (*P. vulgaris* L. var. Rosinha G2) and red kidney beans (*P. vulgaris* var. Linden) have been purified and partially characterized (Whitaker and Sgarbieri, 1981; Sgarbieri and Whitaker, 1981; Wu and Whitaker, 1990). They all have similar amino acid composition and other molecular properties. In this paper, we describe comparative studies on these inhibitors to show that these inhibitors are closely related, with high homology.

Homology among proteins of different plant species provides important information to the study of biology and plant sciences. Such information is used to learn the evolutional processes of proteins of different organisms. For example, the degree of homology of cytochrome *c* from different plants and animals is used to evaluate the closeness of different species in the evolutional process. The information on primary structures of plant proteinase inhibitors enabled Weder (1985) to construct phylogenetic relationships among different species and varieties of legumes. The phylogenetic information can be used in plant taxonomy and may be important for plant breeding. However, determination of primary structures of proteins is very time-consuming and often requires sophisticated instruments. Weder (1981, 1985), therefore, employed electrophoretic patterns of proteinase inhibitors and the activity ratios of trypsin inhibitor/chymotrypsin inhibitor to survey large numbers of legumes and used the information in their taxonomy. Here we also describe a convenient procedure for peptide mapping, suitable for proteinase inhibitors of high cysteine contents. It should provide a complementary method for comparison of proteinase inhibitors in addition to electrophoresis patterns and amino acid sequences.

MATERIALS AND METHODS

Materials. Three inhibitors were purified from Brazilian pink beans (var. Rosinha G2) according to the method of Whitaker and Sgarbieri (1981) and were designated B(A), B(B), and B(C). Four inhibitors were purified from red kidney beans (var. Linden) as described earlier (Wu and Whitaker, 1990) and were designated R(A), R(B1), R(B2), and R(C). These inhibitors are homogeneous as judged by polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulfate, isoelectric focusing, and FPLC anion-exchange chromatography on a Mono-Q column.

Bovine pancreas trypsin (type III), N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BANA), *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA), acrylamide, sodium dodecyl sulfate, and 5,5′-dithiobis(2-nitrobenzoic acid) were from Sigma Chemical Co. α-Chymotrypsin was from Worthington Biochemical Corp. *N,N′*-Methylenebis(acrylamide) and ammonium persulfate were from Bio-Rad Laboratories. All the chemicals were of analytical grade. Deionized water was used in all the experiments.

Methods. *Trypsin and α-Chymotrypsin Assays.* Trypsin and chymotrypsin activities in the absence and presence of inhibitors were determined at 30 °C by using N^{α}-benzoyl-DL-arginine *p*-nitroanilide and *N*-glutaryl-L-phenylalanine *p*-nitroanilide as substrates, respectively (Erlanger et al., 1961, 1966).

Determination of Binding Stoichiometry. The binding stoichiometry of the inhibitors to different enzymes was determined by activity titration. The determination of molar concentrations of inhibitors and enzymes was described earlier (Wu and Whitaker, 1990).

K_d Determination. The inhibition constants of inhibitors to trypsin and chymotrypsin were determined according to the method of Bieth (1974) using *N*-CBZ-L-tyrosine *p*-nitrophenyl ester (ZTNE) and N^{α}-CBZ-L-lysine *p*-nitrophenyl ester (ZLNE) as the substrates of chymotrypsin and trypsin, respectively. These nitrophenyl ester substrates provided the needed sensitivity to

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determine K_d values of $\sim 10^{-10}$ M, unlike the anilide substrates used previously (Wu and Whitaker, 1990).

Cleavage of Disulfide Bonds and Blocking of Half-Cystine Residues of the Inhibitors. The inhibitors had to be denatured before tryptic and chymotryptic digestion occurred. Denaturation of the inhibitors was carried out by cleaving the disulfide bonds and blocking the half-cystine residues according to a method similar to that described by Thannhauser and Scheraga (1985). The procedure was as follows: inhibitors were dissolved (10 mg/mL) in a solution containing 7.5 mM 2-nitro-5-thiosulfobenzoate [prepared according to the method of Thannhauser et al. (1987)], 0.4 M sodium sulfite, and 6 M guanidine hydrochloride in 50 mM glycine buffer (pH 9.0). The solution was left to stand at room temperature for 30 min. An aliquot (0.4 mL) of 50% acetic acid was added to each milliliter of the solution, and the modified inhibitors were separated from the reagent and other compounds on a Sephadex G-25 gel filtration column (1.5 × 18 cm) equilibrated with 0.1 M acetic acid and lyophilized. This procedure completely sulfonated the inhibitor as judged by the number of disulfide bonds cleaved (Thannhauser et al., 1987) and yielded a homogeneous product as shown by a single peak in ion-exchange chromatography on FPLC. Inhibitors so modified had no remaining activity against either trypsin or chymotrypsin.

Digestion of Inhibitors. Modified inhibitors were dissolved in 0.1 M NH_4HCO_3 buffer (pH 8.0) containing 0.5 mM $CaCl_2$, and 1.5% (w/w) trypsin or chymotrypsin was added. The tryptic mixtures were incubated at 35 °C for 4 h, and the chymotryptic mixtures were incubated for 5 h. Results of preliminary studies indicated that the above lengths of incubation time were sufficient for complete digestion.

Peptide Mapping on Ion-Exchange Chromatography. One hundred micrograms of tryptic or chymotryptic digests was loaded to an analytical anion-exchange column (Mono-Q HR5/5) equilibrated with 0.02 M Tris buffer (pH 8.10). After the column was washed with 5 mL of starting buffer, a linear gradient of 0–0.7 M NaCl in the above buffer (35 mL) was applied. The eluted peptides were detected by absorbance at 214 nm. Flow rate was 1.0 mL/min.

Peptide Mapping by Reverse-Phase HPLC. Peptide mapping by reverse-phase HPLC was carried out on a Rainin ODS column (0.45 × 25 cm) or an Altex ODS column (0.45 × 25 cm). Proteolytic digests (40–60 μg) were loaded to the column equilibrated with starting buffer containing 49 mM KH_2PO_4 and 5.4 mM phosphoric acid. After a 5-min elution with the starting buffer, a linear gradient of 0–80% acetonitrile in 45 min was applied. Flow rate was 0.6 mL/min. Column temperature was 30 °C. All peptides eluted before 40 min. Eluents were monitored at 220 nm.

Determination of N-Terminal Residues. N-Terminal residues of the inhibitors were determined by using (dimethylamino)-azobenzene isothiocyanate (DABITC) as the coupling reagent as described by Chang (1983a). The DABTHs of the end-terminal amino acids were identified by two-dimensional micro-TLC analysis on 3 × 3 cm polyamide plates as described by Chang (1983b).

N-Terminal Sequencing. The amino acid sequence at the N terminus of Brazilian pink bean inhibitor B [B(B)] was determined by gas-phase protein sequencer (Applied Biosystems 470A).

RESULTS

Binding Stoichiometry and Inhibition Constants. All three Brazilian pink bean (BPB) inhibitors and three of the four red kidney bean (RKB) inhibitors inhibited both trypsin and chymotrypsin simultaneously. One RKB inhibitor [R(B1)] inhibited only trypsin. By use of specific substrates, the inhibition of trypsin could be measured in the presence of chymotrypsin and vice versa. In this way, binding of all the inhibitors to trypsin and chymotrypsin was shown to be independent. The results on stoichiometry determination indicated that inhibitor R(B1) binds only to one trypsin molecule; all the other inhibitors bind to one trypsin and one chymotrypsin molecules independently (Table I). The apparent less than unit binding of some BPB inhibitors probably indicates that the binding site to one enzyme (trypsin or chymotrypsin) can bind to another enzyme (chymotrypsin or trypsin) competitively. In the presence of excess of another enzyme, the binding to the measured enzyme would appear to be less.

Table I. Binding Stoichiometry and K_d of Red Kidney Bean Inhibitors[a] and Brazilian Pink Bean Inhibitors to Trypsin and Chymotrypsin[b]

	no. of enzymes bound/inhibitor		K_d, E-10 M	
inhibitor	Tr	CT	to Tr	to CT
R(A)	1 (1.09)[c]	1 (0.91)	7.4	8.9
R(B1)	1 (0.71)	0 (0.13)	53.7	
R(B2)	1 (0.81)	1 (0.61)	7.7	10.7
R(C)	1 (1.02)	1 (0.87)	5.9	8.5
B(A)	1 (0.55)	1 (0.87)	5.2	5.8
B(B)	1 (0.86)	1 (1.01)	7.6	16.9
B(C)	1 (0.57)	1 (0.45)	4.8	24.8

[a] Data from Wu and Whitaker (1990). [b] BANA and GPNA were used in binding stoichiometry measurement as substrates for trypsin and chymotrypsin, respectively. ZLNE and ZTNE were used in K_d measurement as substrates for trypsin and chymotrypsin, respectively. [c] Figures in parentheses are experimental values.

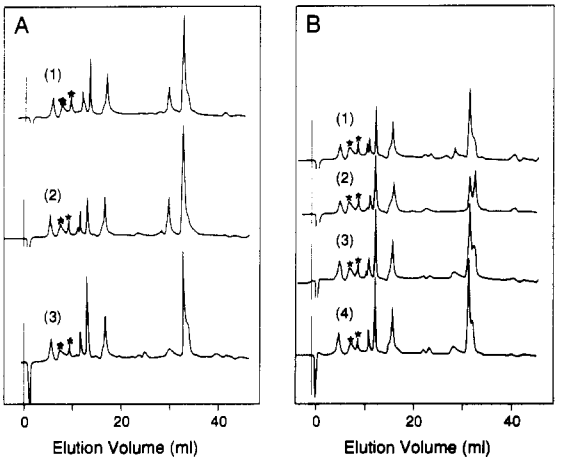

Figure 1. Tryptic peptides of BPB and RKB inhibitors on FPLC anion-exchange column (Mono-Q HR 5/5): (a) chromatograms of tryptic digests of BPB inhibitors, (1) B(A), (2) B(B), (3) B(C); (b) chromatograms of tryptic digests of RKB inhibitors, (1) R(A), (2) R(B1), (3) R(B2), (4) R(C). Peaks labeled with stars were derived from buffer.

Measurement of inhibition constants showed that binding constants of the Brazilian pink bean inhibitors to trypsin and chymotrypsin were similar to those of red kidney bean inhibitors. The dissociation constants (K_d) of trypsin–inhibitor complexes and chymotrypsin–inhibitor complexes are all in the range 0.48–5.37 nM (Table I).

Peptide Mapping of the Inhibitors. Peptide mapping of the seven inhibitors was carried out to learn whether these inhibitors, similar in molecular weight and amino acid composition, have similar amino acid sequences. Comparison of peptide maps of different inhibitors on an FPLC anion-exchange column are shown in Figures 1 and 2. The elution patterns of tryptic and chymotryptic digests of all the inhibitors are very similar on the anion-exchange column, indicating similar distribution of charged amino acid residues among the inhibitors.

Inhibitors from BPB had very similar chromatograms on the ion-exchange column to the corresponding RKB inhibitors [e.g., B(A) to R(A) and B(B) to R(B2), etc.], indicating that there was homology between inhibitors from BPB and RKB. There were bigger differences among

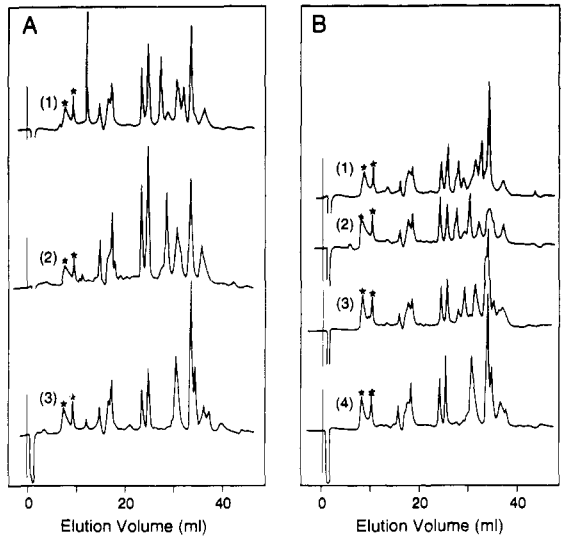

Figure 2. Chymotryptic peptides of BPB and RKB inhibitors on FPLC anion-exchange column (Mono-Q HR 5/5): (a) chromatograms of chymotryptic digests of BPB inhibitors, (1) B(A), (2) B(B), (3) B(C); (b) chromatograms of chymotryptic digests of RKB inhibitors, (1) R(A), (2) R(B1), (3) R(B2), (4) R(C). Peaks labeled with stars were derived from buffer.

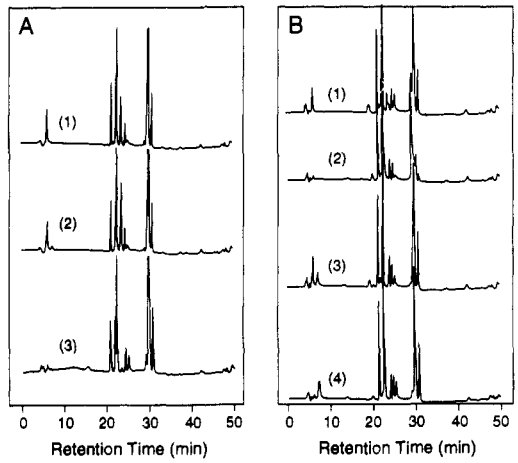

Figure 3. Tryptic peptide mapping of BPB and RKB inhibitors on RP-HPLC (Rainin ODS column): (a) comparison of chromatograms of tryptic peptides of BPB inhibitors, (1) B(A), (2) B(B), (3) B(C); (b) comparison of chromatograms of tryptic peptides of RKB inhibitors, (1) R(A), (2) R(B1), (3) R(B2), (4) R(C).

chymotryptic digests than among tryptic digests according to their FPLC chromatograms. This may be due to the lower specificity of chymotrypsin than trypsin.

The chromatograms of the inhibitor digests on the HPLC reverse-phase (RP-HPLC) column were also quite similar (Figures 3 and 4). As in the anion-exchange chromatograms, there were greater variations among chymotryptic digests than among tryptic digests and BPB inhibitors were very similar to the corresponding RKB inhibitors. Generally, the peptide maps on the RP-HPLC showed more differences among the inhibitors than on the FPLC anion-exchange column. This was partly due to the higher resolution power of RP-HPLC and partly due to different separation modes.

Pinder and Gratzer (1972) derived an equation for quantitatively determining the degree of congruence of two ribosomes by analyzing their nuclease degradation patterns by polyacrylamide gel electrophoresis. Calvert and Gratzer (1978) applied the same equation to determine the degree of similarity between two proteins by their peptide mapping patterns on polyacrylamide electrophoresis gel. Results of peptide mapping on anion-exchange chromatography and RP-HPLC can be analyzed by the same method. Table II shows an analysis of the common peaks in peptide mapping among the BPB inhibitors. Chromatography of tryptic peptides on a Mono-Q anion-exchange column indicated that six of the eight peaks of inhibitor B(B) and six of the seven peaks of inhibitor B(C) had retention times identical with those of the peaks of inhibitor B(A) (Table II). In a similar manner, chromatography of chymotryptic peptides on the Mono-Q column indicated that 8 of the 10 peaks of inhibitor B(B) and 9 of the 12 peaks of inhibitor B(C) had retention times identical with those of peaks of inhibitor B(A). Similar

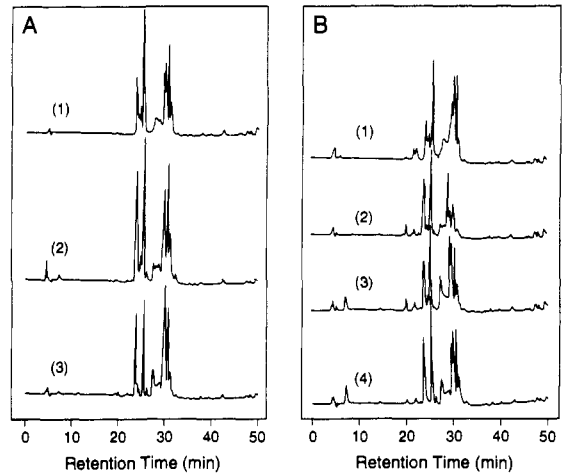

Figure 4. Chymotryptic peptide mapping of BPB and RKB inhibitors on RP-HPLC (Rainin ODS column): (a) comparison of chromatograms of chymotryptic peptides of BPB inhibitors, (1) B(A), (2) B(B), (3) B(C); (b) comparison of chromatograms of chymotryptic peptides of RKB inhibitors, (1) R(A), (2) R(B1), (3) R(B2), (4) R(C).

Table II. Statistical Comparison of Chromatographic Patterns from Peptide Mapping of BPB Inhibitors[a]

chromatogr method	inhibitor	no. of peaks	no. of common peaks[c]	$P[X \geq X(\text{obs})]$[b] $N = 40$	$N = 60$
Mono-Q, tryptic	B(A)	7			
	B(B)	8	6	4.8E-5	
	B(C)	7	6	1.3E-5	
Mono-Q, chymotryptic	B(A)	12			
	B(B)	10	8	2.3E-4	
	B(C)	12	9	1.3E-4	
HPLC, tryptic	B(A)	8			
	B(B)	7	7		2.1E-8
	B(C)	8	5		5.0E-4
HPLC, chymotryptic	B(A)	8			
	B(B)	10	7		2.4E-6
	B(C)	8	7		1.6E-7

[a] Analysis of results in Figures 1–4 to show statistical significance and degree of similarity among BPB inhibitors; all comparisons were made to B(A). [b] Total probability of finding the degree of congruence equal to or higher than the observed values on a random basis. The probabilities were calculated by using the equation [derived by Pinder and Gratzer (1972)]

$$P(X) = [m!n!(N-m)!(N-n)!]/[N!x!(m-x)!(n-x)! \times (N-m-n+x)!]$$

where m and n are the numbers of peaks in each of the two chromatograms for comparison, x is the number of common peaks, and N is the maximum number of statistically resolved and distinguishable peaks in a chromatogram. [c] For FPLC anion-exchange chromatography peaks within ±1% of total elution time were considered the same. For HPLC, peaks that differ in less than ±0.15 min in retention time were considered the same.

Table III. Statistical Comparison of Chromatographic Patterns from Peptide Mapping of RKB Inhibitors[a]

chromatogr method	inhibitor	no. of peaks	no. of common peaks[c]	$P[X \geq X(\text{obs})]^b$ $N = 40$	$N = 60$
Mono-Q, tryptic	R(A)	7			
	R(B1)	7	5	5.9E-4	
	R(B2)	8	7	4.3E-7	
	R(C)	6	6	1.8E-6	
Mono-Q, chymotryptic	R(A)	12			
	R(B1)	13	9	3.7E-4	
	R(B2)	12	9	1.3E-4	
	R(C)	10	7	3.1E-3	
HPLC, tryptic	R(A)	10			
	R(B1)	9	5		3.3E-3
	R(B2)	11	8		2.0E-4
	R(C)	8	6		2.1E-4
HPLC, chymotryptic	R(A)	10			
	R(B1)	8	4		1.9E-2
	R(B2)	11	8		2.6E-6
	R(C)	10	7		6.4E-4

[a] Analysis of results in Figures 1–4 to show statistical significance and degree of similarity among RKB inhibitors; all comparisons were made to inhibitor R(A). [b] Total probability of finding the degree of congruence equal to or higher than the observed values on a random basis; same as defined in footnote *b* for Table II. [c] For FPLC anion-exchange chromatography peaks within ±1% of total elution time were considered the same. For HPLC, peaks, that differ in less than ±0.15 min in retention time were considered the same.

comparisons are shown for the tryptic and chymotryptic peptides separated by HPLC chromatography. The high degree of chromatographic similarity among the inhibitors, relative to inhibitor B(A), is also indicated by $P[X \geq X(\text{obs})]$. The smaller the P value, the more similar are the chromatographic elution patterns of the two proteins. Therefore, inhibitor B(B) showed less chromatographic similarity with inhibitor B(A) than did inhibitor B(C) by Mono-Q chromatography of tryptic and chymotryptic peptides and by HPLC of chymotryptic peptides. However, by HPLC of tryptic peptides, inhibitor B(C) was less similar to inhibitor B(A) than was inhibitor B(B). In all cases, however, the similarity was highly significant, indicating existence of high homology.

Similar analyses of the peptides for the RKB inhibitors are given in Table III. In all cases, there is a high degree of similarity between the other inhibitors and inhibitor R(A). Least similarity seemed to be between inhibitors R(B1) and R(A). The largest difference between inhibitors R(B1) and R(A) was given by the HPLC of chymotryptic peptides; only four of the eight peaks of inhibitor R(B1) were identical in retention time with those of inhibitor R(A).

Comparison between similar inhibitors of the BPB and RKB by HPLC of tryptic and chymotryptic peptides is shown in Table IV. Inhibitors R(A) and B(A) show much chromatographic similarity. The eight peaks of tryptic digest of inhibitor B(A) all have identical retention time with 8 of the 10 peaks of inhibitor R(A). Six of the 8 peaks of chymotryptic digest of inhibitor B(A) have the same retention time as 6 of the 10 peaks of inhibitor R(A). By similar comparisons, inhibitors R(B1) and B(B) show similarity to inhibitor R(B2); inhibitor B(C) shows similarity to inhibitor R(C). All the comparisons were with higher than 99.9% confidence (P values lower than 0.001; Table IV), clearly indicating that the inhibitors from the two beans are homologous and thus closely related. Interestingly, inhibitor R(B1) shows less homology to inhibitor R(B2) than does inhibitor B(B). Such a result

Table IV. Statistical Comparison of HPLC Peptide Mapping Patterns between RKB and BPB Inhibitors[a]

method of digestion	inhibitor	no. of peaks	no. of common peaks[c]	$P[X \geq X(\text{obs})]^b$ $N = 40$	$N = 60$
tryptic	R(A)	10			
	B(A)	8	8	5.9E-7	1.8E-8
	R(B2)	11			
	R(B1)	8	5	2.2E-2	3.3E-3
	B(B)	7	6	7.2E-4	5.9E-5
	R(C)	8			
	B(C)	8	6	1.8E-4	1.5E-5
chymotryptic	R(A)	10			
	B(A)	8	6	1.2E-3	1.0E-4
	R(B2)	11			
	R(B1)	8	5	2.2E-2	3.3E-3
	B(B)	10	8	7.9E-5	2.6E-6
	R(C)	10			
	B(C)	8	8	5.9E-7	1.8E-8

[a] Analysis of results in Figures 3 and 4 to show statistical significance and degree of similarity between RKB and BPB inhibitors. [b] Total probability of finding the degree of congruence equal to or higher than the observed values on a random basis; same as defined in footnote *b* for Table II. [c] Peaks that differ in less than ±0.15 min in retention time were considered the same.

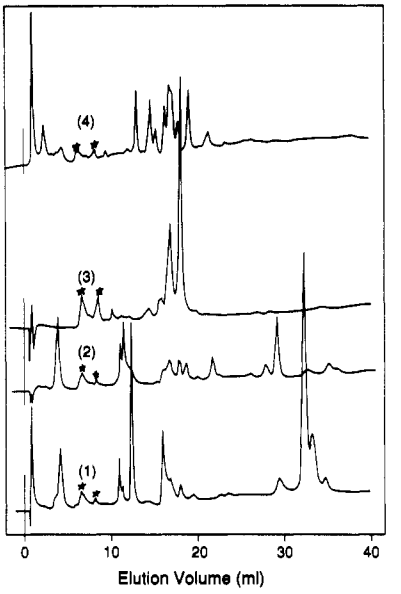

Figure 5. Tryptic peptide mapping of different proteinase inhibitors by FPLC anion-exchange chromatography (Mono-Q column, HR5/5): (1) tryptic peptides of B(B); (2) tryptic peptides of BBI; (3) tryptic peptides of LBI; (4) tryptic peptides of STI. Peaks labeled with stars were derived from buffer.

is consistent with the fact that, although R(B1) and R(B2) have very similar charged groups and behaved similarly on isoelectric focusing and ion-exchange chromatography, they differ significantly in their surface hydrophobicity since they can be separated by hydrophobic interaction chromatography (Wu and Whitaker, 1990).

Peptide mapping of two Bowman–Birk inhibitors from soybean and lima bean and one Kunitz inhibitor from soybean, together with one BPB inhibitor [B(B)], was also carried out on both anion-exchange chromatography and RP-HPLC. Their chromatograms are compared in Figures 5 and 6. There is some similarity between B(B) and the Bowman–Birk inhibitors from soybean and lima bean, although the similarity is much less pronounced than that among the BPB and RKB inhibitors. The patterns of

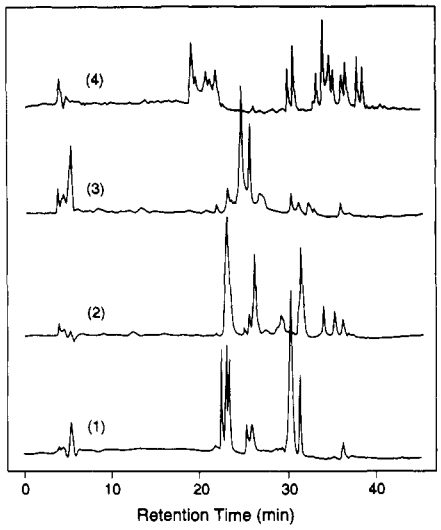

Figure 6. Tryptic peptide mapping of different proteinase inhibitors by RP-HPLC (Altex ODS column): (1) tryptic peptides of B(B); (2) tryptic peptides of BBI; (3) tryptic peptides of LBI; (4) tryptic peptides of STI.

Table V. Statistical Comparison of Chromatographic Patterns of Tryptic Peptides of Different Inhibitors[a]

chromatogr method	inhibitor	no. of peaks	no. of common peaks[c]	$P[X \geq X(\text{obs})]$[b] $N = 40$	$N = 60$
FPLC	B(B)	10			
	BBI	10	5	4.2E-2	7.1E-3
	LBI	5	3	7.9E-2	2.7E-2
	STI	12	2	2.4E-1	3.3E-1
HPLC	B(B)	9			
	BBI	9	5	1.4E-2	2.1E-3
	LBI	10	6	3.1E-3	2.8E-4
	STI	15	3	2.9E-1	2.5E-1

[a] Analysis of results in Figures 5 and 6 to show statistical significance and degree of similarity among different inhibitors. [b] Total probability of finding the degree of congruence equal to or higher than the observed values on a random basis; same as defined in footnote *b* for Table II. [c] For FPLC anion-exchange chromatography peaks within ±1% of total elution time were considered the same. For HPLC, peaks that differ in less than ±0.15 min in retention time were considered the same.

soybean trypsin inhibitor (Kunitz) are completely different from those of the other inhibitors.

Table V shows the analysis of the results on peptide mapping of the four inhibitors from different families. Inhibitor B(B) certainly belongs to the Bowman–Birk inhibitor family since the analysis indicates that it has some homology to both soybean proteinase inhibitor (Bowman–Birk) and lima bean trypsin inhibitor but no homology to soybean trypsin inhibitor (Kunitz).

N-Terminal Amino Acid Residue and Sequence. N-Terminal determination was carried out on native R(C), B(B), and B(C). The results showed that serine was at the N terminus of all three inhibitors.

N-Terminal sequencing was carried out on inhibitor B(B) to 16 cycles. Figure 7 compares the sequence of the 16 amino acid residues with other inhibitors from the Bowman–Birk inhibitor family. The N-terminal sequence of inhibitor B(B) shows high homology to several inhibitors from the Bowman–Birk inhibitor family, giving additional evidence that the inhibitors from BPB and RKB belong to the Bowman–Birk inhibitor family. This also reconfirms the conclusion drawn on the basis of analysis of results from peptide mapping (Tables II–V).

DISCUSSION

Proteinase inhibitors from legumes can generally be grouped into two families: Kunitz inhibitor family and Bowman–Birk inhibitor family (Laskowski and Kato, 1980; Sgarbieri and Whitaker, 1982; Read and James, 1986; Garcia-Olmedo et al., 1987). Kunitz inhibitors are relatively high in molecular weight (above 20 000), are low in cystine content, and have only one reactive site, whereas Bowman–Birk inhibitors are characterized as double-headed inhibitors (inhibiting either two different enzymes or two molecules of the same enzyme) with low molecular weights (8000–13 000) and a high cystine content.

All seven RKB and BPB inhibitors have amino acid composition similar to that of the inhibitors in the Bowman–Birk inhibitor family (Sgarbieri and Whitaker, 1981; Wu and Whitaker, 1990). Similar to RKB inhibitors (Wu and Whitaker, 1990), BPB also gave higher molecular weights as determined under native conditions (results not included). This should be due to formation into dimers under the conditions. Molecular weights of completely denatured inhibitors are in the range 11 000–12 000, similar to other inhibitors in the Bowman–Birk inhibitor family. The previously reported 20 000 molecular weights for BPB inhibitors by SDS–PAGE (Whitaker and Sgarbieri, 1981) were consistent with the observation for RKB inhibitors (Wu and Whitaker, 1990) and are believed not to reflect their real molecular weights. Since soybean Bowman–Birk inhibitor and lima bean inhibitor both showed higher apparent molecular weights on SDS–PAGE following the same procedure as for the RKB and BPB inhibitors, the higher molecular weights on SDS–PAGE may be inherent properties of the Bowman–Birk inhibitor family.

Many proteinase inhibitors from legumes have been studied; however, the fact that the complete amino acid sequences of only a small portion of these inhibitors are known implies that much of the classification/subclassification of the proteinase inhibitors to date is based on their molecular weights, amino acid composition, and information from their inhibitory activities and immunochemical interactions (Sgarbieri and Whitaker, 1982; Tan-Wilson et al., 1987). Chromatographic separation of protein fragments, i.e., peptide mapping, can be used for further characterization and even identification of proteins as well as to answer questions about identity of closely related proteins (Prestidge, 1984; Schroeder, 1984). Peptide mapping has also been used to study the similarities and differences of closely related proteins or subunits of proteins and to identify various posttranslational modifications of proteins (Ma et al., 1980; Roberts et al., 1982; Laber et al., 1989). Therefore, it should be an ideal method for comparison of different proteinase inhibitors and for classification of proteinase inhibitors into different families and subgroups within a family.

Proteinase inhibitors from legumes, especially those of the Bowman–Birk inhibitor family, have a large number of disulfide bonds and very compact structures. Therefore, not only is digestion of native inhibitors understandably impossible [except at the binding site(s)] with the enzymes they inhibit (such as trypsin), but complete digestion of native inhibitors by other proteinases is very difficult. A common approach to peptide mapping of proteinase inhibitors is to reduce all the disulfide bonds and then modify the thiol groups by S-carboxymethylation (Wilson and Laskowski, 1975; Kato et al., 1987; Ary et al., 1988). We adopted another approach: cleave the disulfide bonds and block the thiol groups with sulfite groups in one step by the NTSB reagent. The S-sulfo-modified proteins are relatively stable at neutral and acid pH and have good

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B(B)   Ser-Gly-His-Arg-His-Glu-Ser-Thr-Asp-Glu-Pro-Ser-Glu-Ser-Ser-Lys
LBI    Ser-Gly-His-His-Glu-His-Ser-Thr-Asp-Glx-Pro-Ser-Glx-Ser-Ser-Lys
ABI1   Ser-Gly-His-His-Asp-Glu-Thr-Thr-Asp-Glu-Pro-Ser-Glu-Ser-Ser-Lys
ABI2   Ser-Val-His-His-Asp-Asp-Ser-Ser-Asp-Glu-Pro-Ser-Glu-Ser-Ser-His
MBI    Ser-Ser-His-His-His-Asp-Ser-Ser-Asp-Glu-Pro-Ser-Glu-Ser-Ser-Glu
DE3            Glu-His-His-His-Ser-Thr-Asp-Glu-Pro-Ser-Glu-Ser-Ser-Lys
DE4                His-Glu-His-Ser-Ser-Asp-Glu-Ser-Ser-Glu-Ser-Ser-Lys
GBI                                        Glx-Pro-Ser-Glx-Ser-Ser-Pro
BBI                                        Asp-Asp-Glu-Ser-Ser-Lys
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Figure 7. N-Terminal sequence of inhibitor B(B) and comparison with the N-terminal sequences of other Bowman–Birk inhibitors from beans. The homologous residues of the other inhibitors to inhibitor B(B) are in bold type. LBI is inhibitor IV-IV′ from lima bean (Stevens et al., 1974); ABI1 is inhibitor I-II from azuki bean (Yoshikawa et al., 1979); ABI2 is inhibitor I-A,A′ from azuki bean (Kiyohara et al., 1981); MBI is inhibitor from mung bean (Wilson and Chen, 1983); DE3 and DE4 are inhibitors of *Macrotyloma axillare* (Joubert et al., 1979); GBI is inhibitor II′ from garden bean (Wilson and Laskowski, 1975); BBI is the Bowman–Birk inhibitor from soybean (Odani and Ikenaka, 1972).

solubility due to the added charged groups (Pechere et al., 1958; Thannhauser et al., 1987). Thannhauser et al. (1985) developed a two-dimensional technique in peptide mapping for determination of disulfide pairing using sulfonated protein as starting material. Our work showed that this is a convenient procedure for peptide mapping of cystine-rich proteinase inhibitors. One potential advantage of this procedure is that the blocked thiol groups can be released and will again form disulfide bonds by treatment with a redox buffer at pH above 8, making it an ideal procedure for recovering fragments with activity (Wu and Whitaker, unpublished results).

The statistical analysis for peptide mapping patterns appears to be a valid approach to assess the degree of similarities between two proteins. In their derivation, Pinder and Gratzer (1972) and Calvert and Gratzer (1978) assumed random distribution of size of RNA or peptide fragments on electrophoresis gels. To apply their equation in assessing the degree of homology in chromatographic peptide mapping, one also needs to assume random distribution of the peptide peaks in the chromatograms. This is, however, not necessarily true. In chromatography using gradient elution, very often the peaks are eluted in a certain range of the gradient. There is a very low probability of finding peaks outside the gradient range. Furthermore, increasing the gradient slope will compress the area where the peaks elute. Therefore, a judgment has to be made on the size of the gradient range for complete elution of the peptides, thus determining the maximum number of peaks that might be resolved in that region.

The P values from the equation of Pinder and Gratzer (1972) are very dependent on N, which is essentially a measure of resolution of the chromatographic methods. The figures we used for the calculation of P values (N = 40 for FPLC anion-exchange chromatography and N = 60 for RP-HPLC, on the basis of their resolution ability and gradient ranges) are, in our opinion, minimum numbers for N. The P values calculated on the basis of these figures, therefore, are quite conservative.

The results of peptide mapping indicated that all the RKB and BPB inhibitors were homologous in their amino acid sequences. The inhibitors in this study also have similar amino acid compositions and molecular weights (Whitaker and Sgarbieri, 1981; Sgarbieri and Whitaker, 1981; Wu and Whitaker, 1990), all of which indicate these inhibitors are members of one family. All the inhibitors except inhibitor R(B1) inhibit both trypsin and chymotrypsin and have binding constants similar to those of trypsin and chymotrypsin. The much greater similarity in peptide mapping patterns among the seven inhibitors than that between those inhibitors and two inhibitors from Bowman–Birk inhibitor family suggests that the BPB and RKB inhibitors are very closely related and may belong to one subclass in a family.

Inhibitor R(B1) does not inhibit chymotrypsin, and its binding to trypsin is looser than that of the other six inhibitors isolated in this study, apparently not fitting into the traditional double-headed model for Bowman–Birk inhibitor family. However, on the basis of its amino acid composition, molecular weight, and chromatographic similarities in peptide mapping to the other six inhibitors, inhibitor R(B1) and the other six inhibitors should belong to one family. The lack of chymotrypsin inhibitory activity may be due to the inhibitor having two domains, one of which is active not against chymotrypsin but another enzyme. This would be similar to the inhibitor II from garden bean which belongs to the Bowman–Birk inhibitor family on the basis of its double-headed structure and homology to other Bowman–Birk type inhibitors (Wilson and Laskowski, 1975; Odani and Ikenaka, 1982). It is also possible that one of the domains in inhibitor R(B1) has a putative reactive site. Although domains with an inactive reactive site have not been reported in inhibitors of the Bowman–Birk family, they have been observed in some ovomucoid and ovoinhibitors (Laskowski et al., 1978; Laskowski and Kato, 1980; Kato et al., 1987).

The analytical comparison of one BPB inhibitor with two Bowman–Birk inhibitors and one Kunitz inhibitor showed that BPB inhibitors have significant homology to inhibitors of the Bowman–Birk family and no homology to the Kunitz inhibitor. The result is consistent with the same conclusion drawn from comparison of other properties (Wu and Whitaker, 1990) and sequences (Figure 7).

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