ISOLATION AND CHARACTERIZATION OF A PROTEASE INHIBITOR FROM COMMERCIAL STEM BROMELAIN ACETONE POWDER

Seymour H. Perlstein and Ferenc J. Kézdy

Departments of Biochemistry and Chemistry, University of Chicago, Chicago, Illinois 60637

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Seven closely related protease inhibitors were isolated from commercial bromelain acetone powder in electrophoretically pure form by gel filtration on Sephadex G-75, followed by ion exchange chromatography on DEAE Sephadex at pH 7.55. The inhibitors are proteins of MW 5000-6000, which inhibit competitively the bromelain-catalyzed hydrolysis of CLN ($K_i \approx 10^{-7}$ M). This inhibition is optimal at pH 3 to 4, and it depends upon the ionization of two acidic residues of pK = 4.5 and 5.0. In the acidic pH range the inhibitors are also effective toward papain, ficin and trypsin.

Proteolytic enzyme inhibitors of proteinic nature are ubiquitous in plant and animal tissues (1) where they preseumably act as regulators and protective agents. Previous studies focussed mostly on inhibitors specific toward serine proteases. These inhibitors are also active toward sulfhydryl proteases, but with a reduced efficiency. Because the inhibitor often shows remarkable specificity toward proteases contained in the same tissue, an inhibitor specific for sulfhydryl proteases was sought in the pineapple stem, a rich source of the sulfhydryl protease bromelain.

We observed that the enzymatic activity toward CLN (pH 4.60, $S_o = 2.7 \times 10^{-4}$ M, 10^{-3} M cysteine, 0.1 M KCl, 0.01 M acetate) of a mixture of crude bromelain extracted from the commercial bromelain acetone powder (Pierce Chemical Co.) and the homogeneous enzyme was less than the sum of the activities of the components measured individually, indicating that an inhibitor must be present in the crude extract. When the phenylmercury complex of crude bromelain was subjected to gel filtration (2) on Sephadex G-75, an approximate four-fold increase in total enzymatic activity toward CLN was observed. The concentration of the inhibitor was quantitated by the decrease of the rate of hydrolysis of CLN by bromelain upon addition of aliquots of the eluate. In this way it was possible to show that all the inhibitory activity is associated with a shoulder on the A_{280} profile eluting at an apparent MW of 5 to 10,000 (Fig. 1). The inhibitor fractions were pooled and further purified by ion exchange chromatography on a DEAE-Sephadex column (pH 7.55, 0.1 M Tris-HCl, 0 to 0.2 M NaCl gradient), resulting in the separation of eight peaks, as monitored by the absorbance at 280 nm. The first peak was inactive, followed by seven peaks (inhibitors I to VII) possessing approximately the same specific inhibitory activity. The eluted material in each peak was pure by the criterion of polyacrylamide disc gel electrophoresis with and without added SDS (3, 4) (Fig. 2). The bands were visualized by staining with Coomassie Brilliant Blue R-250, indicating that

Abbreviations used: CLN: p–nitrophenyl N^{α}benzyloxycarbonyl–L–lysinate; BAEE: ethyl N^{α}benzoyl–L–argininate.

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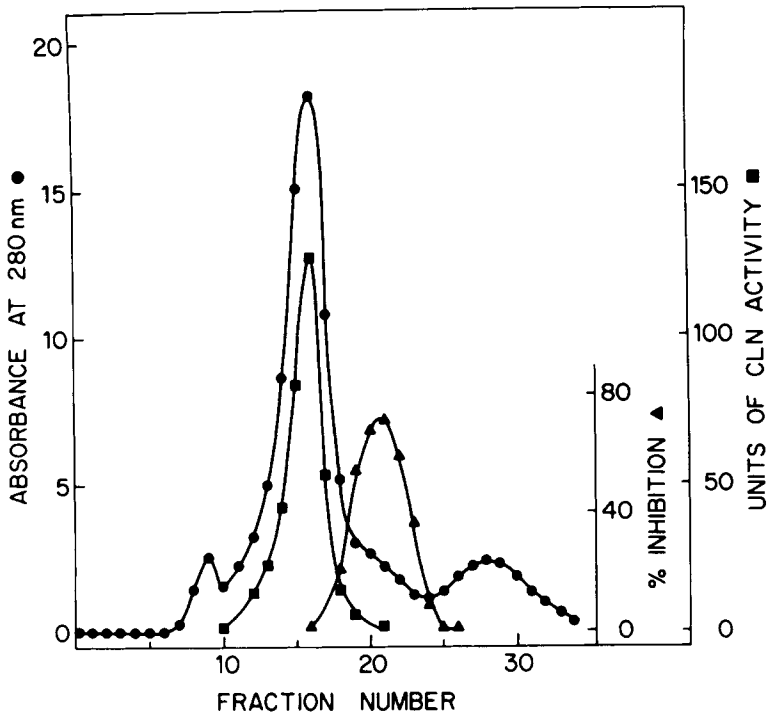

Fig. 1. Gel filtration of crude extract on Sephadex G-75. 2.5 grams of commercial acetone powder were extracted by 10 ml of pH 6.10, 0.10 M phosphate buffer saturated with phenylmercury acetate and centrifuged. The supernatant was applied to 2.5 × 75 cm column of Sephadex G-75 and eluted with the same buffer that was used for extraction. 14.5 ml fractions were collected.

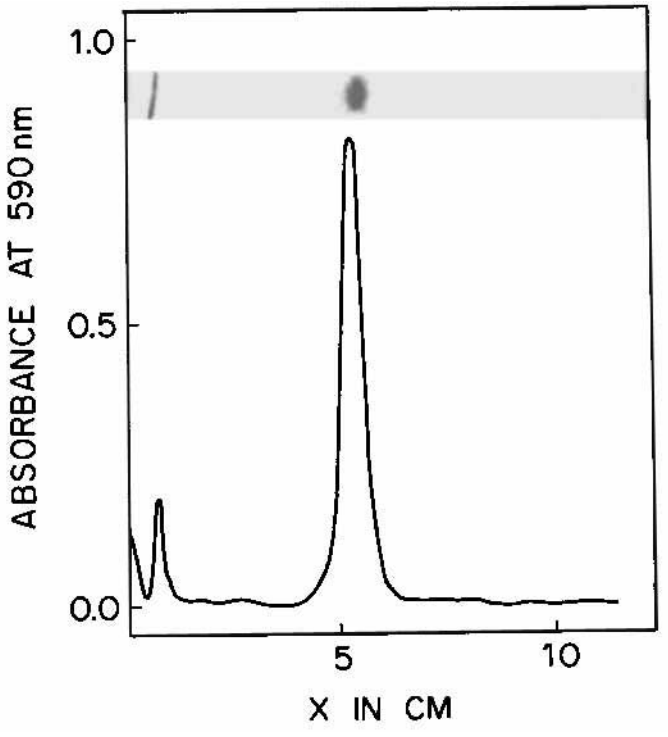

Fig. 2. Polyacrylamide disc gel electrophoresis of inhibitor VII at pH = 9.5. Densitometric scanning of the Coomassie Blue stained gel. Cathode on left.

the isolated inhibitors are proteins. The single band on the SDS gel had an electrophoretic mobility that indicates a molecular weight of less than 10,000. Attempts to stain the electrophoretic gels for carbohydrate with the fuchsin-sulfite stain after periodic acid oxidation (5) yielded negative results. Amino acid analysis of the two major inhibitors III and VII on a BioCal BC200 automatic amino acid analyzer (Table I) indicated that the inhibitor is a protein of low molecular weight, since the amino acids are in the ratios of small integers. The amino acids account for the total weight of the protein within experimental error. The minimum molecular weight is calculated to be 5550 for inhibitor III and 5630 for inhibitor VII. Titration by 5,5'-dithio bis-2-nitrobenzoic acid in 6 M guanidine hydrochloride indicated the absence of free sulfhydryl groups. Finally, the specific absorptivity of Inhibitor VII was found to be $A_{280}^{1\%} = 9.7$ AU at pH 4.6.

The stoichiometry and the kinetic law of the inhibition was established by measuring the rate of hydrolysis of CLN by bromelain II (6) in the presence of increasing amounts of inhibitor VII. With $S_o = 1.05 \times 10^{-5}$ M the condition $S_o \ll K_m$ was satisfied and the rate of liberation of p–nitrophenol, measured at 320 nm on a Cary 15 recording spectrophotometer, obeyed pseudo first-order kinetics. In the case of purely competitive inhibition the experimental first-order rate constant (k_i) is proportional to the free enzyme concen-

TABLE I

	Inhibitor III		Inhibitor VII	
Amino acid	Moles[1]	Nearest integer	Moles[1]	Nearest integer
Aspartic acid + Asparagine	5.0	5	5.8	6
Threonine	2.0	2	2.0	2
Serine	2.7	3	2.7	3
Proline	3.2	3	2.7	3
Glutamic acid + Glutamine	3.8	4	3.8	4
Alanine	2.2	2	2.7	3
Valine	1.8	2	1.8	2
Half-cystine[2]	8.4	8	8.4	8
Methionine[2]	0	0	0	0
Isoleucine	1.5	2	2.3	2
Leucine	2.6	3	3.1	3
Tyroisine	3.2	3	3.6	4
Phenylalanine	1.7	2	2.0	2
Ammonia	3.9	4	1.9	2
Lysine	6.7	7	6.4	6
Histidine	0	0	0	0
Arginine	1.0	1	0	0
Tryptophan[3]	0	0	0	0
Glucosamine[4]	0.3	0	0.3	0
Glycine	2.0	2	2.0	2
Calculated molecular weight		5556		5631

[1] Based on 2 glycines per mole.

[2] The values for these residues are most likely low since no precautionary measures were taken to insure quantitative analysis.

[3] The absence of tryptophan was shown by analysis of UV spectrum.

[4] Fucose, glucose and xylose were found in much less than equimolar amounts by analysis as their borate complexes by Dowex–1 ion-exchange chromatography, kindly performed by Dr. L. Rodén of the Departments of Pediatrics and Biochemistry.

tration (E); $k_i = (k_{cat}/K_m)E$. For a series of experiments with constant enzyme and substrate concentrations in the presence of increasing amounts of inhibitor (I_o), when E_o, I_o and k_i are of the same order of magnitude, one can show that

$$k_i = \frac{(k_i)_{I_o = O}}{1 + \dfrac{I_o}{K_i + (K_m/k_{cat})k_i}} \qquad (1)$$

with $K_i = (E) \cdot (I)/(EI)$ and (EI) the concentration of the inactive enzyme-inhibitor complex. If the inhibitor concentration is G_i in units of g/l, the $I_o = G_i/MW$, and equation (1) can be transformed into

$$G_i k_i / [(k_i)_{I_o = O} - k_1] = MW \cdot K_i + (K_m/k_{cat}) \cdot MW \cdot k_i. \qquad (2)$$

By plotting the left hand side of equation (2) vs. k_i, one should obtain a straight line of slope $(K_m/k_{cat}) \cdot MW$ and an intercept of $MW \cdot K_i$ if the inhibition is competitive and if the inhibitor combines in a mole to mole ratio with the enzyme. From our data at pH = 4.60 (Fig. 3) such a plot indeed yielded a straight line (see insert), thus establishing the competitive nature of the inhibition. The slope and intercept of the line yield MW = 5500 ± 650 and $K_i = (8 \pm 2) \times 10^{-8}$ M. These values were used in calculating the theo-

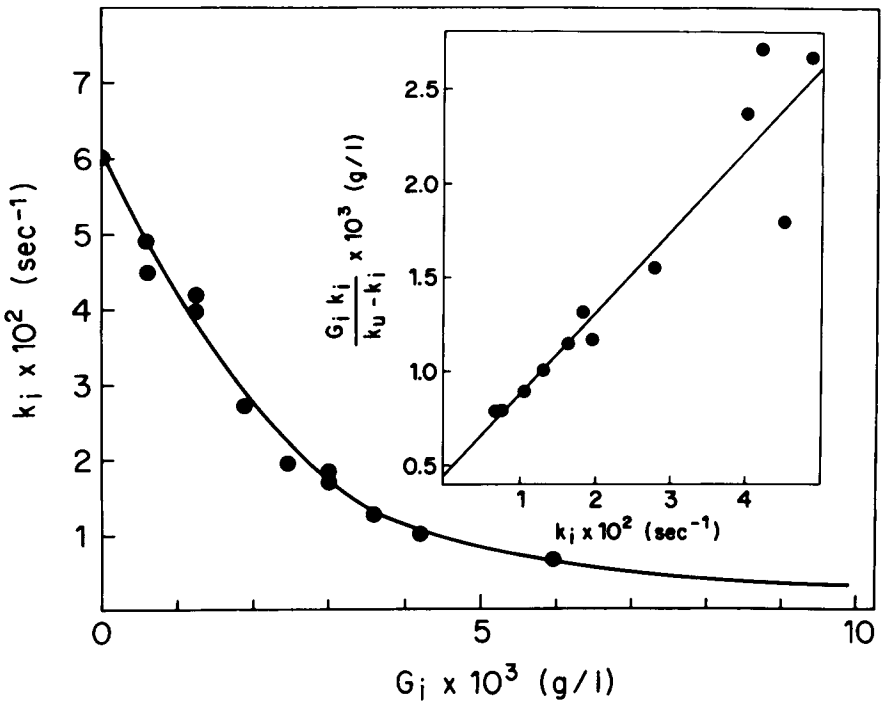

Fig. 3. Pseudo first-order rate constants of the bromelain-catalyzed hydrolysis of CLN as a function of the inhibitor concentration. pH 4.60, 0.1 M KCl, 0.1 M acetate buffer. $E = 4.8 \times 10^{-7}$ M, $CLN_o = 1 \times 10^{-5}$, 10^{-3} M cysteine. Theoretical curve from equation (1) with MW = 5600; $K_i = 8.15 \times 10^{-8}$; $(k_i)_{I_o} = 6.085 \times 10^{-2}$ sec^{-1}. Insert: Plot of experimental data according to equation (2).

retical curve of Fig. 3. The excellent agreement between the MW obtained from these experiments and from the amino acid analysis supports the assumption of a one-to-one complex formation between the enzyme and the inhibitor.

K_i values at different pH's were determined from the inhibition of the bromelain-catalyzed hydrolysis of CLN with the help of equation (1) and the MW determined at pH 4.60. The pH-dependency of K_i is an inverse sigmoid with a pH independent region at pH < 4 and a region where K_i is proportional to $1/(H^+)^2$ at pH > 5. These data are consistent with a scheme in which the protonated form of two acids of apparent pK = 4.45 and 5.03 are required for inhibition (Fig. 4, theoretical line).

Inhibitor VII also decreases the proteolytic activity of bromelain II, as measured by the acid denatured hemoglobin assay (7) modified for pH = 4.6, although the competitive nature of the inhibition could not be ascertained in this system. The bromelain III-catalyzed hydrolysis of CLN was inhibited competitively with a value if $K_i = 10^7$ M. In the acidic pH range the hydrolysis of CLN by trypsin and of BAEE by papain and ficin were inhibited by inhibitor VII with the same approximate value of $K_i = 10^{-5}$ M.

The other inhibitors possess kinetic properties similar to those of inhibitor VII; for example, inhibitor III has a $K_i = 2.7 \times 10^{-7}$ M at pH = 4.6, when measured with the bromelain II-catalyzed hydrolysis of CLN.

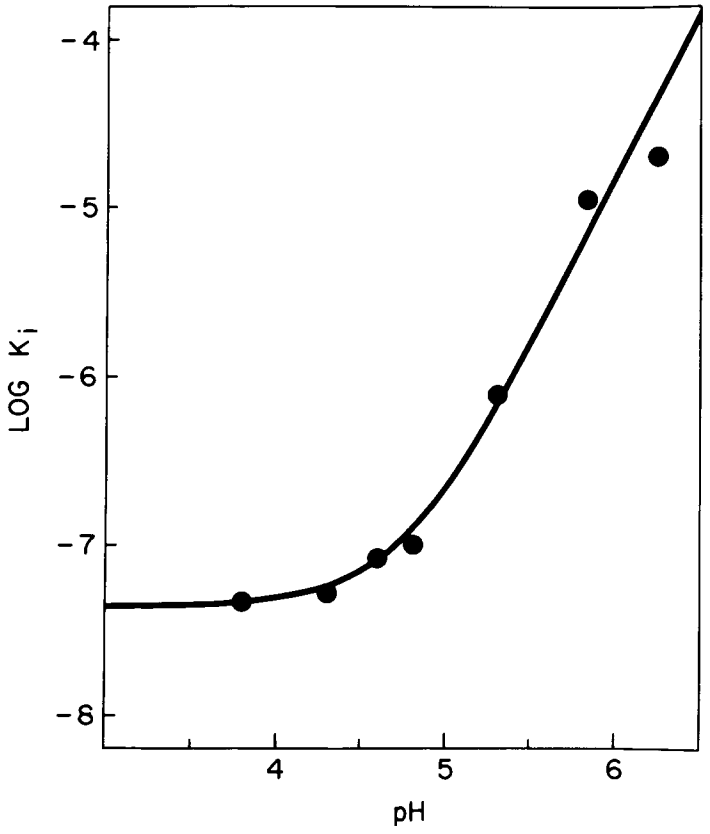

Fig. 4. pH-dependency of K_i. Theoretical curve calculated from equation $K_i = 4.3 \times 10^{-8} \left(1 + \frac{9.3 \times 10^{-6}}{H} + \frac{3.3 \times 10^{-10}}{H^2}\right)$.

The inhibitors are probably a genuine constituent of the pineapple stem, since they are present in the crude extract of the acetone powder in molar concentrations far exceeding the concentration of bromelain. Their presence is also indicated in the Sephadex G-75 gel filtrations previously reported (2, 6) by a peak of UV-absorbing material in the appropriate MW range. The limited pH range for the activity of the inhibitors might explain the fact that their function went undetected. The restricted pH range of activity might be indicative of the protective physiological role of the inhibitor. The low value of K_i (of the order of 10^{-7} M) is suggestive of a covalent enzyme-inhibitor complex, perhaps like the trypsin-soybean trypsin inhibitor complex (8).

Preliminary experiments indicate the presence of similar inhibitors in the commercial crude papain and ficin-containing latex. The further study of these specific sulfhydryl protease inhibitors should yield interesting information concerning the action and role of these plant enzymes.

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