Purification and characterization of multiple forms of the pineapple-stem-derived cysteine proteinases ananain and comosain

Andrew D. NAPPER,*§ S. Paul BENNETT,* Marianne BOROWSKI,* Marcella B. HOLDRIDGE,* Marina J. C. LEONARD,* Elizabeth E. ROGERS,* Yongjun DUAN,† Richard A. LAURSEN,† Bruce REINHOLD‡ and Spencer L. SHAMES*§

*Genzyme Corporation, One Kendall Square, Cambridge, MA 02139, U.S.A., †Department of Chemistry, Boston University, Boston, MA 02215, U.S.A. and ‡Department of Nutrition, Harvard School of Public Health, Boston, 02155, U.S.A.

A mixture of ananain (EC 3.4.22.31) and comosain purified from crude pineapple stem extract was found to contain numerous closely related enzyme forms. Chromatographic separation of the major enzyme forms was achieved after treatment of the mixture with thiol-modifying reagents: reversible modification with 2-hydroxyethyl disulphide provided enzyme for kinetic studies, and irreversible alkylation with bromotrifluoroacetone or iodoacetamide gave enzyme for structural analyses by ^{19}F-n.m.r. and electrospray mass spectrometry respectively. Structural and kinetic analyses revealed comosain to be closely related to stem bromelain (EC 3.4.22.32), whereas ananain differed markedly from both comosain and stem bromelain. Nevertheless, differences were seen between comosain and stem bromelain in amino acid composition and kinetic specificity towards the epoxide inhibitor E-64. Differences between five isolatable alternative forms of ananain were characterized by amidolytic activity, thiol stoichiometry and accurate mass determinations. Three of the enzyme forms displayed ananain-like amidolytic activity, whereas the other two forms were inactive. Thiol-stoichiometry determinations revealed that the active enzyme forms contained one free thiol, whereas the inactive forms lacked the reactive thiol required for enzyme activity. M.s. provided direct evidence for oxidation of the active-site thiol to the corresponding sulphinic acid.

INTRODUCTION

Cysteine proteinases [1–3] have recently attracted renewed interest as they have been implicated in the progression of several human diseases [4,5]. One of the key challenges faced by workers studying cysteine proteinases, especially those of plant origin, has been the characterization of multiple enzyme forms, such as those found in preparations of chymopapain [6–11], ficin [12], stem bromelain [13,14] and the mammalian cathepsins [15]. Recently, the isolation of fully active enzymes from mixtures containing inactivated (usually oxidized) material has been facilitated by the use of either thiol-specific affinity chromatography [16,17] or covalent chromatography [18]. Unfortunately, affinity purification is often neither practical nor economic for the production of the large quantities of enzyme that may be required for pharmaceutical and industrial applications.

One important pharmaceutical application of cysteine proteinases is the enzymic debridement of necrotic tissue from ulcers and burn wounds [19]. Among those proteinases examined as enzymic debriding agents are the pineapple-stem-derived enzymes ananain (EC 3.4.22.31) and comosain [20,21]. A mixture of ananain and comosain suitable for burn-debridement studies may be obtained by conventional cation-exchange chromatography, but further purification is required to obtain enzyme for mechanistic and structural characterization. Rowan et al. [13] used high-resolution cation-exchange chromatography and affinity chromatography on Sepharose–Phe-glycinaldehyde semicarbazone to obtain a highly purified preparation of ananain that was characterized as a new cysteine proteinase distinct from stem bromelain (EC 3.4.22.32), the major pineapple-stem-derived proteinase. Unfortunately, similar chromatographic methods gave only a partial purification of comosain [22], so it has been difficult to determine whether this species represents a distinct enzyme or a minor form of stem bromelain.

To obtain ananain and comosain on a large scale, we employed conventional chromatographic methods similar to those described previously [20,21]. In addition to the expected mixture of the fully active principal forms of ananain and comosain, the enzyme fraction was found to contain numerous active and inactive alternative enzyme forms. We were therefore presented with an opportunity for analysis of the range of species, both active and inactive, that may be present in a single source of enzyme. In this paper, we report a novel method for the separation of ananain and comosain and the major alternative enzyme forms that has allowed us to address two issues in particular. First, we were able to characterize comosain more completely, thereby determining its relationship to ananain and stem bromelain. Secondly, we have been able to identify a number of closely related alternative forms of ananain present after purification. Detailed characterization of these enzyme forms has helped to reveal whether they are products of genetic variation or post-translational modification, or simply artefacts of processing of the pineapple stem extract. The methods used and the conclusions drawn from the present study may also help to further our understanding of the complex behaviour of other cysteine proteinases.

EXPERIMENTAL

Materials

Crude pineapple stem extract was obtained from Enzyme Development Corporation (New York, NY, U.S.A.). Peptide-*p*-

Abbreviations used: E64, L-3-carboxy-*trans*-2,3-epoxypropionylleucylamido-(4-guanidino)butane; BCA, bicinchoninic acid; HEDS, 2-hydroxyethyl disulphide; Bz-, benzoyl; *p*NA, *p*-nitroanilide; Z-, benzyloxycarbonyl; DMF, *N,N*-dimethylformamide; K_i, equilibrium dissociation constant of bound enzyme inhibitor; $k_{inact.}$, rate constant for irreversible inactivation of enzyme by bound inhibitor; $k_{obs.}$, first-order rate constant for time-dependent inactivation of enzyme by inhibitor; TFA, trifluoroacetic acid; e.s.m.s., electrospray mass spectrometry.

§ To whom correspondence should be addressed.

nitroanilide substrates were obtained from Bachem Bioscience (Philadelphia, PA, U.S.A.). E64 was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). *p*-Hydroxyphenylglyoxal was synthesized as described previously [23]; for reasons that are not clear, commercially available material from two suppliers (Pierce and Wako) gave an unacceptably high background in the gelatinolytic assay described below. Other reagents were purchased from Sigma, Aldrich, Lancaster Synthesis and Pierce, and were of the highest commercially available purity.

Protein determination

Protein concentrations were determined either by the biuret assay [24] or with the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, U.S.A.). BSA was used as standard in both assays. On the basis of biuret assay results, an $A^{1\%}_{1cm,280}$ of 24 was assigned to ananain.

Purification of stem bromelain and a mixture of ananain and comosain

Stem bromelain and a mixture of ananain and comosain were purified from crude pineapple stem extract by cation-exchange chromatographic methods based on those of Rowan et al. [20,21]. The fraction of crude pineapple stem powder soluble in sodium acetate, pH 5.0, was eluted from an S-Sepharose column with a step gradient of NaCl. The fraction containing ananain and comosain was applied to a second S-Sepharose column and eluted with NaCl in sodium acetate, pH 5.0, after the column had been washed with Na_2CO_3, pH 9.0. The composition of the starting material and products was analysed by cation-exchange chromatography on a Mono S column (see Figure 1). The purified enzymes were lyophilized in the presence of 12.5 mM cysteine and stored at 4 °C under an atmosphere of nitrogen.

General h.p.l.c. methods

The components of the lyophilized ananain and comosain mixture were analysed by cation-exchange chromatography on a Pharmacia HR 5/5 Mono S column attached to a Hewlett–Packard HP 1050 h.p.l.c. system equipped with a Spectra-Physics u.v.–visible monitor. Samples were eluted at a flow rate of 1 ml/min with a gradient of either 0–0.16 M or 0–0.25 M NaCl in 25 mM sodium borate/HCl, pH 9.0 (Figure 2). For preparative separations, up to 50 mg of protein was eluted from an HR 10/10 Mono S column. Fractions were collected into sufficient 0.5 M sodium acetate, pH 4.3, to give a final pH of 5.0.

Thiol modification and separation of principal and alternative forms of ananain and comosain

The components of the lyophilized ananain and comosain mixture were separated as shown in Figure 2(a). Fractions containing the principal ananain and comosain forms (peak 3 in Figure 2a) and alternative ananain forms (peaks 1 and 2 in Figure 2a) were treated with thiol-modifying reagents to separate the constituent disulphide-modified or alkylated enzyme species. In all cases, a 2–5 mg/ml solution of the enzyme forms was mixed with 0.2 vol. of 0.5 M Hepes, pH 7.5, and a 100-fold molar excess of the appropriate thiol-modifying reagent in one of the following stock solutions: 0.5 M 2-hydroxyethyl disulphide (HEDS) in 10% aq. ethanol, 10% bromotrifluoroacetone in acetone or 0.5 M iodoacetamide in water. Mixtures of enzyme and HEDS were stirred gently for 2 min at room temperature, and those of enzyme and alkylating reagents were allowed to stand for 30 min and then quenched with cysteine (one equivalent relative to alkylating reagent). The constituent enzyme forms were separated as shown in Figures 2(b)–2(d). HEDS-modified comosain was repurified twice using the same gradient (chromatograms not shown) to ensure that all traces of ananain had been removed before kinetic analysis.

SDS/PAGE and Western-blot analysis

Enzyme samples were inactivated by treatment with a 1 M aqueous solution of iodoacetamide (2 μl/mg of protein). SDS/PAGE was carried out under non-reducing conditions using pre-made 10–20% mini-gels (Daiichi Pure Chemicals, Tokyo, Japan). Protein-containing bands were visualized using Pro Blue stain (Enprotech, Hyde Park, MA, U.S.A.) and quantified on an Ultrascan Laser densitometer.

For Western-blot analysis the protein-containing bands were transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). Bands containing ananain or comosain were detected after sequential binding of biotinylated antibody raised against ananain and comosain followed by horseradish peroxidase–streptavidin conjugate (Zymed, South San Francisco, CA, U.S.A.). Antibodies were raised in rabbits immunized with the principal forms of ananain and comosain (peak 3 in Figure 2a). Anti-(stem bromelain) antibody activity was removed from the resulting antiserum by adsorption to a stem bromelain affinity column prepared from Affi-Gel 10 (Bio-Rad) according to the manufacturer's directions. After three passes over the affinity column, the eluate was biotinylated and confirmed to be reactive towards both ananain and comosain but not towards stem bromelain.

Amidolytic activity assays

Amidolytic activities were determined on a Hewlett–Packard HP 8452 diode array spectrophotometer by monitoring the release of *p*-nitroaniline from the appropriate peptide-*p*-nitroanilide (*p*-NA) substrates: Bz-Phe-Val-Arg-*p*NA for ananain and Z-Arg-Arg-*p*NA for comosain [22,25]. Assays were carried out in 1 ml of aq. 10% dimethylformamide (DMF) containing a mixture of 5 mM cysteine, 5 mM EDTA and 0.1 M Hepes at pH 7.5. A mixture of buffer and HEDS-modified enzyme was incubated for 2 min at 25 °C, after which the assay was initiated by addition of substrate in DMF; the final substrate concentration was 2 mM for routine assays and specific activity determinations. The final enzyme concentration was chosen to give a linear change in A_{410} of 0.15–1.0 over 3 min; typically, the principal forms of ananain and comosain were assayed at 2.5 μg/ml and 6.25 μg/ml respectively. The rate of *p*-nitroaniline release was calculated using an ϵ_{410} of 8480 $M^{-1} \cdot cm^{-1}$ [26].

Steady-state kinetic parameters were determined on an HP 89500 ChemStation by Marquardt analysis (26b) of rate data obtained using the amidolytic assay described above, with substrate concentrations ranging from 0.02 to 3.0 mM. The exact concentration of substrate was determined from the absorbance change on complete enzymic hydrolysis. E-64 titration [27] was used to determine the concentration of catalytically active enzyme.

Kinetics of E-64 inactivation

The apparent second-order rate constant $k_{inact.}/K_i$ for enzyme inactivation by E-64 was determined by the method of Tian and

Tsou [28,29]. Using the amidolytic assay described above, stem bromelain and comosain were assayed with 250 μM Z-Arg-Arg-*p*NA, and ananain with 2 mM Bz-Phe-Val-Arg-*p*NA. A mixture of *p*-nitroanilide substrate and E-64 inhibitor in assay buffer was incubated at 25 °C for 2 min, after which the assay was initiated by addition of a portion of enzyme that had been preactivated in assay buffer for 2 min. The concentrations of E-64 inhibitor ranged from 5 to 80 μM for stem bromelain., 1 to 15 μM for comosain and 0.14 to 0.85 μM for ananain. The enzyme concentrations (12.5–100 nM stem bromelain, 20–100 nM comosain and 14–85 nM ananain) were chosen to give a linear absorbance change in the absence of inhibitor. Values of $k_{obs.}$ were derived from the progress curves obtained in the presence of inhibitor using the Guggenheim analysis [28], and the value of $k_{inact.}/K_i$ was determined by fitting a plot of $[I]/k_{obs.}$ versus [I] to eqn. (1),

$$[I]/k_{obs.} = (K_i/k_{inact.})(1+[S]/K_m)+([I]/k_{inact.}) \quad (1)$$

where [I] and [S] are concentration of inhibitor E-64 and substrate respectively [29]. The appropriate substrate K_m was determined separately as described above.

Gelatinolytic activity assays

Proteolytic activity was determined using gelatin as substrate; peptides released on hydrolysis were quantified by measurement of their arginine content using *p*-hydroxyphenylglyoxal. A solution of 12 mg of bovine gelatin (Sigma type I) in 1.2 ml of buffer (0.1 M Hepes, 5 mM cysteine, 5 mM EDTA, 0.001 % Brij 35, pH 7.5) was incubated at 37 °C for 10 min, after which the assay was initiated by the addition of a 50 μl portion of HEDS-modified enzyme to a final concentration of 2 μg/ml. After 20 min at 37 °C, the reaction was quenched by the addition of 315 μl of 100 % trichloroacetic acid, and an 800 μl portion of the supernatant was mixed with 500 μl of 4 M K_3PO_4/HCl, pH 13.5, to give a final pH of 11.5. After incubation in a cuvette for 10 min at 56 °C, 950 μl of the pH-adjusted supernatant was mixed with 50 μl of a solution of *p*-hydroxyphenylglyoxal (100 mg/ml in 0.6 M NaOH). After a further 10 min at 56 °C, the absorbance at 500 nm was measured and corrected for the A_{500} of the corresponding blank derived from a gelatin sample assayed in the absence of enzyme. Arginine content was calculated from a standard plot constructed by *p*-hydroxyphenylglyoxal assay of arginine standards ranging in concentration from 0 to 0.5 mg/ml. One unit of gelatinolytic activity was defined as that amount of enzyme that releases 1 μmol of arginine/min.

Dipeptide cleavage assay

The specificities of stem bromelain, ananain and comosain towards dipeptide substrates were determined using an array of fluorescein-labelled dipeptides immobilized on membrane disks [30]. Enzymic cleavage between the two amino acids in each dipeptide was detected by measurement of the A_{490} of the N-terminal fluorescein-labelled amino acid released from the membrane-bound C-terminus. The disks were attached to pins in a 96-pin block, and the assay was initiated by immersion of the disks in a solution of the HEDS-modified enzyme (5–10 μg/ml) in 0.1 M Hepes, pH 7.5, containing 5 mM cysteine, 5 mM EDTA, and 3 % DMF in a 96-well microtitre plate. At 20 min intervals thereafter, the disks were temporarily removed from the enzyme solutions, and the plate was read on a Bio-Tek BT 2000 MicroKinetics Reader. Relative rates were calculated from the linear increase in A_{490} over an appropriate time interval.

Thiol-stoichiometry determination by ^{19}F-n.m.r.

The number of free thiols in each enzyme form was determined from the ^{19}F-n.m.r. spectrum obtained after treatment with bromotrifluoroacetone [31,32]. Samples isolated by Mono S chromatography were concentrated to 300 μl, and the protein concentration was determined by A_{280} measurement. An exact volume of the protein sample was mixed with ^2H_2O and 35 μl of aq. 4 mM trifluoroacetic acid (TFA) to a total final volume of 700 μl, giving a mixture of 0.2 mM TFA and 2–20 mg/ml protein [0.085–0.85 mM based on an M_r of 23420 for ananain, determined by electrospray mass spectrometry (e.s.m.s.)]. ^{19}F-n.m.r. spectra were obtained at 376.19 MHz on a Varian 400 MHz spectrometer with accumulation of 1000–15000 scans using a delay time of 9.0 s. Chemical shifts are reported in p.p.m. relative to the internal TFA standard. Thiol stoichiometry was calculated from the n.m.r. peak integration of the 3,3,3,-trifluoroacetonyl-labelled protein and the TFA standard.

E.s.m.s.

Iodoacetamide-treated enzyme samples were isolated by Mono S chromatography with sodium borate buffer as described above. Non-volatile buffer salts were removed by eluting samples from an HR 5/5 Mono S column with a 20 min gradient of 0.01–1 M ammonium acetate/acetic acid, pH 5.0. The protein-containing fraction was eluted at approx. 0.7 M ammonium acetate, lyophilized to remove volatile buffer salts, and analysed using a Finnigan MAT TSQ-700 tandem quadrupole spectrometer equipped with an Analytica of Branford electrospray ion source. Protein solutions [approx. 5 pmol/μl in methanol/water (1:1, v/v) containing 0.5 % acetic acid] were infused at a flow rate of 0.75 μl/min and a potential of 4000 V. More than 15 distinct charge states were observed with the most intense corresponding to the adduction of about 20 protons. M_r values were obtained using the standard Finnigan software [33].

Amino acid sequence analysis

Samples were treated with iodoacetamide and lyophilized as described above for e.s.m.s. CNBr cleavage, amino acid analysis and N-terminal sequencing were carried out by Dr. John Leszyk at the Protein Chemistry facility of the Worcester Foundation for Experimental Biology, Shrewsbury, MA, U.S.A.

Study of the effect of air oxidation on ananain

The principal form of ananain was regenerated from the HEDS-modified enzyme by addition of cysteine to a final concentration of 10 mM. Ananain was isolated by Mono S chromatography using the gradient shown in Figure 2(a) and a 1.5 mg/ml solution was incubated at pH 5.0 and 35 °C under air for 30 days. Samples were withdrawn periodically and analysed by Mono S chromatography.

RESULTS

Ananain and comosain were obtained as a mixture containing the principal forms of the two enzymes as well as several isolatable alternative forms. The aims of this study were threefold: first, to develop a simple and effective method for the separation of ananain and comosain; secondly, to characterize the principal ananain and comosain forms; and thirdly, to separate and characterize the ananain-derived alternative enzyme forms. Treatment of the ananain and comosain mixture with thiol-modifying reagents rendered the constituent enzyme forms

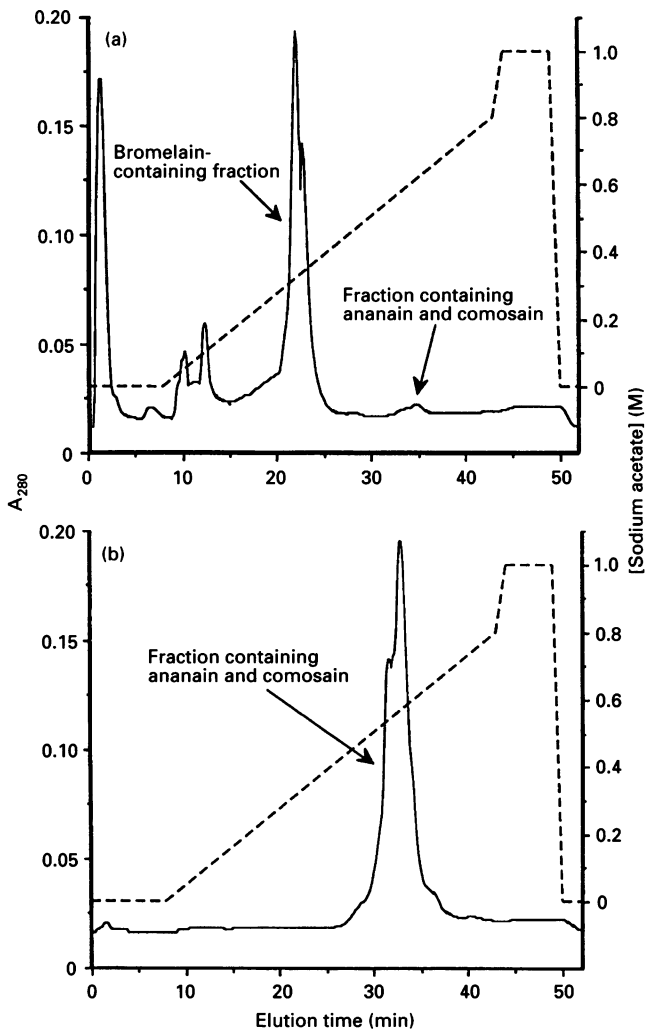

Figure 1 Chromatographic analysis of the purification of a mixture of ananain and comosain from crude pineapple stem bromelain

Cation-exchange chromatograms show (**a**) crude pineapple stem bromelain and (**b**) a mixture of ananain and comosain after purification as described in the Experimental section. Samples were eluted from a Pharmacia HR 5/5 Mono S column using a gradient of 0.01–1.0 M sodium acetate, pH 5.0 (----). ——, A_{280}.

separable by cation-exchange chromatography: a number of different reversible and irreversible modifying reagents resulted in similar elution profiles, so that each enzyme form could be isolated and characterized as either a fully activatable or an irreversibly inactivated derivative as required.

Purification of ananain and comosain

Separation of ananain and comosain mixture from stem bromelain

Stem bromelain and a mixture of ananain and comosain were purified from crude pineapple stem extract by two stages of cation-exchange chromatography on S-Sepharose. Mono S chromatographic analysis of the crude starting material revealed an elution profile (Figure 1a) similar to that reported previously [13,21]. Figure 1(b) shows an analytical chromatogram of the ananain and comosain mixture after purification; purity was judged to be greater than 90% by SDS/PAGE, which revealed a 5:1 ratio of ananain (apparent M_r 23800) to comosain (apparent M_r 24400). The product displayed amidolytic activity towards both Bz-Phe-Val-Arg-*p*NA and Z-Arg-Arg-*p*NA, confirming the presence of ananain and comosain respectively [22].

Purification of the principal forms of ananain and comosain

The ananain and comosain mixture contained a number of enzyme forms revealed by Mono S cation-exchange chromatography at pH 9 (Figure 2a). The principal forms of ananain and comosain were both obtained from the fraction represented by peak 3 in Figure 2(a). Activatable enzyme for kinetic characterization was obtained by treatment of peak 3 with HEDS and separation of HEDS-modified ananain and comosain by Mono S chromatography as shown in Figure 2(d). The alkylated enzymes required for structural characterization were obtained similarly: chromatographic separation of the principal enzyme forms after iodoacetamide alkylation was also as shown in Figure 2(d).

Structural and kinetic characterization of ananain and comosain

Structural characterization of the principal ananain and comosain forms

The aim of the structural characterization of ananain and comosain, and of the kinetic characterization described below, was to determine the relationship of comosain to ananain and to stem bromelain, the major pineapple-stem-derived enzyme. Carbohydrate, amino acid and N-terminal sequence analyses of ananain and comosain were carried out using the principal forms isolated after iodoacetamide alkylation. Comosain was found to have a similar carbohydrate composition to stem bromelain, whereas ananain is not glycosylated (results not shown). The amino acid composition data, shown in Table 1, revealed minimal differences between ananain, comosain and stem bromelain. Stem bromelain appeared to differ somewhat from the other two proteins, particularly in the number of the polar amino acids lysine and arginine, and also in the number of some aliphatic amino acids, for example alanine and isoleucine. However, the significance of such differences will not become apparent until the complete amino acid sequences are known. The N-terminal amino acid sequence data obtained to date revealed close similarity between all three proteins (Figure 3). Our structural data are in agreement with the previously reported N-terminal amino acid sequence of ananain [25] and the amino acid composition and sequence of stem bromelain [34].

Kinetic characterization of the principal ananain and comosain forms

Gelatinolytic assay of the principal ananain and comosain forms gave specific activities of 27.6 ± 0.6 and 29.5 ± 0.4 units/mg respectively, indicating that the two enzymes have similar proteolytic activity towards gelatin. In contrast, ananain and comosain showed marked differences in specificity towards peptide-*p*-nitroanilide substrates. This is highlighted in Table 2, which shows the kinetic parameters – $k_{cat.}$, K_m, and the specificity constant $k_{cat.}/K_m$ – obtained with the substrates Bz-Phe-Val-Arg-*p*NA and Z-Arg-Arg-*p*NA. The results also show that the substrate specificity of ananain differs markedly from that of stem bromelain, whereas comosain is closely related to stem bromelain in its behaviour towards the two peptide-*p*-nitroanilide substrates.

We examined the kinetic specificity of the three enzymes further by comparison of their relative activity towards 400

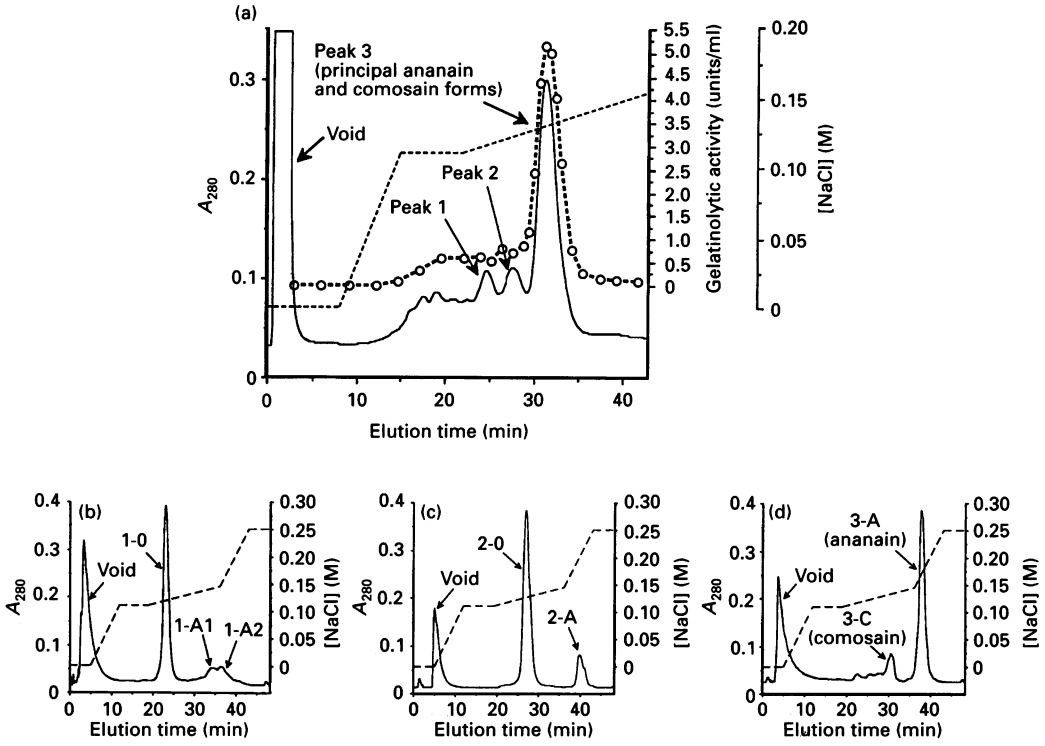

Figure 2 Chromatographic separation of principal and alternative forms of ananain and comosain

Cation-exchange chromatograms were obtained by elution of samples from a Pharmacia HR 5/5 Mono S column with 25 mM sodium borate, pH 9.0, and a gradient of NaCl (– – – –). ——, A_{280}. (**a**) Lyophilized ananain and comosain mixture; ○, gelatinolytic activity. Fractions corresponding to peaks 1, 2 and 3 were treated with HEDS and eluted as shown in (**b**), (**c**) and (**d**) respectively. The enzyme forms are labelled according to the fraction from which they were isolated: (**b**) 1-0, 1-A1 and 1-A2 were from peak 1; (**c**) 2-0 and 2-A were from peak 2; and (**d**) 3-A and 3-C (the principal ananain and comosain forms) were from peak 3. A suffix indicates the amidolytic activity, if any, of each isolated fraction. Thus, 1-A1, 1-A2, 2-A and 3-A had activity characteristic of ananain, 3-C had activity characteristic of comosain, and 1-0 and 2-0 were inactive. Treatment of fractions from (**a**) with either iodoacetamide or bromotrifluoroacetone gave elution profiles indistinguishable from those obtained after HEDS modification.

Table 1 Amino acid composition of ananain, comosain and bromelain

Data are rounded to the nearest whole number. The reported number of cysteine residues in ananain and comosain (*) include six measured as cysteic acid after performic acid oxidation of the iodoacetamide-inactivated enzymes, and the alkylated active-site cysteine residue that is not detected by this analytical method. The tryptophan content was not measured.

	Number of residues found		
Amino acid	Ananain	Comosain	Bromelain
Asx	19	18	18
Thr	8	7	9
Ser	18	17	17
Glx	13	13	16
Gly	24	25	22
Ala	20	20	25
Val	14	13	14
Met	2	3	3
Ile	14	12	17
Leu	9	9	6
Tyr	12	12	14
Phe	5	7	6
His	2	2	1
Lys	11	10	15
Arg	10	11	6
Cys	7*	7*	7

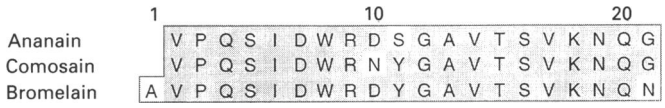

Figure 3 Comparison of N-terminal amino acid sequences of ananain, comosain and bromelain

Residue numbers refer to the numbering scheme of stem bromelain [34].

dipeptides (Figure 4). The results of this study provided a qualitative specificity profile for each of the three enzymes. Stem bromelain and comosain displayed a preference for a polar amino acid in both the P1 and P1′ substrate binding sites. Ananain shared the preference for a polar amino acid in the P1′ site, but differed from the other two enzymes in its acceptance of a much broader range of amino acids in the P1 site. The best substrates for both stem bromelain and comosain were combinations of Glu, Asn, Lys or Arg (P1 site) and Ala, Thr or Ser (P1′ site). For ananain the best substrates consisted of Leu, Met, Cys or Gln (P1 site) in combination with Gln, Asn or Lys (P1′ site). Overall, stem bromelain and comosain displayed very similar specificity towards the dipeptide substrates, suggesting that there are minimal differences between the P1–P1′ portions of the active sites of the two enzymes. (For an explanation of the P1 and P1′ designations, see ref. [3], p. 294.)

In contrast with the behaviour of the enzymes with peptide substrates, both comosain and ananain differed from stem

Table 2 Kinetic parameters of ananain, comosain and bromelain

The concentration of catalytically active enzyme was determined by titration with E-64 [27]. Ananain and comosain regenerated from HEDS-modified enzyme samples were found to be more than 95% active, whereas bromelain samples prepared directly from the lyophilized powder were about 90% active. The error limits of the kinetic data represent ± S.D.

	Bz-Phe-Val-Arg-*p*NA			Z-Arg-Arg-*p*NA			E-64
Enzyme	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)	$k_{inact.}/K_i$ ($M^{-1} \cdot s^{-1}$)
Ananain	10.9 ± 0.2	1.6 ± 0.1	6800	0.13 ± 0.01	11.0 ± 0.9	12.0	1.0×10^5
Comosain	0.24 ± 0.02	1.0 ± 0.1	240	2.93 ± 0.04	0.048 ± 0.003	6.1×10^4	8900
Bromelain	0.13 ± 0.01	1.0 ± 0.1	130	3.30 ± 0.06	0.074 ± 0.006	4.5×10^4	850

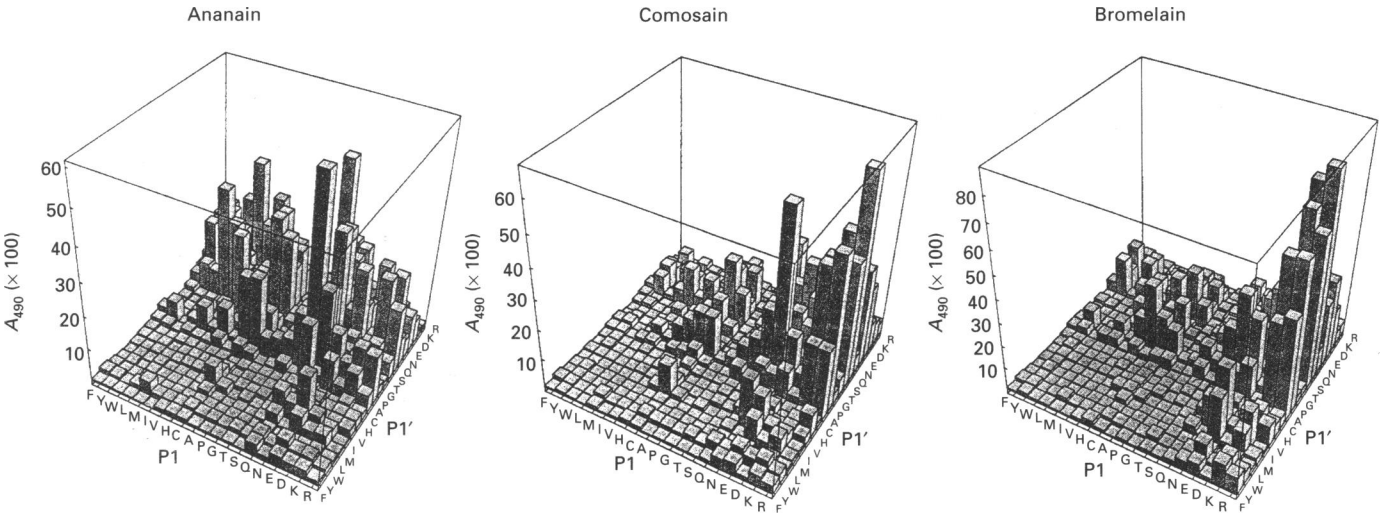

Figure 4 Dipeptide cleavage assay of ananain, comosain and bromelain

The diagrams show a profile of the relative specificities of each enzyme towards dipeptide substrates. The base of each plot consists of an array of 400 squares, representing the 400 dipeptides with which the enzymes were assayed. The axes are labelled in order of increasing amino acid polarity to indicate the P1 and P1′ amino acids in each dipeptide. (For an explanation of the P1 and P1′ designations, see ref. [3], p. 294). The column height represents A_{490} measured after an appropriate time. For a given enzyme, the assay time was the same for all 400 dipeptides, allowing direct comparison of the relative activity of the enzyme towards each dipeptide. Each specificity profile represents the results of a single experiment.

bromelain in their kinetic behaviour towards the irreversible inhibitor E-64. We determined the rate constants, $k_{inact.}/K_i$ by the method of Tian and Tsou [29]. The overall rates of inactivation given by the values of $k_{inact.}/K_i$ revealed significant variation in the reactivity of E-64 with the three enzymes (Table 2). Our values for the E-64 inactivation of stem bromelain and ananain are in agreement with those reported previously [13].

Separation and characterization of alternative forms of ananain

Analysis of the composition of the ananain and comosain mixture

We used kinetic, gel-electrophoretic and immunological analyses to study the enzyme forms derived from the mixture of ananain and comosain revealed by the chromatogram shown in Figure 2(a). The profile of gelatinolytic activity shown in Figure 2(a) revealed proteolytic activity throughout the mixture. A profile of amidolytic activity towards Bz-Phe-Val-Arg-*p*NA (not shown) displayed close correspondence to the gelatinolytic activity profile, suggesting that most of the observed proteolytic activity was ananain-derived. This was confirmed by SDS/PAGE: fractions throughout the chromatogram each consisted mainly of material with the same apparent M_r as the principal ananain form, although some comosain-derived material was also present, ranging from less than 5% in peaks 1 and 2 to 15% in peak 3. Western-blot analysis confirmed that all the species revealed by SDS/PAGE were derived from ananain and comosain and not from stem bromelain.

Separation of principal and alternative forms of ananain

Mixtures of the principal ananain and comosain enzyme forms (peak 3 in Figure 2a) and those of alternative ananain forms (peaks 1 and 2 in Figure 2a) were separated by Mono S chromatography to allow isolation and further characterization of the constituent species. Although the region preceding peak 1 in Figure 2(a) also contained material derived from ananain and comosain as revealed by Western-blot analysis, we did not attempt to separate this complex mixture of minor species. Treatment of fractions corresponding to peaks 1, 2 and 3 with thiol-modifying reagents rendered the constituent enzyme forms separable by a second stage of Mono S chromatography, as shown in Figures 2(b)–2(d). Three different thiol modifications

Table 3 Kinetic and structural parameters of ananain and comosain enzyme forms

Designations of the enzyme forms are those used in Figures 2(b)–2(d). Specific activities were determined against Bz-Phe-Val-Arg-*p*NA, except where indicated otherwise. Thiol stoichiometry and M_r values are from ^{19}F-n.m.r. and e.s.m.s. respectively. For those species with one free thiol, the parent M_r was calculated from the mass obtained by e.s.m.s. by subtraction of 57 mass units for replacement of Cys-SCH_2CONH_2 by Cys-SH. For those species with no reactive thiol, the e.s.m.s. samples are not alkylated on iodoacetamide treatment, so the parent M_r is the same as that determined by e.s.m.s. Relative intensity denotes peak heights in each mass-transformed spectrum relative to the height of the largest peak assigned as 100%.

Enzyme form	Specific activity (μmol/min per mg)	Thiol stoichiometry	Parent M_r	Relative intensity (%)
Ananain-derived				
3-A	18	0.9	23420	100
2-0	0	0†	23499	100
2-A	18	0.8	23420	100
			23580	40
1-0	0	0†	23452	100
1-A1	17	1.0	23420	100
			23580	40
1-A2	18	0.8	23420	60
			23580	100
Comosain-derived				
3-C	7.0*	1.0	24509	100
			23569	60

* Amidolytic activity determined using Z-Arg-Arg-*p*NA substrate.
† The limit of detection corresponded to a thiol stoichiometry of < 0.05.

were used: reversible modification with HEDS for activity assays; alkylation with bromotrifluoroacetone [31,32] for ^{19}F-n.m.r. determination of the number of free thiols; and alkylation with iodoacetamide for structural studies, including M_r determination by e.s.m.s. and amino acid sequence analysis.

Characterization of principal and alternative forms of ananain

Table 3 shows the amidolytic activity, thiol stoichiometry and relative molecular masses of the enzyme forms isolated as shown in Figures 2(b)–2(d). The data provide further evidence that the active and inactive species isolated from peaks 1 and 2 in Figure 2(a) represent alternative enzyme forms closely related to ananain: amidolytic activity towards the Z-Arg-Arg-*p*NA substrate and a higher M_r clearly distinguish comosain from the other enzyme forms. The results of ^{19}F-n.m.r. analysis of the products of bromotrifluoroacetone treatment show that the thiol stoichiometry correlates with the amidolytic activity of the HEDS-modified enzyme forms. The principal ananain and comosain forms each showed one free thiol; in addition, those other species designated 'A' with activity equal to ananain also revealed a single reactive thiol. In all cases the ^{19}F-n.m.r. resonance was at -9.31 ± 0.02 p.p.m. In contrast, those species with no amidolytic activity (labelled '0') gave no ^{19}F-n.m.r. resonance, indicating the absence of the reactive active-site thiol required for enzyme activity.

We employed e.s.m.s. to look for structural differences between the various ananain-derived enzyme forms. Accurate mass determinations were made after iodoacetamide treatment and lyophilization from ammonium acetate; Table 3 shows the calculated M_r values of the parent non-alkylated enzyme forms. The results show the principal ananain form and the inactive variants thereof (peaks 1-0 and 2-0 in Figure 2) each to be a single species with a clearly defined M_r. In contrast, m.s. analysis of the three alternative active ananain-like samples (peaks 1-A1, 1-A2 and 2-A in Figure 2) revealed a mixture of two species common to all three samples. Thus there are two alternative active ananain-like species which are eluted in a broad poorly resolved peak beneath both peaks 1 and 2 in Figure 2(a).

To look for minor amino acid variation that might account for the mass differences between the ananain-derived enzyme forms, we obtained partial sequences of intact protein and peptide fragments obtained after CNBr digestion. N-terminal sequencing revealed that all five ananain-derived forms possessed the same N- and C-termini: VPQSI and DPLYPTLQS respectively. These results suggest that the observed mass differences were the result of modifications elsewhere in the protein. A readily observable example of such a modification is oxidation of the active-site thiol to give the inactive enzyme form represented by peak 1.0 in Figure 2(b): a solution of ananain in the presence of air was completely converted over a period of 30 days into material identical with peak 1-0. Also, in the absence of stabilizing agents, the proportion of peak 1-0 in the lyophilized ananain and comosain mixture increased gradually in the presence of air.

DISCUSSION

Purification of ananain- and comosain-derived enzyme forms

As reported previously, fractionation of crude pineapple stem extract gave a mixture of ananain and comosain [22]. We have now developed a novel purification step in which the two enzymes may be readily separated from this mixture after reversible HEDS modification. HEDS treatment of the principal enzyme forms (peak 3 in Figure 2a) caused ananain to be eluted at higher salt concentration, whereas the elution of comosain was virtually unchanged (Figure 2d). Similarly, we were able to separate several alternative ananain forms (Figures 2b and 2c). The effect of disulphide modification on the ion-exchange elution profile of another group of cysteine proteinases, those from papaya latex, has been reported previously [35]; in that instance, however, there was no improvement in chromatographic separation.

Structural and kinetic comparison of ananain, comosain and stem bromelain

Rowan et al. [13,25] have previously shown that ananain is an enzyme distinct from pineapple stem bromelain. Therefore the main focus of our study was to determine the relationship of comosain to ananain and stem bromelain. Comosain apparently differs markedly from ananain but closely resembles stem bromelain. By e.s.m.s., we determined M_r values of 24509 and 23420 for the principal forms of comosain and ananain respectively. (The M_r of comosain reported here is that of the major form shown in Table 3; we did not attempt to isolate and separately characterize the two comosain-derived forms revealed by e.s.m.s.) These M_r values are comparable with an M_r of 24395 determined for stem bromelain (T. Edmunds, personal communication). It appears, however, that there are some structural differences between comosain and stem bromelain, as demonstrated by amino acid composition (Table 1) and also immunoblotting, by which comosain was detected with an antibody preparation shown to be devoid of anti-(stem bromelain) antibody activity.

In addition to showing some unique structural features, comosain also displays certain kinetic characteristics that distinguish it from stem bromelain. From both the dipeptidase

assay (Figure 4) and the kinetic data obtained with substrates Bz-Phe-Val-Arg-*p*NA and Z-Arg-Arg-*p*NA (Table 2), ananain displays a distinct substrate-specificity profile, whereas the specificity profiles of comosain and stem bromelain are closely matched. However, the kinetics of E-64 inactivation of comosain and stem bromelain appear to reveal a significant difference between the two enzymes. The values of $k_{inact.}/K_i$ presented in Table 2 show that the active-site directed inhibitor E-64 is 10-fold more reactive towards comosain than stem bromelain; in fact, the difference in reactivity between these two enzymes is no less than that between comosain and ananain. The differences in inhibitor specificity between comosain and stem bromelain taken together with the structural differences revealed by immunoblotting and amino acid analysis suggest possible justification for classifying comosain as a distinct enzyme.

Characterization of alternative forms of ananain

We focused our attention on the ananain-derived enzyme forms contained in peaks 1, 2 and 3 in Figure 2(a) as these were readily separable, and therefore provided us with an opportunity for characterization of a representative selection of the multiple forms derived from a single cysteine proteinase. With the exception of the principal form of comosain, contained in peak 3, we did not attempt to identify comosain-derived species. Table 3 summarizes the information obtained by study of the five ananain forms (three active and two inactive) separated by cation-exchange chromatography.

The two alternative active ananain forms clearly have a fully functional active site, and also identical N- and C-termini to the parent ananain form, so the differences are apparently the result of minor structural variations elsewhere in the protein. The active species with M_r 23420 may be the product of deamidation of one or more asparagine residues in the principal ananain form, as the two materials have the same M_r. The alternative species was eluted first on cation-exchange chromatography, which is consistent with a decrease in net positive charge that is expected on deamidation. We considered the possibility that the active species with M_r 23580 might have arisen by glycosylation of ananain with a single sugar. However, monosaccharide analysis revealed that, in common with the principal ananain form, the species with M_r 23580 was not glycosylated.

The inactive ananain form with M_r 23452 (peak 1-0 in Figure 2b) is apparently the product of oxidation of the principal ananain form. Peak 1-0 has no free thiol, is catalytically inactive and has an M_r that is 32 mass units greater than that of ananain, suggesting that oxidation of ananain gives the corresponding sulphinic acid (i.e. Cys-SH is converted into Cys-SO_2H). The alternative form with M_r 23499 (peak 2-0 in Figure 2c) also has no free thiol and a corresponding lack of catalytic activity. Therefore oxidation of the active-site thiol in this enzyme form probably accounts for some of its increased mass relative to ananain.

For definitive structural assignment of the alternative enzyme forms with M_r values 23420, 23499 and 23580, we await the determination of the complete amino acid sequences. Nevertheless, the evidence to date allows us to speculate on the origins of the various species.

The origin of comosain and the multiple forms of ananain

The evidence presented in this paper reinforces the conclusion of Rowan et al. [22] that stem bromelain, ananain and comosain represent three genetically distinct cysteine proteinases, although it is unclear what significance we should attribute to the presence of comosain in the pineapple stem at a level less than 1 % of that of stem bromelain. The possibility that the primary source of comosain might be the pineapple fruit has been ruled out by Rowan et al. [13,22] on the basis of chromatographic and immunological analyses of both crude pineapple stem bromelain and the corresponding fruit enzyme. In addition, there appear to be significant differences between the amino acid composition of comosain reported herein (Table 1) and the composition of each of the fruit bromelain enzyme forms analysed by Ota et al. [14].

In contrast with the above, the multiple forms of ananain appear to be the product of a single gene. The question here is whether these species arise from post-translational modifications in the plant, or whether they are artifacts arising during processing of the pineapple stem extract. We have observed oxidation of purified ananain to the corresponding sulphinic acid with M_r 23452 but not to the other inactive oxidized form with M_r 23499; the latter species may nevertheless arise at an early stage of the purification process. It is possible, however, that all the alternative forms arise within the plant. We have observed a remarkable consistency in the chromatographic profiles of lyophilized ananain and comosain mixtures prepared over a 2-year period from several different lots of crude pineapple stem powder. The alternative ananain form with M_r 23420 may result from ageing of the enzyme in the plant, as deamidation of asparagine residues commonly occurs spontaneously *in vivo* [36].

Applicability of this work to the analysis of other cysteine proteinases

The techniques used in this study may be applicable to other cysteine proteinases, for example to provide further confirmation of the relationship between stem bromelain, fruit bromelain and comosain. We have been able to use chemical modification in the dual role of separation tool and analytical method. Active-site thiol alkylation provided material for n.m.r., e.s.m.s. and other structural studies, allowing us to identify a number of closely related ananain species. Incorporation of a fluorine label allowed us to determine the thiol stoichiometry of the enzyme forms and gives the potential for study of changes in the active-site environment [31]. E.s.m.s. allowed us to detect mass differences that were not detectable by gel electrophoresis; for example, we were able to detect oxidation of a single thiol in a protein with an M_r of 23420, and to distinguish the product sulphinic acid readily from other possibilities (e.g. Cys-SOH, Cys-SO_3H). Straightforward means of identifying the different forms of a given enzyme might facilitate their unambiguous assignment as products of genetic variation or post-translational modification, or as processing artifacts, such as oxidation products.

Portions of this work were supported by National Science Foundation Grants DIR-8900216 (to R.A.L.) and DIR-9002024 (to B.R.). We thank Dr. William Christopher, Dr. Mike Hayes, Lee Sherman and Edward Nichols for preparation of the ananain and comosain enzyme mixture, Dr. Yhang Zhang for assistance in enzyme characterization, Lisa Bruce for gel and immunological analyses, and the Genzyme Structural Protein Chemistry Group for amino acid and carbohydrate analyses. We are grateful to Dr. Tim Edmunds for critical review of this manuscript, and to Dr. Gordon Lowe (Oxford University, Oxford, U.K.) and Dr. David Buttle (Strangeways Research Laboratory, Cambridge, U.K.) for helpful discussions.

REFERENCES

1 Brocklehurst, K., Willenbrock, F. and Salih, E. (1987) in Hydrolytic Enzymes (Neuberger, A. and Brocklehurst, K., eds.), pp. 39–158, Elsevier, Amsterdam
2 Baker, E. N. and Drenth, J. (1987) in Biological Macromolecules and Assemblies (Jurnak, F. A. and McPherson, A., eds.), pp. 314–368, Wiley, New York
3 Lowe, G. (1976) Tetrahedron **32**, 291–302
4 Dando, P. M., Brown, M. A. and Barrett, A. J. (1993) Biochem. J. **294**, 451–457

5 Ménard, R., Khouri, H. E., Plouffe, C., Laflamme, P., Dupras, R., Vernet, T., Tessier, D., Thomas, D. Y. and Storer, A. C. (1991) Biochemistry **30**, 5531–5538
6 Buttle, D. J. and Barrett, A. J. (1984) Biochem. J. **223**, 81–88
7 Buttle, D. J., Dando, P. M., Coe, P. E., Sharp, S. L., Shepherd, S. T. and Barrett, A. J. (1990) Biol. Chem. Hoppe-Seyler **371**, 1083–1088
8 Korodi, I., Asboth, B. and Polgar, L. (1986) Biochemistry **25**, 6895–6900
9 Barrett, A. J. and Buttle, D. J. (1985) Biochem. J. **228**, 527
10 Brocklehurst, K., Salih, E., McKee, R. and Smith, H. (1985) Biochem. J. **228**, 525–526
11 Polgar, L. (1984) Biochem. J. **221**, 555–556
12 Friedenson, B. and Liener, I. E. (1972) Arch. Biochem. Biophys. **149**, 169–179
13 Rowan, A. D., Buttle, D. J. and Barrett, A. J. (1988) Arch. Biochem. Biophys. **267**, 262–270
14 Ota, S., Muta, E., Katahira, Y. and Okamoto, Y. (1985) J. Biochem. (Tokyo) **98**, 219–228
15 Polgar, L. and Csoma, C. (1987) J. Biol. Chem. **262**, 14448–14453
16 Polgar, L. (1981) Biochim. Biophys. Acta **658**, 262–269
17 Lynn, K. R. (1977) Anal. Biochem. **77**, 33–38
18 Brocklehurst, K., Carlsson, J. and Kierstan, M. P. J. (1985) Top. Enzyme Fermentat. Biotechnol. **10**, 146–188
19 Silverstein, P., Maxwell, P. and Duckett, L. (1987) in The Art and Science of Burn Care (Boswick, J. A., ed.), pp. 75–81, Aspen, Rockville, MD
20 Rowan, A. D., Christopher, C. W., Kelley, S. F., Buttle, D. J. and Ehrlich, H. P. (1990) Burns **16**, 243–246
21 Rowan, A. D. and Buttle, D. J. (1992) U.S. Patent 5,106,621
22 Rowan, A. D., Buttle, D. J. and Barrett, A. J. (1990) Biochem. J. **266**, 869–875
23 Fodor, G. and Kovacs, O. (1949) J. Am. Chem. Soc. **71**, 1045–1048
24 Layne, E. (1957) Methods Enzymol. **3**, 450–451
25 Rowan, A. D. (1989) The Pineapple Proteinases: Characterization and Clinical Use, Ph.D. Thesis, The British Library, London
26 Delmar, E. G., Largman, C., Brodrick, J. W. and Geokas, M. C. (1979) Anal. Biochem. **99**, 316–320
26a Marquardt, D. W. (1963) J. Soc. Indust. Appl. Math. **11**, 431–441
27 Zucker, S., Buttle, D. J., Nicklin, M. J. H. and Barrett, A. J. (1985) Biochim. Biophys. Acta **828**, 196–204
28 Knight, C. G. (1986) in Proteinase Inhibitors (Barrett, A. J. and Salvesen, G. S., eds.), pp. 42–44, Elsevier, Amsterdam
29 Tian, W.-X. and Tsou, C.-L. (1982) Biochemistry **21**, 1028–1032
30 Duan, Y. and Laursen, R. A. (1994) Anal. Biochem., **216**, 431–438
31 Bendall, M. R. and Lowe, G. (1976) Eur. J. Biochem. **65**, 481–491
32 Donald, L. J., Crane, B. R., Anderson, D. H. and Duckworth, H. W. (1991) J. Biol. Chem. **266**, 20709–20713
33 Mann, M., Meng, C. K. and Fenn, J. (1989) Anal. Chem. **61**, 1702–1708
34 Ritonja, A., Rowan, A. D., Buttle, D. J., Rawlings, N. D., Turk, V. and Barrett, A. J. (1989) FEBS Lett. **247**, 419–424
35 Dubois, T., Jaquet, A., Schnek, A. G. and Looze, Y. (1988) Biol. Chem. Hoppe-Seyler **369**, 733–740
36 Kossiakoff, A. A. (1988) Science **240**, 191–195

Received 3 November 1993/7 February 1994; accepted 3 March 1994