Purification and Characterization of a Stable Cysteine Protease Ervatamin B, with Two Disulfide Bridges, from the Latex of *Ervatamia coronaria*

Suman Kundu, Monica Sundd, and Medicherla V. Jagannadham*

Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India

Latex of the medicinal plant *Ervatamia coronaria* was found to contain at least three cysteine proteases with high proteolytic activity, called ervatamins. One of these proteases, named ervatamin B, has been purified to homogeneity using ion-exchange chromatography and crystallization. The molecular mass of the enzyme was estimated to be 26 000 Da by SDS−PAGE and gel filtration. The extinction coefficient ($\epsilon^{1\%}_{280\ nm}$) of the enzyme was 20.5 with 7 tryptophan and 10 tyrosine residues per molecule. The enzyme hydrolyzed denatured natural substrates such as casein, azoalbumin, and azocasein with a high specific activity. In addition, it showed amidolytic activity toward *N*-succinyl-alanine-alanine-alanine-*p*-nitroanilide with an apparent K_m and K_{cat} of 6.6 ± 0.5 mM and 1.87×10^2 s^{-1}, respectively. The pH optima was 6.0−6.5 with azocasein as substrate and 7.0−7.5 with azoalbumin as substrate. The temperature optimum was around 50−55 °C. The enzyme was basic with an isoelectric point of 9.35 and had no carbohydrate content. Both the proteolytic and amidolytic activity of the enzyme was strongly inhibited by thiol-specific inhibitors. Interestingly, the enzyme had only two disulfide bridges versus three as in most plant cysteine proteases of the papain superfamily. The enzyme was relatively stable toward pH, denaturants, temperature, and organic solvents. Polyclonal antibodies raised against the pure enzyme gave a single precipitin line in Ouchterlony's double immunodiffusion and typical color in ELISA. Other related proteases do not cross-react with the antisera to ervatamin B showing that the enzyme is immunologically distinct. The N-terminal sequence showed conserved amino acid residues and considerable similarity to typical plant cysteine proteases.

Keywords: *Plant latex; cysteine protease; plant endopeptidase; Ervatamia coronaria; ervatamin A, B, C*

INTRODUCTION

In view of the various medicinal and other applications (Anonymous, 1952) of different parts of the indigenous flowering plant *Ervatamia coronaria*, search for important biochemical constituents revealed the presence of a highly stable cysteine protease in its latex (Sundd et al., 1998). Cysteine proteases from plant latices have mostly been reported in multiples. Papaya (*Carica papaya*) latex has been shown to contain four structurally and functionally distinct cysteine proteases (Glazer and Smith, 1971; Robinson, 1975; Lynn, 1979). Likewise, the latices of *Ficus glabrata* and *Ficus carica* contain a number of cysteine proteases which are chromatographically and electrophoretically distinct (Sgarbieri et al., 1964). Latex of *Calotropis gigantea* also has been shown to contain four cysteine proteases: calotropin FI, FII (Abraham and Joshi, 1979), DI, and DII (Pal and Sinha, 1980). However, the reason for such multiplicity of proteases has not been extensively highlighted.

Such reports about multiplicity of proteases have prompted further screening of the latex of *E. coronaria*, and during the process at least two more cysteine proteases have been identified. Proteases isolated from a single species of plant exhibit clear differences in amino acid composition, peptide map, and electrophoretic and other properties (Jones and Glazer, 1970; Pal and Sinha, 1980). The possibility has been excluded that these multiple forms of proteases would have arisen by autodigestion from a common precursor or as artifacts of the preparation procedure (Jones and Glazer, 1970; Sengupta et al., 1984). In this article, purification and characterization of one of the cysteine proteases are reported.

Though these proteases show considerable sequence homology to the plant cysteine protease papain and seem to belong to this superfamily, they revealed some interesting features distinct from each other and from other cysteine proteases of this superfamily. They seem to be potential endopeptidases with probable implications in food industry and medicine (Sundd et al., 1998). Spectroscopic studies of one of the proteases regarding the conformational transitions under various conditions (Kundu et al., 1999) also reveal novel observations distinct from papain (Edwin and Jagannadham, 1998), studied in our laboratory, indicating that these proteases might have a different folding pattern. In this light, it is essential to pursue studies on these enzymes relative to papain and other well-known proteases. Such studies on comparative biochemistry may provide better insight into the structure−function relationship of proteases.

* To whom correspondence should be addressed (tel, 91-542-316606; fax, 91-542-317217; e-mail, jvm@banaras.ernet.in).

10.1021/jf990661j CCC: $19.00
Published on Web 01/29/2000

MATERIALS AND METHODS

Materials. Fresh latex was obtained from young stems of the plant *E. coronaria*. SP-Sepharose was purchased from Pharmacia. TEMED, HEPES, BSA, RNase A, hen egg white lysozyme, azocasein, azoalbumin, glycerol, DTNB, DTT, Gu-HCl, urea, *o*-phenanthroline, EDTA, EGTA, leupeptin, SBTI, NEM, β-mercaptoethanol, PMSF, hemoglobin, Coomassie brilliant blue R-250, all synthetic substrates, Freund's complete and incomplete adjuvants, agarose, Tween-20, *o*-phenylenediamine, goat anti-rabbit IgG horseradish peroxidase conjugate, and other standard proteins were obtained from Sigma Chemical Co. Ampholine carrier ampholytes were from LKB. Coomassie brilliant blue G250 was from Eastman Kodak. TFA was obtained from Applied Biosystems. Acetonitrile was of HPLC grade. PVDF membrane was from Millipore. All other chemicals were of highest purity available commercially. Sodium tetrathionate ($Na_2S_4O_6 \cdot 2H_2O$) was synthesized by the method of Gilman et al. (1946). Papain was purified from dried latex purchased from Enzochem, India, by the method of Kimmel and Smith (1957). Hemoglobin was denatured with urea before assay as described (Sarath et al., 1989).

Proteinase Purification. All purification steps were performed at 4 °C unless specified otherwise.

Step 1: Gum Removal. Latex was collected from young stems in 0.01 M Na-acetate buffer, pH 5.0, containing 5 mM sodium tetrathionate and frozen at −20 °C for 24 h. The latex was thawed to room temperature and centrifuged at 17000*g* for 20 min to remove gum and other debris. The resulting clear supernatant was used in the next step.

Step 2: Cation-Exchange Chromatography. Chromatography was performed at room temperature. Protein solution from the previous step was applied to a SP-Sepharose fast flow ion-exchange column preequilibrated with 0.01 M acetate buffer, pH 5.0, containing 5 mM sodium tetrathionate. The column was washed with the same buffer until no protein was detected in the eluate. Bound proteins were eluted at a flow rate of 7 mL/min, initially with 0.3 M NaCl and then with a linear gradient of NaCl from 0.3 to 1.0 M over 15 column volumes. Fractions of 4 mL were monitored by absorbance at 280 nm and assayed for enzyme activity on azocasein, and their purity was assessed by SDS−PAGE. Three active peaks designated as A, B, and C were obtained. Active fractions of peak A and homogeneous, active fractions of peak C were pooled and concentrated separately by 80% ammonium sulfate saturation followed by dialysis against 0.01 M Na-phosphate buffer, pH 7.0. The clear protein solutions were sterile filtered and stored at 4 °C for further use.

Step 3: Crystallization. Active fractions of peak B were pooled and subjected to 80% ammonium sulfate saturation. Precipitation was allowed for 12−14 h, and a very thick suspension of the protein pellet (∼30−35 mg/mL), collected by centrifugation, was dialyzed against 750 mL of 0.01 M Na-phosphate buffer, pH 8.0, containing 5 mM sodium tetrathionate with changes of the dialysis buffer (750 mL) at an interval of 11−12 h. Within 6−7 h tiny crystals appeared in the dialysis bag and crystallization was complete within 2 days when the crystals settled at the bottom of the dialysis bag. The crystals thus formed were collected by centrifugation at 17000*g* for 30 min at 4 °C, dissolved in 0.01 M sodium acetate buffer, pH 5.0, and dialyzed against the same. The clear protein solution was sterile filtered and stored at 4 °C for further use.

Protein Concentration. Protein concentration was determined spectrophotometrically as well as by Bradford assay using BSA as standard (Bradford, 1976).

Enzyme Activity Assays. Routinely, the general proteolytic activity of the enzyme was monitored using denatured substrates such as casein, hemoglobin, azoalbumin, and azocasein by the method of Arnon (1970) with some modifications (Sundd et al., 1998). Per tube 2−4 μg of enzyme was used and the reaction was terminated by an addition of 0.5 mL of 10% TCA. The control assay was performed without any enzyme in the reaction mixture and used as reference.

Assay for Enzymatic Hydrolysis of Peptidyl-*p*-Nitroanilide. The amidolytic activities of the enzyme were studied using BAPA, L-alanine-alanine-*p*-nitroanilide, succinyl-phenylalanine-*p*-nitroanilide, L-glutamyl-*p*-nitroanilide, L-alanine-*p*-nitroanilide, *N*-succinyl-alanine-alanine-alanine-*p*-nitroanilide, and L-leucine-*p*-nitroanilide by the method of Arnon (1970) with some modifications (Sundd et al., 1998). The reaction mixture contained 5−25 μg of active enzyme in 0.5 mL buffer and 0.5 mL of the peptidyl-*p*-nitroanilide (5−20 mM). The liberated *p*-nitroaniline was monitored spectrophotometrically by measuring absorbance at 410 nm against a blank sample containing no enzyme and an extinction coefficient of 8800 M^{-1} cm^{-1} (Erlanger et al., 1961) was used to calculate the initial reaction velocity.

pH Optima. The reaction mixture was composed of 0.5 mL of enzyme solution equilibrated for 30 min at room temperature in an appropriate buffer of desired pH and 0.5 mL of 1% hemoglobin or 0.6% azocasein or azoalbumin solution at the same pH. The assay was done in the presence of an activator at 37 °C as described above. Enzyme assay below pH 4.0 could not be done with azoalbumin or azocasein as substrate due to its insolubility. However, the enzyme activity was checked below pH 4.0 using denatured hemoglobin (Sarath et al., 1989). Blank determinations were done simultaneously at all pH's without enzyme.

Temperature Optima. The effect of temperature on the activity of the enzyme was studied using 0.6% azoalbumin solution as substrate in 0.1 M Tris-HCl, pH 8.0, prepared at specified temperatures. The enzyme was equilibrated in the same buffer at different temperatures in the range of 15−80 °C for 15 min and an appropriate aliquot was assayed at the same temperature as described above. Blank determinations were done at the respective temperatures without enzyme.

Effects of Activators. The effects of various thiol-specific activators such as β-mercaptoethanol, DTT, cysteine, and glutathione on the proteolytic activity of the enzyme were measured. Enzyme of concentration 3 μg/mL was incubated for 15 min with activators in the concentration range 0−40 mM and a sample was assayed by the method described above.

Effects of Inhibitors. Inhibition of the hydrolysis of a substrate by the enzyme was investigated using sodium tetrathionate, iodoacetamide, PMSF, mercuric chloride, PCMB, leupeptin, EDTA, EGTA, SBTI, *o*-phenanthroline, and NEM. The tetrathionate-inactivated enzyme was activated by 0.05 M β-mercaptoethanol at room temperature in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.002 M EDTA for 30 min. The activator was removed subsequently by dialysis against 0.1 M acetic acid at 4 °C with frequent changes of the dialysate. In each case, 1 μM enzyme was incubated in the presence of increasing concentrations of inhibitors in 0.1 M Tris-HCl buffer, pH 8.0, for 20 min at 37 °C and assayed with azocasein as substrate. A control assay was done with enzyme solution without inhibitors and the resulting activity was considered as 100%.

Effects of Substrate Concentration on Reaction Velocity. Both natural and synthetic substrates were used to study the effects of substrate concentration on the reaction velocity of enzymatic hydrolysis at pH 8.0 and 37 °C. In the case of natural substrate, 2.5 μg of the enzyme was used and the concentration of azoalbumin was increased from 3 to 75 μM. For synthetic substrate, 15 μg of the enzyme was assayed using *N*-succ-Ala-Ala-Ala-*p*-NA in the concentration range of 2−33 mM. The assays were performed as described above. Blank determinations were done simultaneously at the specified substrate concentrations without enzyme. In each case, a Lineweaver−Burk plot was plotted and the values of the Michaelis−Menten constant (K_m) and the maximum reaction velocity (V_{max}) were calculated (Segel, 1976). The value of the catalytic constant (K_{cat}) was estimated by dividing the V_{max} value by the amount of enzyme and assuming that 26 mg of protein (M_r ∼26 000) represents 1 μmol of enzyme (Giovanni-De-Simone et al., 1997).

Electrophoresis. SDS−PAGE using 15% polyacrylamide gel was done by the method of Laemmli (1970) for assesment of homogeneity and estimation of molecular mass (M_r) of the purified enzyme. To avoid protein degradation during boiling,

sodium tetrathionate-treated samples (Shapira and Arnon, 1969) were boiled in nonreducing SDS treatment buffer and then reboiled under reducing conditions. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. Ovotransferrin (76 kDa), albumin (66.25 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (30 kDa), myoglobin (17.2 kDa), and cytochrome *c* (12.3 kDa) were used as molecular weight standards.

Gel Filtration. Molecular mass (M_r) of the purified enzyme was estimated by gel filtration as described by Fish et al. (1969) on a TSK G2000SW (from LKB) gel filtration column fitted to an LKB Bromma HPLC system provided with a variable wavelength detector. Reduced and blocked proteins were used (Sundd et al., 1998).

Isoelectric Focusing. The isoelectric point (p*I*) of the enzyme was determined by isoelectrofocusing on polyacrylamide gels as described (Ramesh and Rao, 1980) with some modifications. Electrophoretic runs were carried out with ampholine carrier ampholytes, pH 9−11. The polyacrylamide gel was prepared as follows. A solution of acrylamide (8.66 g) and *N*,*N*′-methylenebisacrylamide (0.268 g) was prepared in distilled water to a final volume of 25 mL and filtered. Carrier ampholyte (0.45 mL), 25% glycerol (1.8 mL), and water (5.25 mL) were mixed with 1.5 mL of acrylamide solution. The gel solution was deaerated, TEMED (7.5 μL) and 20% ammonium persulfate (15 μL) were added, and the solution was cast in tubes (5 mm × 125 mm) with a layer of water on the top. Anodic and cathodic chambers, flushed with nitrogen gas before electrophoresis, contained 0.01 M HEPES and 0.01 M triethanolamine, respectively. The gels were prerun at a constant voltage of 300 V for 30 min at room temperature. Samples (∼100 μg) containing 10% (v/v) ampholine and 25% glycerol were loaded on the gels and electrophoresed for 3 h at the same voltage. After complete run, the gels were stained with the stain fixative containing 0.04% (w/v) Coomassie G250 in 6% (w/v) perchloric acid (Merril, 1990). An equivalent gel isoelectrophoresed under identical conditions was sliced into 1-cm pieces and the pH measured in water after a 15-min equilibrium.

Determination of Extinction Coefficient. The extinction coefficient of the enzyme was calculated by dry weight (Glazer and Smith, 1961) and spectrophotometric (Aitken and Learmoth, 1997) methods. In the dry weight method the enzyme was activated by β-mercaptoethanol, inhibited with excess of iodoacetamide, and dialyzed against water. From a stock solution of the enzyme in water, several solutions of different concentrations were prepared by serial dilution, absorbance of these solutions was measured at 280 nm, and the samples were dried in an oven at 108−110 °C. The dry weight of the protein in each sample was measured. The extinction coefficient was calculated using Beer−Lambert's law. In the spectrophotometric method, the extinction coefficient was calculated from the given formula by Aitken and Learmoth (1997).

Tyrosine and Tryptophan Content. The tyrosine and tryptophan contents of the enzyme were measured spectrophotometrically using the method of Goodwin and Morton (1946). The absorbance spectra of the enzyme in 0.1 M NaOH was recorded between 300 and 220 nm using a Beckman DU 640 B spectrophotometer and the absorbance values at 280 and 294.4 nm were deduced from the spectra. The standard formula (Goodwin and Morton, 1946) was used to estimate the tryptophan and tyrosine contents. To validate these measurements, similar contents of papain, ribonuclease, and lysozyme were determined simultaneously.

Free and Total Sulfhydryl Content. Ellman's method (1959) was used to determine the free and total sulfhydryl content of the enzyme. For the free sulfhydryl group measurement, the purified enzyme was activated with 0.01 M β-mercaptoethanol in 0.05 M Tris-HCl, pH 8.0, for 15 min and then dialyzed against 0.1 M acetic acid. Similarly, for the measurement of total sulfhydryl content the enzyme was reduced in the presence of 6 M GuHCl (Riddles et al., 1983). The free cysteine and total sulfhydryl contents were measured by the DTNB reaction (Creighton, 1989). To validate these measurements, similar contents of papain, ribonuclease, and lysozyme were also measured.

Measurement of Carbohydrate Content. In view of the reports that some plant proteases such as calotropin FI and FII (Abraham and Joshi, 1979) and stem bromelain (Takahashi et al., 1973) are glycoproteins, the carbohydrate content of this plant protease was measured by phenol−sulfuric acid method (Hounsell et al., 1997). A solution of the enzyme in water in the concentration range of 1−10 μg/10 μL was taken in a microtiter plate. To each well was added 25 μL of 4% aqueous phenol. After 5 min 200 μL of H_2SO_4 was added and the increase in absorbance was measured at 492 nm. A standard plot was generated with known concentrations of galactose and the unknown values of the sample were extrapolated.

Stability Studies. The ability of the purified enzyme to retain its activity under various hostile conditions was studied by exposing the enzyme to those conditions. The enzyme was incubated in buffers of various pH's ranging from 0.5−12.0 and at higher concentrations of GuHCl, urea, SDS, acetonitrile, methanol, and ethanol for 24 h or more. The remaining proteolytic activity of the enzyme was measured at 37 °C, using aliquots withdrawn at different time intervals, with azocasein as substrate, as described previously. Similarly, the enzyme was incubated at different temperatures for 15 min and assayed at 37 °C. The assay was done by the method described above.

Polyclonal Antibodies. Antibodies to the purified enzyme were raised in a male albino rabbit (about 1.25 kg). The enzyme in 10 mM acetate buffer, pH 5.5, was emulsified with an equal volume of Freund's complete adjuvant and injected (200 μg of enzyme) subcutaneously at multiple sites. After 1 week a booster dose of 350 μg of enzyme was administered as an emulsion formed with Freund's incomplete adjuvant. Three more similar doses were administered at intervals of 10, 7, and 15 days. After 7 days of the last dose the rabbit was bled through the marginal ear vein. Blood was allowed to clot initially for 1 h at room temperature and later for 12 h at 4 °C. Supernatant was collected by centrifugation. Preimmune serum was obtained from the rabbit before the first injection of antigen. All sera were stored at −20 °C. The presence of antibodies was confirmed by immunoassays.

Immunoassays. Ouchterlony's double immunodiffusion was performed as described by Ouchterlony and Nilsson (1986); 1% agarose in phosphate-buffered saline containing 0.02% sodium azide was solidified in Petri dishes and appropriate holes were punched into it. Various antigens (40 μg) and 100 μL of antiserum were loaded in the wells and left at room temperature for 24 h. Control assays were performed with preimmune serum.

An indirect ELISA was performed (Friguet et al., 1989) to check cross-reactivities of various antigens with antiervatamin B serum. Wells of a microtiter plate were coated with 1 μg/mL ervatamin B, ervatamin C, and papain (100 μL/well) in 50 mM sodium carbonate buffer, pH 9.6, and incubated overnight at 4 °C. The wells were washed thoroughly with phosphate-buffered saline, pH 8.0, containing 0.05% Tween-20 (PBS-T) and coated with 5% BSA to avoid nonspecific adsorption. After 1−2 h of incubation at 37 °C, the wells were washed thoroughly and primary antibody to ervatamin B appropriately diluted in PBS-T was added at 100 μL/well. The plate was incubated at 37 °C for 1−2 h and washed as before. The goat anti-rabbit IgG, linked to horseradish peroxidase, was diluted 5000 times in PBS-T and added to the wells. After 1−2 h of incubation at 37 °C, the wells were washed as before. Subsequently, 100 μL of substrate solution prepared by dissolving 10 μL of H_2O_2 and 9 mg of *o*-phenylenediamine in 25 mL of 0.1 M citrate-phosphate buffer, pH 5.0, was added and left at 37 °C for 10 min. The color developed was measured in a microtiter plate reader at 490 nm. Preimmune serum was used as a negative control.

Amino-Terminal Sequence Analysis. The homogeneous enzyme, at a concentration of 0.2−0.25 mg/mL, was dialyzed extensively against autoclaved distilled water at 4 °C. Aceto-

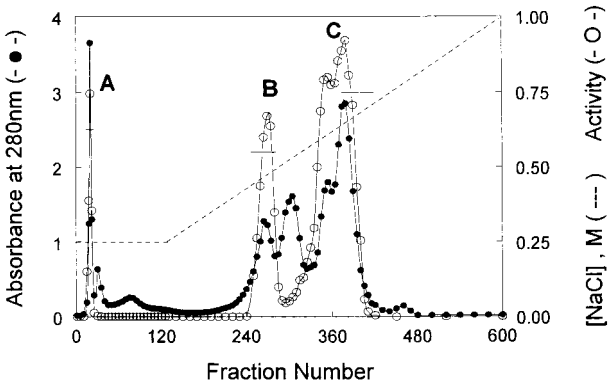

Figure 1. Chromatography of ervatamins on SP-Sepharose fast flow. Crude latex of *E. coronaria* (see Materials and Methods) was applied to a SP-Sepharose fast flow column equilibrated with 0.01 M acetate buffer, pH 5.0, and washed extensively with the same buffer before the bound proteins were eluted with 0.3 M NaCl followed by a linear gradient of 0.3−1.0 M salt. Fractions were collected at a flow rate of 7 mL/min and assayed for protein content (A_{280}) (●) and proteolytic activity (○) against azocasein. Activity peaks were pooled as indicated by horizontal lines, concentrated, and processed further.

nitrile and TFA were added to the dialyzed sample to a final concentration of 60% and 0.1%, respectively. The resulting solution was freeze-dried on a Virtis lyophilizer and sequenced on an Applied Biosystems 477A protein sequenator by the method of Matsudaira (1987).

RESULTS

Purification. The purification of one cysteine protease, previously called ervatamin, from the latex of *E. coronaria* has been reported (Sundd et al., 1998), and the purification procedure revealed another fraction with some proteolytic activity. It was suggested that they may be isozymes of each other. Here the purification protocol has been modified and optimized to identify at least three cysteine proteases and purify two of them to homogeneity. In the modified protocol the crude latex was processed without prior ammomium sulfate precipitation. Chromatography on a cation-exchange column yielded five peaks (Figure 1). No proteolytic activity was found in the unbound eluate, and this has not been included in the figure. The first two peaks contained fractions eluted by 0.3 M NaCl, and fractions eluted by the liner salt gradient gave the successive three peaks. Only peaks A, B, and C (Figure 1) were active (A, B, and C designate the position of the active peaks on elution from the ion-exchange column). Activity measurements showed that the majority of the total activity loaded on the column was eluted in peaks B and C. Peak A showed two major bands and needed to be processed further. Since this peak is active it should have at least one cysteine protease. Fraction B was purified to homogeneity by crystallization, and the crystals formed during purification diffract X-rays to 2.8 Å (Chakrabarti et al., 1999). Under similar conditions fraction A or C did not crystallize. Homogeneity and intactness of the pure enzyme were judged by SDS−PAGE as shown in Figure 2. The homogeneity was further confirmed by gel filtration on HPLC which showed single symmetrical peaks for fractions B and C (data not shown). The enzymes in fractions B and C were called ervatamins B and C. The enzyme purified previously corresponds to ervatamin C (Sundd et al., 1998).

The purification results are summarized in Table 1. It is worth mentioning that if latex was collected in 0.01 M sodium phosphate buffer, pH 7.0, instead of 0.01 M acetate buffer, pH 5.0, then the overall yield of ervatamin B is reduced by 46.0%. Hence, throughout the purification 0.01 M acetate buffer, pH 5.0, containing 5 mM sodium tetrathionate was used. The enzyme at higher concentrations was stored for further use in the same buffer. Generally, the proteases are prone to autolysis. In the case of ervatamin B, the autolytic character was checked over a period of time in the presence and absence of sodium tetrathionate, and it was found that the inactive enzyme does not undergo autolysis (data not shown).

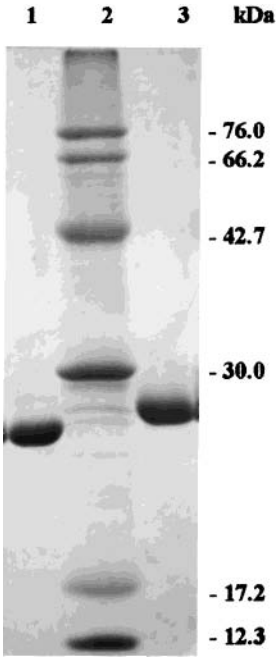

Figure 2. SDS−PAGE of purified ervatamins. Reversibly inactive enzymes (20 μg) were electrophoresed in 15% polyacrylamide gels under reducing conditions: lane 1, ervatamin C; lane 2, protein markers; lane 3, ervatamin B. Ovotransferrin, albumin, ovalbumin, carbonic anhydrase, myoglobin, and cytochrome *c* were used as standards, and their molecular weights are indicated beside the gels. The proteins were stained with Coomassie brilliant blue R-250.

Table 1. Purification of Proteases from *E. coronaria*

	total prot (mg)	total act (units[a])	specific act (units/mg)	recovery (%)
1. crude extract	1447.80	3981.45	2.75	100.00
2. pool B	237.85	796.80	3.35	16.43
3. ervatamin B (crystallized)	115.09	633.01	5.50	7.95
4. ervatamin C	120.32	421.13	3.50	8.31

[a] Definition of unit: 1 unit of enzyme activity is defined as the amount of enzyme which, under the assay conditions described, gives rise to an increase of 1 absorbance at 440 nm/min of digestion. Azocasein was used as substrate, and the assay was done as described in Materials and Methods.

Physical Properties. Ervatamin B showed a single band in SDS−PAGE with an estimated molecular mass (M_r) of 27 039 Da (Figure 2). The molecular mass obtained by gel filtration was 24 915 Da (data not shown). For all purposes an average M_r of 26 000 was assigned to this enzyme. The extinction coefficient measured by dry weight and spectrophotometric methods was 20.5. Like many other cysteine proteases, ervatamin B was found to contain no detectable carbohydrate. Ervatamin B also showed a single band on isoelectrofocusing (Figure 3) with an approximate p*I* of

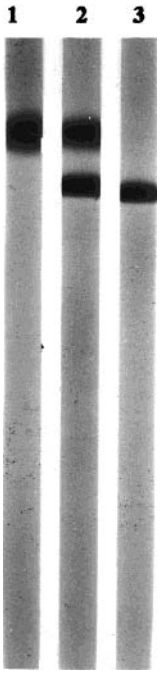

Figure 3. Isoelectric focusing of ervatamins in tube gels. Polyacrylamide gels (5%) were prepared in glass tubes, and electrophoretic runs were carried out with ampholine carrier ampholytes, pH 9−11. The anolyte was 0.01 M HEPES, and the catholyte was 0.01 M triethanolamine. Samples (100 μg) containing 10% ampholine and 25% glycerol were loaded on the gels and electrophoresed for 3 h at a constant voltage of 300 V. The gels were stained with Coomassie G-250: tube 1, ervatamin B; tube 2, ervatamins B and C; tube 3, ervatamin C.

Table 2. Specific Activities of Ervatamin B on Various Substrates

	specific activity			
enzyme	casein[a] (units/mg)	azocasein[a] (units/mg)	azoalbumin[a] (units/mg)	*N*-succ-Ala-Ala-Ala-*p*-NA[b] (mol/min/mol)
ervatamin B	9.0 ± 0.5	5.5 ± 0.5	7.5 ± 0.5	12.45 ± 1.0

[a] Definition of unit and specific activity: 1 unit of enzyme activity is defined as the amount of enzyme which, under the assay conditions described, gives rise to an increase of 1 absorbance at 280 or 440 nm/min of digestion. The specific activity is the number of units of activity/mg of protein. The assays were done as described in Materials and Methods at pH 8.0 and 37 °C. The final concentrations of substrates used were 0.5% casein and azocasein and 0.25% azoalbumin. [b] Definition of specific activity: the specific activity is defined as the number of mol of product released/min of reaction/mol of enzyme. The assays were done as described in Materials and Methods at pH 8.0 and 37 °C.

9.35, while ervatamin C showed a p*I* of 9.53. The p*I* values of ervatamins B and C are higher than that of papain which has a p*I* value of 8.8 (Sumner et al., 1993).

Substrate Specificity. The enzyme ervatamin B hydrolyzed denatured natural substrates such as casein, azocasein, and azoalbumin with high specific activity (Table 2) relative to that of ervatamin C and some reported proteases as well as the other proteases being worked out in our laboratory (data not shown). Unlike ervatamin C, it showed amidolytic activity toward *N*-succ-Ala-Ala-Ala-*p*-nitroanilide (Table 2). However, ervatamin B did not show any activity toward the other synthetic substrates (mentioned in Materials and Methods). The exact reason for the activity of ervatamin B specifically toward *N*-succ-Ala-Ala-Ala-*p*-nitroanilide among other synthetic substrates is not known.

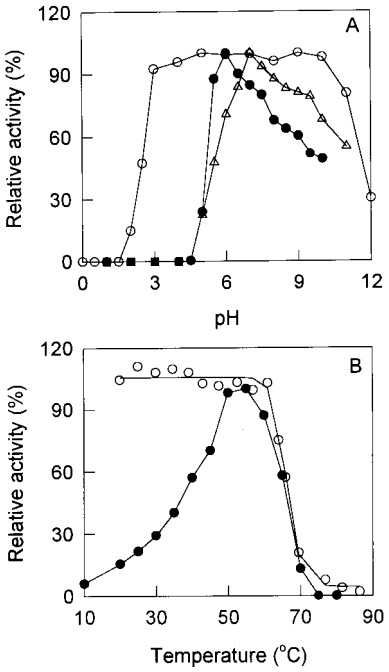

Figure 4. (A) Effects of pH on activity and stability of ervatamin B. Assay solution consisted of 3 μg of activated enzyme in 0.5 mL of buffer, and 0.5 mL of substrate was added. The enzyme was incubated in a solution of the required pH for 30 min at room temperature, and activity was measured at the same pH. As azoalbumin and azocasein are insoluble below pH 4.0, 2% (w/v), denatured hemoglobin was used as substrate in the low pH range: activity toward azoalbumin (△), azocasein (●), and denatured hemoglobin (■); effects of pH on stability of ervatamin B (○). The purified enzyme was incubated at the required pH for 24 h at room temperature, and an aliquot was used for assay at 37 °C and pH 8.0 as described in Materials and Methods. Before assay the enzyme was activated by β-mercaptoethanol. In both cases the buffers used were: 0.1 M KCl-HCl (pH 0.5−1.5); 0.1 M glycine-HCl (pH 2.0−3.5); 0.1 M acetate (pH 4.0−5.5); 0.1 M phosphate (pH 6.0−7.5); 0.1 M Tris-HCl (pH 8.0−10.0); and 0.1 M carbonate (pH 10.5−12.5). (B) Effects of temperature on activity (●) and stability (○) of ervatamin B. Assay solution consisted of 3 μg of activated enzyme in 0.5 mL of buffer and 0.5 mL of 0.6% azoalbumin. The enzyme was incubated in 0.1 M Tris-HCl buffer, pH 8.0, at the required temperature for 15 min, and activity was measured at the same temperature. For stability measurements, the enzyme was incubated in buffer at the required temperature for 15 min, and activity was measured at 37 °C and pH 8.0.

pH and Temperature Optima. The optimum pH for the hydrolysis of azocasein by ervatamin B was 6.0−6.5 as shown in Figure 4A. Half-maximum activity was obtained at pH 5.25 and 10.0. In contrast, with azoalbumin as substrate, the maximum proteolytic activity for the enzyme was at pH 7.0−7.5 (Figure 4A) and half-maximum activity was at pH 5.5 and 10.5. Overall, the enzyme showed a broad spectrum of proteolytic activity in the pH range 5.5−10.0. No activity could be detected below pH 4.5. The hydrolysis of azoalbumin by ervatamin B was optimal around 50−55 °C as shown in Figure 4B and half-maximum activity was observed at 38 and 67 °C.

Effects of Activators. Relative activity of the enzyme at different concentrations of thiol-specific activators such as cysteine, β-mercaptoethanol, DTT, and glutathione was investigated (data not shown). It appears that at least 7.5−10 mM concentration of the activator is necessary to show the maximum activity under the given conditions. In the case of DTT and

Table 3. Effect of Various Compounds on the Proteolytic Activity of Ervatamin B toward Denatured Natural Substrates

compounds	concentration	relative act[a] (%)
none		100
PMSF	40 μM	95.7
NEM	50 μM	29.8
PCMB	8 μM	8.97
SBTI	100 μg/mL	100
EDTA	1 mM	100
EGTA	1 mM	100
o-phenanthroline	1 mM	100
mercuric chloride	2 μM	5.73
iodoacetamide	40 μM	9.63
leupeptin	8 μM	13.11
sodium tetrathionate	40 μM	17.38

[a] Enzyme activity was measured as described in Materials and Methods.

cysteine, however, a lower concentration was enough to result in the same activation.

Effects of Inhibitors. The minimum amount of effective inhibitor required for maximum inhibition is reported in Table 3. The activity was inhibited considerably by all the thiol-specific inhibitors studied but not by serine-specific inhibitors such as PMSF. The enzyme activity was not inhibited by EDTA and SBTI.

Effects of Substrate Concentration on Reaction Velocity. The effects of increasing substrate concentration on reaction velocity follow the typical Michaelis−Menten equation (Figure 5). With azoalbumin as substrate the apparent K_m obtained from the Lineweaver−Burk plot was $25 \pm 3 \times 10^{-6}$ M (inset of Figure 5A). With *N*-succ-Ala-Ala-Ala-*p*-nitroanilide as substrate the apparent K_m was 6.6 ± 0.5 mM (inset of Figure 5B). This value is of the order of the K_m of papain (5.06 ± 0.40 mM) with BAPA as substrate at pH 8.28 (Mole and Horton, 1973). The values of V_{max} and K_{cat} for ervatamin B were $5.9 \pm 0.5 \times 10^{-6}$ M min^{-1} and 1.87×10^2 s^{-1}, respectively. With the synthetic *p*-nitroanilide, the enzymatic activity increased with increasing substrate concentration up to 15 mM and then decreased with further increase of substrate concentration (Figure 5B).

Specific Amino Acid Residues. The tyrosine and tryptophan contents of the protein were 10 (measured value 9.95) and 7 (measured value 7.04), respectively. Under the same experimental conditions ribonuclease, papain, ervatamin C, and lysozyme yielded the reported values. The tryptophan content was the same as that for ervatamin C (7) but different from that for papain (5), while the content of tyrosine was different from those of both ervatamin C (16) and papain (19) (Sumner et al., 1993). The total sulfhydryl content of the protein was found to be 5 (measured value 5.32) with one (measured value 1.15) free cysteine and the other four forming two disulfides. The cysteine content is less than that of ervatamin C and from most of the identical plant cysteine proteases reported. Thus, ervatamin C (Sundd et al., 1998), papain (Sumner et al.,1993), asclepain B5 (Brockbank and Lynn, 1979), calotropin DI, DII, and FI (Pal and Sinha, 1980), papaya proteinase 3,4 (Sumner et al., 1993), and actinidin (Baker, 1977) all have three disulfide bridges. Chymopapain (Sumner et al., 1993) and ficin (Englund et al., 1968) have eight sulfhydryl groups, while stem bromelain (Takahashi et al., 1973) has nine.

Stability. Like papain, even after prolonged exposure the enzyme retained its activity over the pH range 3.0−10.5 as shown in Figure 4B. At pH 2.5 half of the initial

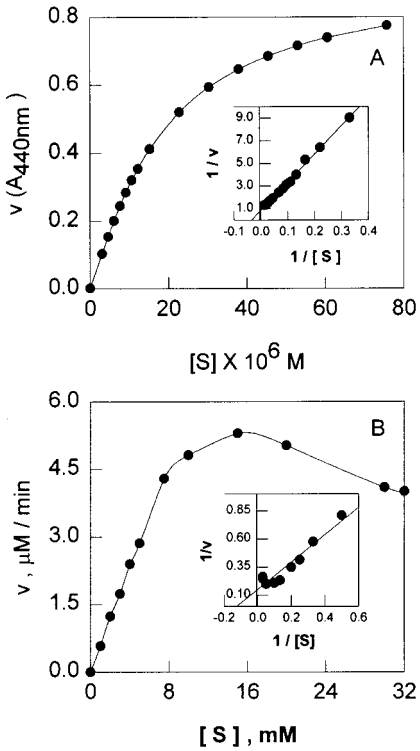

Figure 5. Effect of substrate concentrations on reaction velocity of ervatamin B. (A) To 2.5 μg of the enzyme in 0.5 mL of 0.1 M Tris-HCl, pH 8.0, containing 0.05 M β-mercaptoethanol was added 0.5 mL of azoalbumin in the concentration range 3−75 μM. After 30 min the reaction was terminated by addition of 0.5 mL of 10% TCA. The supernatant, in each case, was mixed with an equal volume of 0.5 M NaOH, and the absorbance at 440 nm was considered as the reaction velocity. Inset: Lineweaver−Burk plot. K_m was calculated according to the Michaelis−Menten equation. (B) To 15 μg of the enzyme in 0.5 mL of 0.1 M Tris, pH 8.0, containing 0.05 M β-mercaptoethanol was added 0.5 mL of the synthetic substrate in the concentration range 2−33 mM. The digestion was allowed to proceed for 30 min at 37 °C, and the reaction was terminated by the addition of 0.2 mL of 30% acetic acid. The liberated *p*-nitroaniline was measured spectrophotometrically at 410 nm. An extinction coefficient of 8800 M^{-1} cm^{-1} was used to calculate the initial reaction velocity. Inset: Lineweaver−Burk plot. K_m was calculated according to the Michaelis−Menten equation.

activity was lost. Thermostability of the enzyme was also examined by measuring the residual activity after 15 min of incubation at various temperatures as shown in Figure 4B. Full activity was retained almost up to 62 °C. Further, the enzyme was stable in 8 M urea and 2.5 M GuHCl at neutral pH, in 40% acetonitrile, 70% ethanol, and 50% methanol. However, it was unstable in SDS.

Polyclonal Antibodies and Immunoassays. The presence of polyclonal antibodies in anti-rabbit serum was checked by immunodiffusion. Precipitin lines start appearing after about 10−12 h of incubation at room temperature and are distinctly visible by about 24−30 h. One merging precipitin band devoid of spur formation (Figure 6A) was obtained when antiervatamin B serum was loaded in the central well surrounded by ervatamin B antigen in the peripheral wells, indicating the purity of ervatamin B and that the antibodies are specific to the enzyme. A control experiment was also performed with preimmune serum in the central well surrounded by ervatamin B antigen in the peripheral wells, and no precipitin line was observed (data not shown). Antisera to ervatamin B did not cross-react with papain or

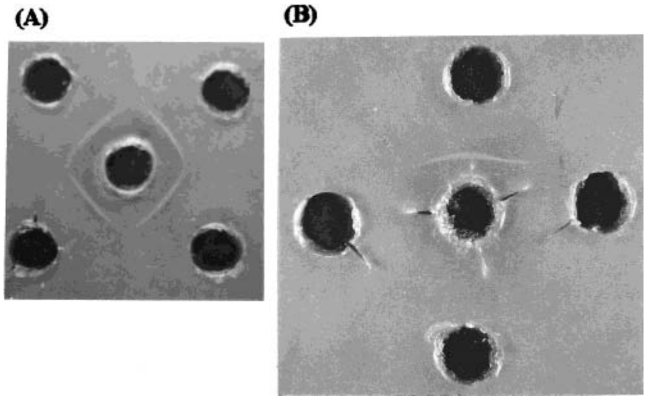

Figure 6. Ouchterlony's double immunodiffusion. (A) Immunodiffusion of ervatamin B (peripheral wells) against antiervatamin B serum (central well). (B) Immunodiffusion of ervatamin B (upper well), ervatamin C (right well), preimmune serum (bottom well), and papain (left well) against antiervatamin B serum (central well). 1% agarose in phophate-buffered saline was poured into Petri dishes and allowed to gel. Wells, punched in the plate and arranged as above, contained 40 μg of antigen and 100 μL of antiervatamin B serum or preimmune serum. The plates were left at room temperature for about 24 h.

ERVATAMIN B	L	P	S	F	V	D	W	X	S	K	G	A	V	N	S	I	K	N	Q	K	Q
PAPAIN	I	P	E	Y	V	D	W	R	Q	K	G	A	V	T	P	V	K	N	Q	G	S
ERVATAMIN C	L	P	E	Q	I	D	W	R	K	X	G	A	V	T	P	V	W	N	Q	G	W
ASCLEPAIN	L	P	N	S	I	D	W	R	Q	K	N	V	V	F	P	I	K	N	Q	G	G
BROMELAIN	V	P	Q	S	I	D	W	R	N	Y	G	A	V	T	S	V	K	N	Q	N	P

Figure 7. Comparison of the N-terminal amino acid sequence of ervatamin B with known cysteine proteases. Abbreviations of amino acids follow the alphabetical system.

ervatamin C (Figure 6B) suggesting that the antigenic determinants of the latter two cysteine proteinases differed from those of ervatamin B. Normal rabbit serum, however, did not give any precipitin line with the anti-enzyme serum (bottom well in Figure 6B) or papain and ervatamin C.

Typical color development in indirect ELISA due to antigen−antibody (ervatamin B and its antibodies) complex formation also indicates the presence of antibodies to ervatmin B in the serum of immunized rabbit. Insignificant color development in the case of ervatamin C and papain shows the absence of cross-reactivities of papain or ervatamin C with antiervatamin B.

Amino-Terminal Sequence. The N-terminal sequence of the first 21 amino acid residues of ervatamin B has been compared to those of ervatamin C (Sundd et al., 1998) and papain (Mitchel et al., 1970). It showed about 47% sequence similarity to ervatamin C and 57% to papain. The sequence similarity is shown in Figure 7. The N-terminal sequence of other known plant cysteine proteases such as asclepain (Lynn et al., 1980) and bromelain (Goto et al., 1980) have also been shown to indicate the conserved amino acid residues in these enzymes.

DISCUSSION

At least three cysteine proteases have been identified in the latex of the valuable plant *E. coronaria* thus demonstrating the phenomenon of multiplicity of cysteine proteases in plant latices. The multiple forms of ervatamins probably exist in vivo and are not products of self-proteolysis of one or more native species.

A simple purification procedure has been standardized to simultaneously purify two cysteine proteinases, ervatamins B and C, in good yield. Such an economic purification procedure combined with the easy availability of the latex makes large-scale preparation of the enzymes feasible allowing an extensive study of its various aspects and hence probable applications. Endopeptidases such as papain and stem bromelain have been extensively used for medicine, brewing wine, and food industries (Kaneda et al., 1997). In fact 60% of the enzymes used in industry are proteolytic, and hence the study of proteolytic enzymes such as ervatamins B and C is important.

The low yield of the enzyme when processed with buffer of pH 7.0 instead of pH 5.0 might be due to the low solubility of this enzyme near neutrality and at low ionic strength. This property of the enzyme has been used for its crystallization which yields pure ervatamin B. The specific activity of crude latex was quite high because the proteolytic assay was not specific for ervatamin B or C but due to all the proteolytic enzymes present in it. This may give a wrong impression about the fold-purification of the enzyme in terms of specific activity, if each of the purified enzymes is compared to the crude. Further, if the total specific activity of all the enzymes together is compared to that of the crude, the purification fold is very high. A single protein band in SDS−PAGE and tube gel isoelectric focusing, a single symmetrical peak in gel filtration, and a single precipitin line in Ouchterlony's double immunodiffusion shows the purity of the enzyme.

The enzyme showed proteolytic activity toward natural substrates, and the activity was inhibited by thiol-specific inhibitors but not by serine-specific inhibitors or metal chelators. It was maximally activated by various reducing and chelating reagents. The estimated molecular mass of ervatamin B (26 000 Da) was in the range of molecular mass (20 000−35 000 Da) reported for other plant cysteine proteases (Turk et al., 1997). N-Terminal sequence of the first 21 residues showed that the amino acid residues common to known plant cysteine proteases were also conserved in the sequence of ervatamin B. All these suggest that the proteinase belongs to the class of cysteine proteases.

Ervatamin B, like some other cysteine proteases but unlike ervatamin C, showed amidolytic activity in addition to proteolytic activity. However, unlike papain and ficin it categorically cleaves *N*-succ-Ala-Ala-Ala-*p*-nitroanilide among other synthetic anilides. It does not hydrolyze BAPA, an ideal substrate for papain, ficin, and other plant endopeptidases. Thus, the substrate specificity of ervatamin B seems to be different from those of ervatamin C, papain, and other plant cysteine proteases.

Ervatamin B, like ervatamin C, resists inhibition by proteinaceous inhibitors such as SBTI which is present in protein-rich foods such as soybeans. Hence, it can be used as a proteolytic enzyme in food industries and may have important implications (Kaneda et al., 1997). However, this is the first report of general characterization of the enzyme, and a detailed study in terms of its applications is being explored.

The optimal pH for enzymatic hydrolysis of azocasein and azoalbumin by the enzyme was different. Such a difference in pH optimum with different substrates was also observed in the case of the cysteine protease gingipains R (Potempa et al., 1998) isolated from

Porphyromonas gingivalis. Thus, ervatamin B has a broad spectrum of proteolytic activity. The plant cysteine protease actinidin has a broad pH optimium of pH 5.0−7.0 (McDowall, 1970). The pH optimum of papain, chymopapain, and asclepain is at neutral pH.

The substrate saturation curve obtained with the synthetic substrate showed an interesting feature, where the enzymatic activity decreases when the concentration of the substrate is increased beyond a certain concentration. This could be due to product inhibition or nonspecific peptide−peptide interaction of the substrate molecules resulting in more resistance toward cleavage by ervatamin B.

Ervatamin B is a fairly stable proteinase and resembles papain in its stability toward pH, strong denaturants, temperature, and organic solvents. It showed high stability in 8 M urea, 50% methanol, 70% ethanol, and 40% acetonitrile. It was unstable in SDS but stable in 2.5 M GuHCl, up to a temperature of 62 °C and over a broad pH range of 3.0−10.5. However, ervatamin B is less stable than ervatamin C (Sundd et al., 1998).

An interesting property of ervatamin B is the presence of only five sulfhydryl groups in the molecule. Plant endopeptidases of the papain superfamily mostly showed higher sulfhydryl content of 7, 8, or 9 except in asclepain A3 (Brockbank and Lynn, 1979) and calotropin FII (Pal and Sinha, 1980). The presence of only five sulfhydryl groups with two disulfide bridges in ervatamin B is unique. It is interesting to note that inspite of the presence of only two disulfide bonds, as opposed to three in other plant cysteine proteases, ervatamin B is relatively stable. Study of the structural aspects which contribute to the stability of this enzyme can throw more light in this respect. It is of relevance to learn whether the differences in stability of ervatamins B and C, two cysteine proteases from the same source, are a reflection of the differences in their disulfide content.

Polyclonal antibodies specific to ervatamin B have been raised, and they can be used in turn to purify the enzyme and as a probe for various conformational studies. Immunoassays performed proved that antiervatamin B serum does not cross-react with ervatamin C, papain, and probabaly other related cysteine proteases. Thus ervatamin B is immunologically distinct.

Analysis of the N-terminal amino acid sequence of the first 21 residues of ervatamin B showed considerable similarity to papain. It thus seems apparent that ervatamin B, like ervatamin C, probably shares an ancestral gene with papain.

It is thus evident that ervatamins B and C are cysteine proteases of the papain superfamily which catalyze similar reactions but are distinct from each other in many respects. Thus they differ in their molecular mass, isoelectric point, amidolytic activity, sulfhydryl content, tyrosine content, solubility, and N-terminal sequence. Such differences in their properties could be better analyzed from a knowledge of their three-dimensional structure. Ervatamins B and C seem to provide an excellent system for crystallographic studies, and the crystallization and preliminary X-ray analysis of the proteases having been reported (Chakrabarti et al., 1999), an effort in this direction would provide insight into the structure−function relationship.

ABBREVIATIONS USED

TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; GuHCl, guanidine hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; BAPA, benzoylarginine *p*-nitroanilide; ELISA, enzyme-linked immunosorbent assay.

ACKNOWLEDGMENT

The authors thank Prof. Dinakar M. Salunke, National Institute of Immunology, India, for his help in determination of N-terminal residues.

LITERATURE CITED

Abraham, K. I.; Joshi, P. N. Studies on proteinases from *Calotropis gigantea* latex. I. Purification and some properties of two proteinases containing carbohydrate. *Biochim. Biophys. Acta* **1979**, *568*, 111−119.

Aitken, A.; Learmonth, M. Protein determination by UV absorption. In *The Protein Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1997; pp 3−6.

Anonymous. Ervatamia. In *The Wealth Of India*; CSIR: New Delhi, 1952; Vol. III, pp 192−193.

Arnon, R. Papain. *Methods Enzymol.* **1970**, *19*, 226−244.

Baker, E. N. Structure of actinidin: Details of the polypeptide chain conformation and active site from an electron density map at 2.8 Å resolution. *J. Mol. Biol.* **1977**, *115*, 263−277.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Brockbank, W. J.; Lynn, K. R. Purification and preliminary characterization of two asclepains from the latex of Asclepias syriaca L. (milkweed). *Biochim. Biophys. Acta* **1979**, *578*, 13−22.

Chakrabarti, C.; Biswas, S.; Kundu, S.; Sundd, M.; Jagannadham, M. V.; Dattagupta, J. K. Crystallization and preliminary X-ray analysis of ervatamin B and C, two thiol proteases from Ervatamia coronaria. *Acta Crystallogr. Section D* **1999**, *D55*, 1074−1075.

Creighton, T. E. Disulfide bonds between protein residues. In *Protein Structure− Practical Approach*; Creighton, T. E., Ed.; IRL Press: Oxford, 1989; pp 155−160.

Edwin, F.; Jagannadham, M. V. Sequential unfolding of papain in molten globule state *Biochem. Biophys. Res. Commun.* **1998**, *252*, 654−660.

Ellman, L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.

Englund, P. T.; King, T. P.; Craig, L. C.; Walti, A. Studies on ficin. I. Its isolation and characterization. *Biochemistry* **1968**, *7*, 163−175.

Erlanger, B. F.; Kokowsky, N.; Cohen, N. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* **1961**, *95*, 271−278.

Fish, W. W.; Mann, K. G.; Tanford, C. The estimation of polypeptide chain molecular weights by gel filtration in 6M guanidine hydrochloride. *J. Biol. Chem.* **1969**, *244*, 4989−4994.

Friguet, B.; Djavadi-Ohaniance, L.; Goldberg, M. E. Immunochemical analysis of protein conformation. In *Protein Structure−A Practical Approach*; Creighton, T. E., Ed.; IRL Press: Oxford, 1989; pp 287−310.

Gilman, A.; Philips, F. S.; Koelle, E. S.; Allen, R. P.; John, St. E. The metabolic reduction and nephrotoxic action of tetrathionate in relation to a possible interaction with sulfhydryl compounds. *Am. J. Physiol.* **1946**, *147*, 115−123.

Giovanni-De-Simone, S.; Aguiar, A. S.; Gimenez, A. R.; Novellino, K.; Moura, R. S. de. Purification, properties, and

N-terminal amino acid sequence of a Kallikrein-like enzyme from the venom of Lachesis muta rhombeata (Bushmaster). *J. Protein Chem*. **1997**, *16*, 809−818.

Glazer, A. N.; Smith, E. L. Phenolic hydroxyl ionization in papain. *J. Biol. Chem*. **1961**, *236*, 2948−2951.

Glazer, A. N.; Smith, E. L. Papain and other plant sulfhydryl proteolytic enzymes. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. 3, pp 501−546.

Goodwin, W.; Morton, R. A. The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem. J*. **1946**, *40*, 628−632.

Goto, K.; Takahashi, N.; Murachi, T. Structural studies on stem bromelain. *Int. J. Pept. Protein Res*. **1980**, *15*, 335−341.

Hounsell, E. F.; Davies, M. J.; Smith, K. D. Chemical methods of analysis of glycoprotein. In *The Protein Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1997; pp 633−634.

Jones, I. K.; Glazer, A. N. Comparative studies on four sulfhydryl endopeptidases ("Ficins") of ficus glabrata latex. *J. Biol. Chem*. **1970**, *245*, 2765−2772.

Kaneda, M.; Yonezawa, H.; Uchikoba, T. Purification and some properties of a protease from the sarcocarp of musk melon. *Biosci. Biotechnol. Biochem*. **1997**, *61*, 2100−2102.

Kimmel, J. R.; Smith, E. L. The properties of papain. *Adv. Enzymol*. **1957**, *19*, 267−334.

Kundu, S.; Sundd, M.; Jagannadham, M. V. Structural characterization of a highly stable cysteine protease ervatamin C. *Biochim. Biophys. Res. Commun*. **1999**, in press.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 6850−6855.

Lynn, K. R. A purification and some properties of two proteases from papaya latex. *Biochim. Biophys. Acta* **1979**, *569*, 193−201.

Lynn, K. R.; Yaguchi, M.; Roy, C. Homologies of the N-terminal sequences of asclepains and papain. *Biochim. Biophys. Acta* **1980**, *624*, 579−80.

Matsudaira, P. Sequence from picomole quantities of proteins electroblotted onto poly(vinylidene difluoride) membrane. *J. Biol. Chem*. **1987**, *262*, 10035−10038.

McDowall, M. A. Anionic proteinase from Actinidia chinensis. Preparation and properties of the crystalline enzyme. *Eur. J. Biochem*. **1970**, *14*, 214−221.

Merril, C. R. Gel-staining techniques. In *Guide to Protein Purification*; Deutscher, M. P., Ed.; Academic Press: San Diego, CA, 1990; pp 477−488.

Mitchel, R. E. J.; Chaiken, I. M.; Smith, E. L. The complete amino acid sequence of papain. Additions and corrections. *J. Biol. Chem*. **1970**, *245*, 3485−3492.

Mole, J. E.; Horton, H. R. Kinetics of papain-catalyzed hydrolysis of α-N-benzoyl-L-arginine-*p*-nitroanilide. *Biochemistry* **1973**, *12*, 816−822.

Ouchterlony, O.; Nilsson, L. A. Immunodiffusion and immunoelectrophoresis. In *Handbook of Experimental Immunology*; Weir, D. M., Herzerberg, L. A., Blackwell, C., Herzerberg, L. A., Eds.; Blackwell: Oxford, 1986; Vol. 1, pp 32.1−32.50.

Pal, G.; Sinha, N. K. Isolation, crystallization, and properties of Calotropins DI and DII from Calotropis gigantea. *Arch. Biochem. Biophys*. **1980**, *202*, 321−329.

Potempa, J.; Mikolajczyk-Pawlinska, J.; Brassell, D.; Nelson, D.; Thogersen, I. B.; Enghild, J. J.; Travis, J. Comparative properties of two cysteine proteases (Gingipains R), the products of two related but individual genes of Porphyromonas gingivalis. *J. Biol. Chem*. **1998**, *273*, 21648−21657.

Ramesh, K. S.; Rao, N. A. Purification and physicochemical, kinetic and immunological properties of allosteric serine hydroxymethyltransferase from monkey liver. *Biochem. J*. **1980**, *187*, 623−636.

Riddles, P. W.; Blakeley, R. L.; Zerner, B. Reassessment of Ellman's reagent. *Methods Enzymol*. **1983**, *91*, 49−60.

Robinson, G. W. Isolation and characterization of papaya peptidase A from commercial chymopapain. *Biochemistry* **1975**, *14*, 3695−3700.

Sarath, G.; Motte, R. S. de la.; Wagner, F. W. Protease assay methods. In *Proteolytic Enzymes-A Practical Approach*; Beynon, R. J., Bond, J. S., Eds.; IRL Press: Oxford, 1989; pp 25−55.

Segel, I. H. Enzymes. In *Biochemical Calculations*, 2nd ed.; John Wiley and Sons: New York, 1976; pp 208−323.

Sengupta, A.; Bhattacharya, D.; Pal, G.; Sinha, N. K. Comparative studies on calotropins DI and DII from the latex of Calotropis gigantea. *Arch. Biochem. Biophys*. **1984**, *232*, 17−25.

Sgarbieri, V. C.; Gupte, S. M.; Kramer, D. E.; Whitaker, J. R. Ficus enzymes I. Separation of the proteolytic enzymes of Ficus carica and Ficus glabrata latices. *J. Biol. Chem*. **1964**, *239*, 2170−2177.

Shapira, E.; Arnon, R. Cleavage of one specific disulfide bond in papain. *J. Biol. Chem*. **1969**, *244*, 1026−1032.

Sumner, I. G.; Harris, G. W.; Taylor, M. A. J.; Pickersgill, R. W.; Owen, A. J.; Goodenough, P. W. Factors affecting the thermostability of cysteine proteinases from Carica papaya. *Eur. J. Biochem*. **1993**, *214*, 129−234.

Sundd, M.; Kundu, S.; Pal, G. P.; Medicherla, J. V. Purification and characterization of a highly stable cysteine protease from the latex of Ervatamia coronaria. *Biosci. Biotechnol. Biochem*. **1998**, *62*, 1947−1955.

Takahashi, N.; Yasuda, Y.; Goto, K.; Miyake, T.; Murachi, T. Multiple molecular forms of stem bromelain. Isolation and characterization of two closely related components, SB1 and SB2. *J. Biochem*. **1973**, *74*, 355−373.

Turk, B.; Turk, V.; Turk, D. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol. Chem*. **1997**, *378*, 141−150.

Received for review June 18, 1999. Revised manuscript received November 19, 1999. Accepted November 22, 1999. The financial assistance from University Grants Commission (S.K.) and Council of Scientific and Industrial Research (M.S.), Government of India, in the form of research fellowships and Department of Biotechnology, Government of India, for infrastructural facilities is acknowledged.

JF990661J