J. Biochem. 124, 457-461 (1998)

The Amino Acid Sequences of Isoforms of the Bromelain Inhibitor from Pineapple Stem[1]

Ken-ichi Hatano,* Masaru Tanokura,† and Kenji Takahashi‡,[2]

*Department of Biophysics and Biochemistry, Graduate School of Science, and †Biotechnology Research Center, The University of Tokyo, Bunkyo-ku, Tokyo 113-0034; and ‡School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392

Received for publication, April 9, 1998

Eight bromelain inhibitor (BI) isoform fractions were isolated from pineapple stem, and then submitted to conventional amino acid sequencing after performic acid oxidation and subsequent separation of the resulting light and heavy chains. The results revealed that all fractions exhibited microheterogeneity, containing at least two major components, but that all the isoinhibitors have a common double-chain structure (M_r = *ca.* 5,700-5,900) with five disulfide bonds and similar amino acid sequences. Notably, Fraction BI-VIII exhibited less than 40% of the specific inhibitory activity toward stem bromelain as compared with the other inhibitor fractions. This fraction was a mixture of two isoforms, BI-VIII(1) and BI-VIII(2), the latter lacking the arginine or glutamine residue at the COOH-terminus of the light chain. Furthermore, the oxidized light chain of BI-III, used as a representative normal isoinhibitor, was found to exhibit significant inhibitory activity, whereas the oxidized light chain of BI-VIII(2) lacking the COOH-terminal Arg or Gln showed only very low inhibitory activity. Therefore, the major bromelain-inhibitory site was indicated to be the COOH-terminal residue, Arg or Gln, of the light chain. This is consistent with the three-dimensional structure model constructed by computer modeling for the hypothetical complex between BI-VI and papain, a close homolog of bromelain.

Key words: amino acid sequence, bromelain inhibitor, computer modeling, cysteine proteinase inhibitor, isoinhibitor.

Bromelain inhibitor (BI) in pineapple stem is one of the cysteine proteinase inhibitors, which inhibits stem bromelain most strongly among various proteinases (*1*). It comprises various isoinhibitors and was separated into at least seven isoform fractions by DEAE-Sephadex chromatography (*2, 3*). The primary structure was first reported by Reddy *et al.* (*2*) for bromelain isoinhibitors in Fraction BI-VII, which were each shown to be composed of a 10 to 11-residue light chain and a 41-residue heavy chain cross-linked by five disulfide bonds. More recently, we determined the primary structure of another isoinhibitor, BI-VI (*3*), and elucidated its disulfide pairing, and secondary and tertiary structures by nuclear magnetic resonance (NMR) spectroscopy (*3-5*). The results led us to the unexpected finding that BI-VI exhibits structural similarity with the Bowman-Birk trypsin/chymotrypsin inhibitor from soybeans, suggesting that they might have evolved from a common ancestor (*4*). On the other hand, BI-VI exhibited little similarity in structure with known cysteine proteinase inhibitors, such as cystatin (*6*).

The primary structures of other isoinhibitor fractions, however, remained undetermined, and it was thought to be necessary to elucidate all the primary structures of the major isoinhibitors to understand the overall structural relationship and the reason for the extensive microheterogeneity of bromelain inhibitors (BIs). The present study was undertaken accordingly, and the primary structures of essentially all the major BI fractions obtained on DEAE-Sephadex chromatography were analyzed. The results revealed the occurrence of over twenty major isoinhibitors in pineapple stem. This paper describes the results and discusses the structural basis of this microheterogeneity together with the nature of the inhibitory site of BI.

EXPERIMENTAL PROCEDURES

Materials—The 4-methylcoumarin-7-amide (MCA) derivative of *tert*-butyloxycarbonyl (Boc)-Leu-Arg-Arg and leupeptin were purchased from Peptide Institute. Acetone powder of pineapple stem was obtained from Sigma Chemical. All other chemicals were of analytical grade.

Purification of Bromelain Inhibitors—From a crude extract of the acetone powder of pineapple stem, BIs were isolated by gel filtration on a Sephadex G-50 column (2.8× 120 cm), and fractionated by ion-exchange chromatogra-

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed: Phone: +81-426-76-7146, Fax: +81-426-76-7149

Abbreviations: BI, bromelain inhibitor; Boc, *tert*-butyloxycarbonyl; HPLC, high performance liquid chromatography; MCA, 4-methylcoumaryl-7-amide; H, heavy chain; L, light chain; NMR, nuclear magnetic resonance; 3D, three-dimensional.

phy on a DEAE-Sephadex column (2.2×40 cm) essentially as described previously (*3*). During the course of purification, the inhibitory activity was determined essentially by the method of Heinrikson and Kézdy (*7*).

Amino Acid Sequence Determination—A sample (0.4 mg) of each BI fraction was oxidized with performic acid (*8*), and the resulting two chains were separated by reversed-phase HPLC as reported previously (*3*). Amino acid analysis was performed with a derivatizer/analyzer model 421/172 (Applied Biosystems). Amino acid sequences were determined with a protein sequencer model 473A/476A (Applied Biosystems).

Assaying of Inhibitory Activity toward Stem Bromelain—The inhibitory activities of each BI fraction, and its oxidized light and heavy chains were measured by the following method, which exhibited higher sensitivity and accuracy than the previously used method (*7*). This assay was performed at 25°C. To a solution (1.485 ml) comprising 1 mM 2-mercaptoethanol, 40 nM preactivated enzyme, and 10 μM inhibitor in McIlvaine's wide-range buffer, pH 4.6, was added 15 μl of 10 mM Boc-Leu-Arg-Arg-MCA in dimethyl sulfoxide. The increase in fluorescence intensity was recorded at intervals of 10 s for 10 min to determine the amount of 7-amino-4-methylcoumarin released, with an excitation wavelength of 370 nm and an emission wavelength of 460 nm, and a Hitachi fluorescence spectrophotometer F-2000.

Computer Modeling of the Three-Dimensional (3D) Structure of a BI-VI·Papain Complex—The 3D structure of a BI-VI·papain complex was constructed based on the results of X-ray crystallographic analysis of a papain·leupeptin complex (*9*). The coordinates for BI-VI and papain were derived from the Brookhaven Protein Data Bank under the file names, 1BI6 and 1POP, respectively. Construction of iterative visual models was carried out with an IRIS Indigo^2 color graphics workstation using the software package of MidasPlus (UCSF Computer Graphics Laboratory). Assuming the same interaction mode as that of leupeptin, the COOH-terminus of the light chain of BI-VI was located near the sulfur atom of the active site Cys^{25} of papain, and the hydrophobic side chain of Leu^{10} in the light chain was close to those of Val^{133} and Val^{157} in the S_2 hydrophobic pocket (*9*) of papain, so that the former came in van der Waals contact with the latter.

RESULTS AND DISCUSSION

Isolation of Bromelain Isoinhibitors, and Separation of the Light and Heavy Chains—The elution pattern of BIs from a DEAE-Sephadex column is shown in Fig. 1. The result was roughly consistent with that reported in our previous paper (*3*), except that the peaks of BI-III and IV appeared to be much higher than those reported previously, and that additional minor protein peaks (designated as BI-IV′ and BI-VIII) were separated from the BI-IV and BI-VII peaks, respectively. This BI-VIII fraction exhibited much lower specific inhibitory activity than the other isoinhibitor fractions. The differences in elution pattern may be due to differences in the lots and the amounts of the starting material used and/or the slower flow rate used in the present study. At this stage, each BI fraction appeared to be homogeneous, as judged on SDS-PAGE (data not shown), although all these fractions were shown to be heterogeneous in the subsequent sequence studies. Further separation of the multiple isoinhibitors in each fraction, however, has been difficult so far.

Each BI fraction was then oxidized with performic acid to dissociate each inhibitor molecule into light and heavy chains. The resulting chains were separated from each other by HPLC. The HPLC pattern of each oxidized BI fraction was similar to that of BI-VI described previously (*3*), except that Fraction BI-VIII yielded two separate light chain peaks, designated as L-VIII(1) and L-VIII(2), and that Fraction V failed to give any discrete peaks of peptides for some unknown reason. For Fraction V, therefore, no further analysis was performed.

Amino Acid Sequences of Isoinhibitors—Each oxidized light and heavy chain fraction was then submitted to amino acid analyses (data not shown) and amino acid sequencing. Since the sequences of the isoinhibitors in Fraction VII had been reported previously (*2*), no sequencing was performed on this fraction. The results revealed that all fractions were heterogeneous, containing at least two major peptides. However, their sequences could be deduced, although they were analyzed as mixtures, since their contents in each fraction were fairly different. The amino acid sequences thus deduced are shown in Table I. Generally, the light chains were each composed of 10- or 11 amino acid residues, and the heavy chains of 40 or 41 residues. Sequence variations were observed at position 1 (deletion or Thr) and position 11 (Arg, Gln, or deletion) in the light chain, and at position 1 (deletion, Glu, pyrGlu, or Asp), position 8 (Thr or Ala), and position 20 (Lys or Thr) in the heavy chain, based on the numbering of BI-VI. Except for these variations, all the inhibitors had the same amino acid sequence and, therefore, they were approximately 90% identical with each other in sequence. The disulfide bond arrangements of all BIs are thought to be identical with those of BI-VI (*4*), since their half-cystine positions are conserved at the same positions as in BI-VI (Fig. 2). This indicates therefore that

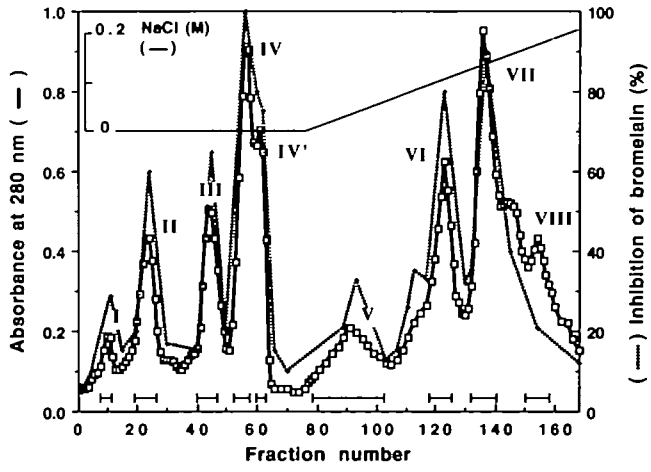

Fig. 1. **Fractionation of bromelain isoinhibitors by chromatography on DEAE-Sephadex A-25.** Approximately 180 mg of the protein inhibitor fraction isolated from pineapple stem acetone powder by gel filtration was applied to a DEAE-Sephadex (2.2×40 cm) column equilibrated with 0.1 M Tris/HCl buffer (pH 7.55), and eluted with the same buffer, and then with a linear gradient of NaCl, from 0 to 0.2 M, in the same buffer. The fraction size was 6 ml. The fractions under the bars were pooled.

TABLE I. **Amino acid sequences of the oxidized light and heavy chains of bromelain isoinhibitors.**

Peptide	Amino acid sequence[a]	Relative molar ratio[b]	
		Position 1	Position 8
Light chain			
	1 10		
L-I	Δ ACSECVCPLR		
L-II	(T)ACSECVCPLR	Δ>T (8:2)	
L-III	(T)ACSECVCPLR	T>Δ (7:3)	
L-IV	(T)ACSECVCPLR	Δ>T (7:3)	
L-IV′	(T)ACSECVCPLR	T>Δ (6:4)	
L-VI	T ACSECVCPLR		
L-VII(1)[c]	(T)ACSECVCPL**Q**	T>Δ (8:2)	
L-VII(2)[c]	(T)ACSECVCPL**R**	T>Δ (8:2)	
L-VIII(1)	(T)ACSECVCPL**Q**	T>Δ (9:1)	
L-VIII(2)	(T)ACSECVCPLΔ	T>Δ (9:1)	
Heavy chain			
	1 10 20		
H-I	ΔEYKCYCT/ADTYSDCPGFCK**K**		T>Δ (7:3)
H-II	E**E**YKCYCT/ADTYSDCPGFCK**K**		T>A (8:2)
H-III	E/DEYKCYCT/ADTYSDCPGFCK**K**	E>D (9:1)	T>A (7:3)
H-IV	E/DEYKCYCT/ADTYSDCPGFCK**T**	E>D (9:1)	T>A (8:2)
H-IV′	E/DEYKCYCT/ADTYSDCPGFCK**T**	E>D (9:1)	T>A (6:4)
H-VI	E**E**YKCYCT/ADTYSDCPGFCK**T**		T>A (7:3)
H-VII[c]	D/∠EEYKCYCA/TDTYSDCPGFCK**K**	D>∠E (6:4)	A>T (6:4)
H-VIII	E/DEYKCYCT/ADTYSDCPGFCK**K**	E>D (6:4)	T>A (7:3)
	21 30 40		
H-I-VIII	CKAEFGKYICLDLI SPNDCVK		

[a]The amino acid sequences are shown using one letter notations for amino acids. The numbering is based on that of BI-VI. (T) indicates that Thr is partially deleted. Cysteine was found as cysteic acid. ∠E stands for pyroglutamic acid residue. The residues at positions where a substitution and/or a deletion were found are shown in boldface. [b]Approximate values estimated from the yields of phenylthiohydantoin derivatives of amino acids obtained on Edman degradation. Δ, deletion. [c]Data from Ref. *2*; the molar ratio of L-VII(1) and L-VII(2) was reported to be approximately 7:3, and, in addition, an isoform with Glu at position 1 was reported to be present in a small amount. The molar ratio of L-VIII(1) and L-VIII(2) was found to be approximately 5:5 in the present study.

the higher order structures of BIs would be essentially identical among the isoinhibitors.

The above variations may result in various combinations of light and heavy chains, and each of the BI-I through BI-IV′ fractions is thought to contain at least two, or possibly more, isoinhibitors, and the BI-VII and BI-VIII fractions at least four, or possibly more, isoinhibitors. However, the net charges of the deduced sequences of these BIs appear to be consistent with the order of elution from the DEAE-Sephadex column.

The two chains would have probably been formed through posttranslational proteolytic processing from a single-chain precursor. It seems most likely that before this processing, the light chain constituted the NH_2-terminal region of the inhibitor, which was followed by the heavy chain. The 3D structure of BI-VI deduced in our NMR studies (*4*) is consistent with this notion; in this structure, the COOH-terminal Arg of the light chain is situated close to the NH_2-terminal Glu of the heavy chain, whereas the NH_2-terminal Thr of the light chain is fairly far away from the COOH-terminal Lys of the heavy chain. The processing enzyme may be the target-proteinase, stem bromelain, itself. In this case, BI as a single-chain precursor would first form a complex with the proteinase. This binding, however, may not be tight enough for complete inhibition and therefore would allow the bound enzyme to hydrolyze the peptide bond connecting the two chains. Although the Arg-X and Gln-X bonds may not be the most preferred sites of cleavage by bromelain (*10*), this cleavage is not inconsistent with the specificity of stem bromelain. Indeed, the enzyme is known to hydrolyze the Arg-Ala bond in the sequence, -Ser-Arg-Arg-Ala-Gln-, of glucagon (*11*), and the Gln-$CySO_3H$ and Gln-Leu bonds in the oxidized insulin A chain (*12*), and Boc-Leu-Arg-Arg-MCA was shown to be a good substrate for the enzyme in the present study.

It is interesting to note that no single-chain inhibitor was found in the acetone powder of pineapple stem. This may be due to the extensive action of stem bromelain, which coexists in a large quantity with the inhibitor in pineapple stem. One-residue deletions were observed in some of the isoinhibitors. At present, it is not clear whether this is due to a difference at the gene level or due to the action of exopeptidases. However, the latter possibility seems most likely since the deletions were found only at the NH_2- or COOH-termini of the two chains. In that case, the deletions at the COOH-terminus of the light chain of BI-VIII and at the NH_2-terminus of the heavy chain of BI-I should have occurred after conversion of the corresponding single-chain precursors to the two-chain forms.

Assuming that the NH_2-terminal residues are originally Thr in the light chains and Glu or Asp in the heavy chains, and that the deletions of these residues are due to the action of certain exopeptidases, one has to assume the presence of at least ten, *i.e.* possibly more, different genes. These genes are thought to have been formed through repeated gene duplications and subsequent mutations in the course of molecular evolution. All the mutations can be explained by a point mutation at a single base: Asp↔Glu (U/C↔A/G), Thr↔Ala (A↔G), Lys↔Thr (A↔C), and Arg↔Gln (A↔G).

Inhibitory Activities of the Isolated Light and Heavy Chains—As can be seen in Fig. 1, the specific inhibitory

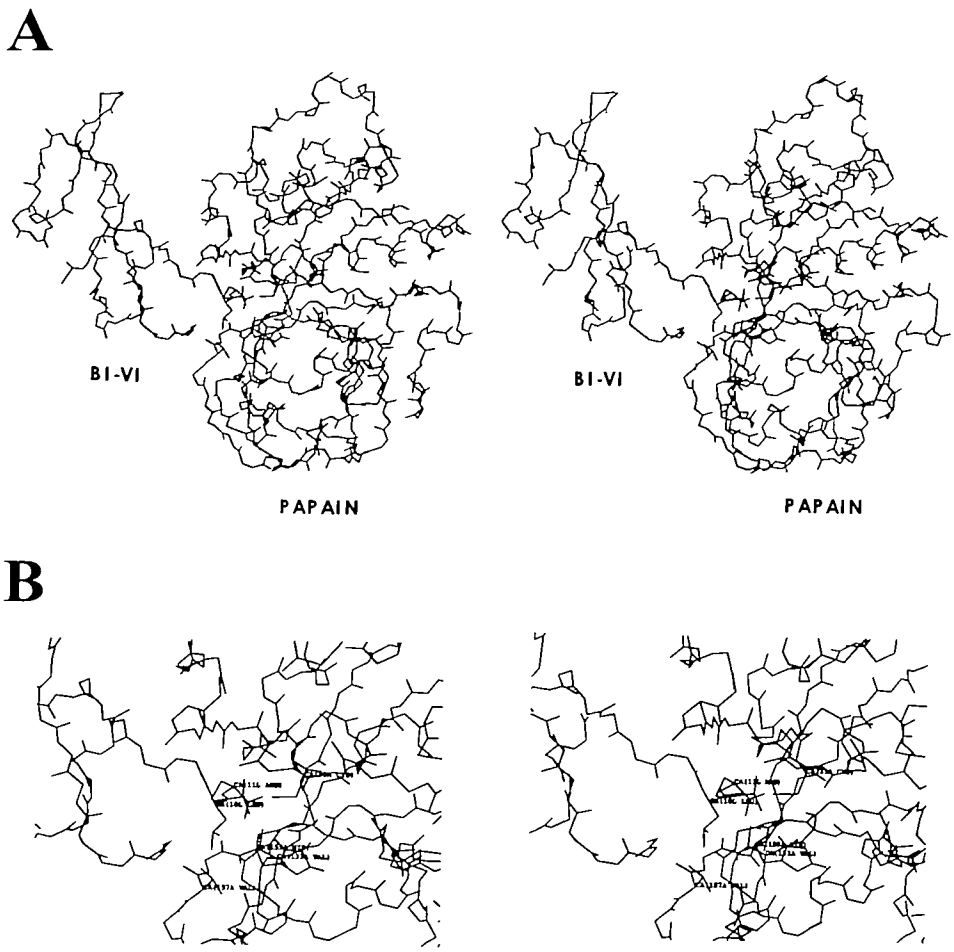

Fig. 2. **Stereo views of a 3D structure model of a complex of BI-VI and papain constructed by computer modeling.** (A) An overall view from the NH_2-terminal side of the heavy chain of BI-VI. (B) A magnified view of the active site cleft region. The side chains are shown for the catalytic residues, Cys^{25} and His^{159}, and Val^{133} and Val^{157}, in the enzyme, and the COOH-terminal residues, Arg^{11} and Leu^{10}, in the light chain of BI-VI. For the other residues, only the backbone atoms are shown.

activities of the inhibitor fractions appear to be essentially the same except for those of Fractions VII and VIII; the specific activities of Fractions VII and VIII appeared to be approximately 70% and less than 40%, respectively, of those of the other inhibitor fractions. These results indicate that the deletions at the NH_2-termini of the light and heavy chains, and the substitutions in the heavy chains do not significantly affect the inhibitory activities of BIs, whereas the deletions at the COOH-termini of the light chains may have a marked effect on their activities. To investigate this point further, we chose the BI-III and BI-VIII fractions as typical examples, and investigated the inhibitory activities of their oxidized light and heavy chain fractions under the conditions of a molar ratio of enzyme:inhibitor:substrate of 1:250:2,500. Under these conditions, Fractions BI-III and BI-VIII inhibited stem bromelain by 99 and 98%, respectively, and both heavy chain (H-III and H-VIII) fractions inhibited it by 21%. On the other hand, Fraction L-III inhibited it by 76%, Fraction L-VIII(1) by 44%, and Fraction L-VIII(2) by 14%. Thus, the isolated light chain with Arg^{11} (L-III) or Gln^{11} [L-VIII(1)] exhibited significant bromelain-inhibitory activity, whereas the light chain lacking the COOH-terminal Arg^{11} or Gln^{11} [L-VIII(2)] retained very low inhibitory activity. These results indicate that Arg^{11} and Gln^{11} in the light chain are directly involved in the bromelain-inhibitory activity, although Arg^{11} seems to be more favorable than Gln^{11} for the inhibitory activity. This is consistent with the fact that Fraction VII exhibited somewhat lower specific inhibitory activity than the other inhibitor fractions except for Fraction VIII (Fig. 1). Therefore, BIs are thought to have a major inhibitory site at the COOH-terminus of the light chain. In this connection, it is interesting to note that under the same conditions, stem bromelain was inhibited completely by leupeptin, whose structure (*N*-acetyl-Leu-Leu-Arg-al) somewhat resembles that of the COOH-terminal regions of the light chains of BIs.

Computer Modeling of a BI-VI·Papain Complex—Stem bromelain belongs to the papain superfamily of cysteine proteinases (*13*); papain (*14*) and stem bromelain (*15*) are both composed of a 212-residue polypeptide chain cross-linked by three disulfide bonds at essentially the same locations and are about 40% identical in sequence. Functionally important residues, such as Cys^{25}, His^{159}, and several other residues at the subsites, including Val^{133} and Val^{157}, are largely conserved in stem bromelain (*i.e.*, Cys^{26}, His^{158}, Val^{132}, and Leu^{156}). Therefore, the 3D structure of stem bromelain is assumed to be very similar to that of papain. However, neither the 3D structure of stem bromelain nor its complex with BI has been solved by X-ray crystallography. On the other hand, we recently determined the solution structure of BI-VI by NMR spectroscopy (*4*). The 3D structure of a papain-leupeptin complex has been determined by X-ray crystallography (*9*), and papain is known to be inhibited by BI (*7*). Therefore, based on these data, we constructed a 3D structure model of a

complex of papain with BI-VI by computer modeling, as shown in Fig. 2. In the solution structure of BI-VI, the residues, especially Arg^{11}, of the COOH-terminal region of the light chain protrude into the solvent from one edge of the BI molecule, thus having a favorable location for serving as the inhibitory site. Indeed, this COOH-terminal region (about 10 Å in length) fitted well into the catalytic site region of papain. In this connection, it is interesting to note that the depth of the active site cleft of papain was previously estimated to be approximately 10 Å by affinity labeling with spin-labeled diazo reagents (*16*). The B-domain of BI-VI (*4*), in addition, could also interact well with the active site cleft region; this interaction would further strengthen the binding of BI with the enzyme and the specificity of the inhibition. No other part of BI could interact so well with the active site cleft region of papain. Therefore, the mode of binding of BI with stem bromelain would probably be similar to this model. To come to a definite conclusion, however, the 3D structure of a BI-bromelain complex must be determined.

REFERENCES

1. Perlstein, S.H. and Kézdy, F.J. (1973) Isolation and characterization of a protease inhibitor from commercial stem bromelain acetone powder. *J. Supramol. Struct.* **1**, 249-254
2. Reddy, M.N., Keim, P.S., Heinrikson, R.L., and Kézdy, F.J. (1975) Primary structural analysis of sulfhydryl protease inhibitors from pineapple stem. *J. Biol. Chem.* **250**, 1741-1750
3. Hatano, K., Kojima, M., Tanokura, M., and Takahashi, K. (1995) Primary structure, sequence-specific ^1H NMR assignments and secondary structure in solution of bromelain inhibitor VI from pineapple stem. *Eur. J. Biochem.* **232**, 335-343
4. Hatano, K., Kojima, M., Tanokura, M., and Takahashi, K. (1996) Solution structure of bromelain inhibitor VI from pineapple stem: structural similarity with Bowman-Birk trypsin/chymotrypsin inhibitor from soybean. *Biochemistry* **35**, 5379-5384
5. Hatano, K., Tanokura, M., and Takahashi, K. (1996) Tertiary structure comparison of bromelain inhibitor VI from pineapple stem with bovine pancreatic trypsin inhibitor. *Proc. Jpn. Acad.* **72**, 104-107
6. Bode, W., Engh, R., Musil, D., Thiele, V., Huber, R., Karshikov, A., Brzin, J., Kos, J., and Turk, V. (1988) The 2.0 Å X-ray crystal structure of chicken egg white cystatin and it possible mode of interaction with cysteine proteinases. *EMBO J.* **7**, 2593-2599
7. Heinrikson, R.L. and Kézdy, F.J. (1976) Acidic cysteine protease inhibitors from pineapple stem. *Methods Enzymol.* **45**, 740-751
8. Hirs, C.H.W. (1967) Performic acid oxidation. *Methods Enzymol.* **11**, 197-199
9. Schröder, E., Phillips, C., Garman, E., Harlos, K., and Crawford, C. (1993) X-ray crystallographic structure of a papain-leupeptin complex. *FEBS Lett.* **315**, 38-42
10. Keil, B. (1992) *Specificity of Proteolysis*, Springer-Verlag, Berlin
11. Murachi, T. and Neurath, H. (1960) Fractionation and specificity studies on stem bromelain. *J. Biol. Chem.* **235**, 99-107
12. Kortt, A.A., Hinds, J.A., and Zerner, B. (1974) On the specificity and pH dependence of ficin-catalyzed hydrolyses. Some comparisons with bromelain specificity. *Biochemistry* **13**, 2029-2037
13. Rawlings, N.D. and Barrett, A.J. (1994) Families of cysteine peptidases. *Methods Enzymol.* **244**, 461-486
14. Mitchell, R.E.J., Chaiken, I.M., and Smith, E.L. (1970) The complete amino acid sequence of papain. Additions and corrections. *J. Biol. Chem.* **245**, 3485-3492
15. Ritonja, A., Roman, A.D., Buttle, D.J., Rawlings, N.D., Turk, V., and Barrett, A.J. (1989) Stem bromelain: amino acid sequence and implications for weak binding of cystatin. *FEBS Lett.* **247**, 419-424
16. Nakayama, S., Hoshino, M., Takahashi, K., Watanabe, T., and Yoshida, M. (1981) Spin-labeling study on the depth of the active site of papain. *Biochem. Biophys. Res. Commun.* **98**, 471-475