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Procerain, a stable cysteine protease from the latex of *Calotropis procera*

Vikash Kumar Dubey, M.V. Jagannadham*

Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi, 221005, India

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Abstract

A protease was purified to homogeneity from the latex of medicinal plant *Calotropis procera* (Family-Asclepiadaceae). The molecular mass and isoelectric point of the enzyme are 28.8 kDa and 9.32, respectively. Hydrolysis of azoalbumin by the enzyme was optimal in the range of pH 7.0–9.0 and temperature 55–60 °C. The enzyme hydrolyses denatured natural substrates like casein, azoalbumin, and azocasein with high specific activity. Proteolytic and amidolytic activities of the enzyme were activated by thiol protease activators and inhibited by thiol protease inhibitors, indicating the enzyme to be a cysteine protease. The enzyme named as procerain, cleaves N-succinyl-Ala-Ala-Ala-*p*-nitroanilide but not L-Ala-Ala-*p*-nitroanilide, L-Ala *p*-nitroanilide and N-d-Benzoyl-DL-Arg-*p*-nitroanilide and appears to be peptide length dependent. The extinction coefficient ($\varepsilon^{1\%}_{280nm}$) of the enzyme was 24.9 and it had no detectable carbohydrate moiety. Procerain contains eight tryptophan, 20 tyrosine and seven cysteine residues forming three disulfide bridges, and the remaining one being free. Procerain retains full activity over a broad range of pH 3.0–12.0 and temperatures up to 70 °C, besides being stable at very high concentrations of chemical denaturants and organic solvents. Polyclonal antibodies against procerain do not cross-react with other related proteases. Procerain unlike most of the plant cysteine proteases has blocked N-terminal residue.

Keywords: *Calotropis procera*; Asclepiadaceae; Plant latex; Cysteine protease; Plant endopeptidase; Procerain; Anti-procerain

1. Introduction

Five catalytic types of proteases are recognized in which serine, threonine, cysteine, aspartic or metallo groups play primary roles in enzyme catalysis. The serine, threonine, and cysteine proteases are catalytically very different from the aspartic and metallo-proteases in that the nucleophile of the catalytic site is part of amino acid, whereas it is an activated water molecule in other two types. Forty-one families of cysteine peptidases are recognized, a family is a group in which every member shows a statistically significant homology of amino acid sequence to at least one other member of the family in the part of the molecule that is responsible for peptidase activity (Priolo et al., 2000).

Many proteases have been isolated from latices, fruits and seeds and most of them belong to cysteine super family (Boller, 1986). Cysteine proteases of plants play a major role in intracellular and extra cellular processes such as development and ripening of fruits (Brady, 1985); as nutritional reserve; degradation of storage protein in germinating seeds (Kembhavi et al., 1993, Taylor and Cuming, 1993); activation of proenzymes, and degradation of defective proteins (Rudenskaya et al., 1998). Besides, enzymes in the latex are also involved in protection of the plant against predator attack (Boller, 1986; Smith et al., 1955). The presence of bacteriolytic action in the latices of *Carica papaya* (Howard and Glazer, 1969), *Ficus glabrata* (Glazer et al., 1969) and *Ervatamia coronaria* (Kidwai and Murti, 1963, 1964) confirms the fact that bacteriolytic and

Abbreviations: BAPA; Nα-benzoyl-DL-arginine-*P*-nitroanilide; BSA; bovine serum albumin; EDTA; ethylene diamine tetraacetic acid; EGTA; ethylene glycol-bis (β-amino ethyl ether)-N; N; N′; N′-tetra-acetic acid; DTNB; 5; 5′-dithiobis-(2-nitro benzoic acid); GuHCl; guanidine hydrochloride; NEM; N-ethyl maleimide; PCMB; *p*-chloro mercuri benzoate; PMSF; phenyl methane sulphonyl fluoride; SBTI; soybean trypsin inhibitor; TCA; trichloroacetic acid; E-64; L-trans-epoxy-succinyl-leucylamide-(4-guanidino)-butane; N-[N-(-3 transcarboxyirane-2-carbonyl)-L-leucyl]-agmatine; DFP; di-isopropylfluorophosphate.

* Corresponding author. Tel.: +91-542-2367936; fax: +91-542-2367568.

E-mail addresses: jvm@banaras.ernet.in, jaganmv@satyam.net.in (M.V. Jagannadham).

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proteolytic enzymes act in unison to degrade undesirable proteins.

Cysteine proteases from the plant latex have mostly been reported in multiples. Papaya (*Carica papaya*) latex contains four structurally and functionally distinct cysteine proteases (Glazer and Smith, 1971; 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). Similarly, the latex of *Calotropis gigantea* also has been shown to contain four cysteine proteases, Calotropin FI, FII, DI and DII (Abraham and Joshi, 1979; Pal and Sinha, 1980). These proteases isolated from a single species of plant exhibit clear differences in amino acid composition, peptide map, 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). At least three proteases from *Ervatamia caonaria,* which are distinctly different from each other are also reported from our laboratory earlier (Sundd et al., 1998; Kundu et al., 2000).

Plant sources have yielded many useful endopeptidases, among them calotropins (Abraham and Joshi, 1979), bromelain (Takahasi et al., 1973), papain (Kimmel and Smith, 1954), ficin (Englund et al., 1968) and have been used extensively in food and medicine industry. Besides, some of these proteases have also been used as model systems for studies on structure–function relationships and protein folding problems (Kundu et al., 1999; Edwin and Jagannadham, 1998, 2000). Proteolytic enzymes from plant sources have received special attention in the pharmaceutical industry and biotechnology due to their property of being active over wide ranges of temperature and pH, thus the search for other valuable proteases is always on.

Calotropis procera commonly known as 'Arka' in India, is a popular medicinal plant found through out the tropics of Asia and Africa. Various parts of this plant have been widely used in traditional systems of medicine. An ethanolic extract of the flower of this plant is reported to have anti-microbial, anti-inflammatory, antipyretic, analgesic (Mascolo et al., 1988), anti-cancerous (Smit et al., 1995) and anti-malarial (Sharma and Sharma, 1999, 2001) activities. Likewise, water, ethanol, acetone and some other organic solvent extracts of this plant have insecticidal (Moursy, 1997), larvicidal (Markouk et al., 2000), anti-bacterial and anti-parasitic (Larhsini et al., 1999) activities. In view of these potential applications, it is important to look in to biochemical constituents of the latex to explore the rationale, if any. In the course of test of proteases, the latex of this plant showed caseinolytic activity.

In the present paper, purification, characterization, substrate specificity along with other properties of a protease are reported. According to the cysteine protease nomenclature the protease was named as procerain.

2. Results and discussion

2.1. Purification

After removal of gum, crude latex was subjected to 50% ammonium sulfate fractionation. The clear supernatant was subjected to cation exchange chromatography using a CM-Sepharose fast flow column. The bound proteins were eluted with a linear gradient of 0–0.80 M NaCl. Bound proteins resolved into two peaks assigned as peak A and B (Fig. 1A). From the preliminary experiments, like pH and temperature optima no significant differences could be detected between the two peaks however, some differences in activity towards synthetic substrates were observed. In view of multiplicity of cysteine protease in plant latices of Carica *papaya* (Glazer and Smith, 1971; Lynn, 1979), *Ervatamia coronaria* (Sundd et al., 1998; Kundu et al., 2000), *Calotropis gigantea* (Abraham and Joshi, 1979; Pal and Sinha, 1980), *Ficus glabrata* and *Ficus carica* (Sgarbieri et al., 1964), possibility of two or more distinct proteases, cannot be ruled out.

The protein pool from peak A was found to contain two major and one minor proteins whereas protein pool of peak B was more heterogeneous with three major and two minor proteins (data not shown). For further purification, protein from peak A which is more homogeneous, was loaded to a SP-Sepharose fast flow column. The column was eluted isocratically with 0.2 M NaCl initially and followed by a linear gradient of 0.2–0.6 M NaCl. Bound proteins resolved in to two active peaks I and II (Fig. 1B). Ascending limb of peak I (Fractions 235–262) was found to be pure and resulted as a single band on SDS–PAGE as well as resulted a single symmetric peak on reverse phase HPLC. Thus, the fractions were pooled and concentrated by 80% ammonium sulphate precipitation followed by dialysis at pH 7.0 and stored at 4 °C for further use. However, it is worth mentioning that if proteins after step 2 were directly loaded to SP-Sepharose could not yield purified protease. The purification results are summarized in Table 1.

Further, the specific activity of the crude latex was quite high because of multiple proteases present and the proteolytic assay was not specific for procerain. Thus, the percent recovery in terms of specific activity is about 4.0%.

Simple and economic purification of procerain, together with easy availability of latex, makes the large-scale production of procerain possible, thus enables to explore various industrial as well as biotechnological applications.

2.2. Physical properties

Procerain showed a single band in SDS–PAGE with an estimated molecular mass (M_r) of 28.79 kDa (Fig. 2) and falls is in the range of molecular mass 20–35 kDa reported for other plant cysteine proteases (Turk et al., 1997). In all subsequent measurements of the enzyme such as determination of extinction coefficient, specific amino acid residues (tyr, trp, etc.) an average M_r of 28.8 kDa was assigned to this enzyme. The extinction coefficient of

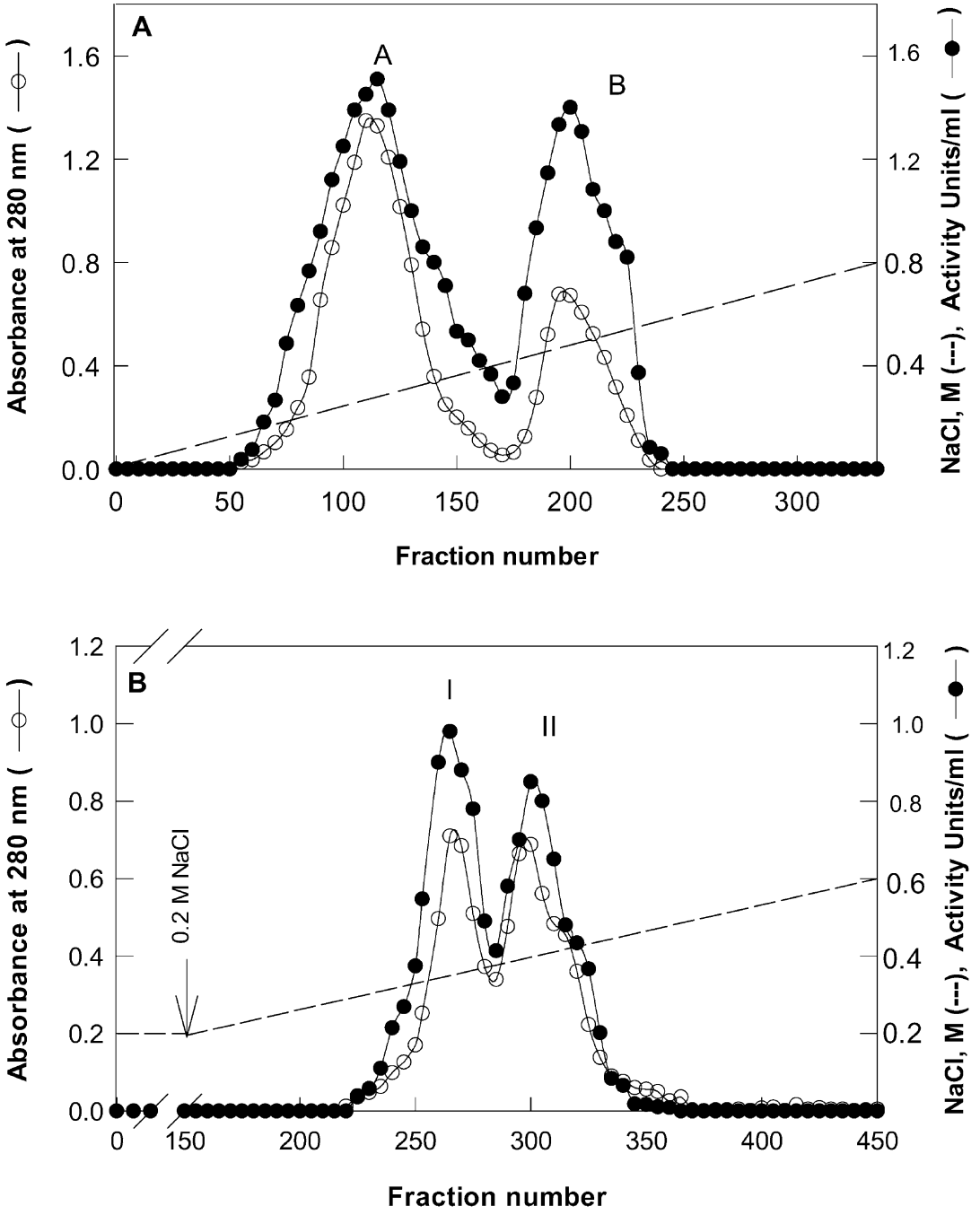

Fig. 1. (A) Chromatography of dialyzed supernatant of 50% ammonium sulphate precipitation on CM-Sepharose: supernatant of 50% ammonium sulphate precipitation (see Experimental section) was applied to a CM-Sepharose fast flow column equilibrated with 0.01 M acetate buffer, pH 4.0 and washed extensively with the same buffer. The bound proteins were eluted with a linear gradient of 0–0.8 M NaCl. The fraction of 6 ml volume were collected at a flow rate 6 ml/min and assayed for protein content (○) and protease activity using casein as a substrate (●). (B) Chromatography of peak A on SP-Sepharose fast flow column: First peak (Peak A) of CM-Sepharose was applied to a SP-Sepharose fast flow column equilibrated with 0.01 M acetate buffer, pH 4.0 and washed extensively with the same buffer. The bound proteins were eluted with 0.2 M NaCl wash followed by a linear gradient of 0.2–0.6 M NaCl. The fraction of 6 ml volume were collected at a flow rate 6 ml/min and assayed for protein content (○) and protease activity using casein as a substrate (●).

Table 1
Purification of procerain from the latex of *Calotropis procera*

Sr.	Step	Total protein (mg)	Total activity (units[a])	Specific activity (units/mg)	Recovery (%)
1.	Crude latex	500.0	2500.0	5.0	100
2.	Ammonium sulfate supernatant (50% cut)	450.0	2354.0	5.23	97
3.	CM-Sepharose (Peak-A)	219.0	1150.0	5.25	43
4.	Procerain	15.0	97.0	6.47	4

[a] Definition of one 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 one absorbancy at 440 nm/min. of digestion. Azoalbumin was used as substrate, and the assay was done as described in Expermental.

procerain ($\varepsilon^{1\%}_{280nm}$) measured by dry weight and spectrophotometric methods was 24.9±0.1 and in the range 15–25, reported for plant cysteine proteases. Procerain was found to contain no detectable carbohydrate, thus the possibility of its being a glycoprotein is ruled out. Although, a few plant cysteine proteases such as calotropin FI, FII (Abraham and Joshi, 1979) and bromelain (Takahasi et al., 1973) are glycoproteins but the biological advantage of the carbohydrate moieties in cysteine proteases is not fully understood. Whereas, in general the clusters of carbohydrate alter the solubility of the proteins with which they are conjugated and in addition the carbohydrate moiety attached to a newly synthesized protein in golgi complex may also influence

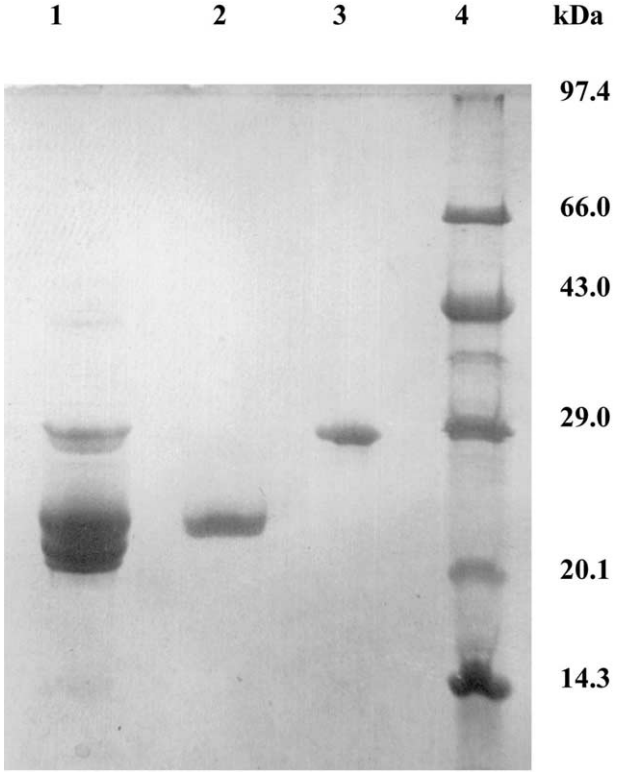

Fig. 2. SDS–polyacrylamide gel electrophoresis of purified procerain: Procerain (12 μg) was electrophoresed on 15% polyacrylamide gel. Lane 1, crude in non-reducing condition; lane 2, procerain in non-reducing condition; lane 3. Procerain in reducing condition; lane 4. Protein markers. The proteins were stained using Coomassie brilliant blue R-250.

the sequence of polypeptide folding events that leads to the tertiary structure (Lehninger et al., 1993). And the carbohydrate content in glycoproteins is helpful in protein stabilization, protection from degradation, control of protein solubility and transport inside the cells. As the present protein is also a protease as well as secreted one it is important to know the glycosylation, if any. Procerain showed single band also on isoelectric focussing with approximate pI of 9.32 hence, there must be predominance of basic amino acid residues on the surface of enzyme. The basic nature was also observed in papain 8.75, ficin 9.0 (Englund et al., 1968), ervatamin C 9.54 (Sundd et al., 1998), and in the case of stem bromelain 9.55 (Murachi, 1976), indicating that all the proteins are basic and distinct from each other. However, there are some plant proteases rich in acidic amino acids. Acidic isoelectric points 3.0 and 3.11 in the case of actinidin and asclepain respectively were reported (Mc Dowall, 1970).

2.3. Specific amino acid residues

The total cysteine content of procerain was found to be seven (measured value 7.14) with one free (measured value 1.15) cysteine and the other six forming three disulfides. The cysteine content and disulfide bridges are similar to most of cysteine proteases like ervatamin C (Sundd et al., 1998), papain (Sumner et al., 1993), asclepain B5 (Brockbank and Lynn, 1979), calotropin DI, DII (Pal and Sinha, 1980) and actinidin (Baker, 1977) but lesser than ficin (Englund et. al., 1968) and chymopapain (Sumner et al., 1993) which consist of two free and remaining six cysteines forming three disulfide bridges. Cysteine residues lesser than procerain are also reported in ervatamin B (Kundu et al., 2000), asclepain A3 (Brockbank and Lynn, 1979), and calotropin FII (Pal and Sinha, 1980) where out of five total cysteine residues one is free and remaining four form two disulfide bridges. The total numbers of tryptophan and tyrosine residues in the enzyme were estimated to be 8 and 20, respectively and are different from papain as it has five tryptophans and 19 tyrosines. Under same experimental conditions ribonuclease and lysozyme yielded the reported values. Although, the number of Trp and Tyr content of procerain is similar to the other

cysteine proteases, their determination is a must for calculation of the extinction coefficient of the enzyme and is useful in spectroscopic studies, which largely depend on the number of Trp and Tyr residues in the molecule as well as their location.

2.4. Protease activity

Procerain exhibited protease activity towards denatured natural substrates such as casein, azocasein, azoalbumin and haemoglobin with high specific activity. The

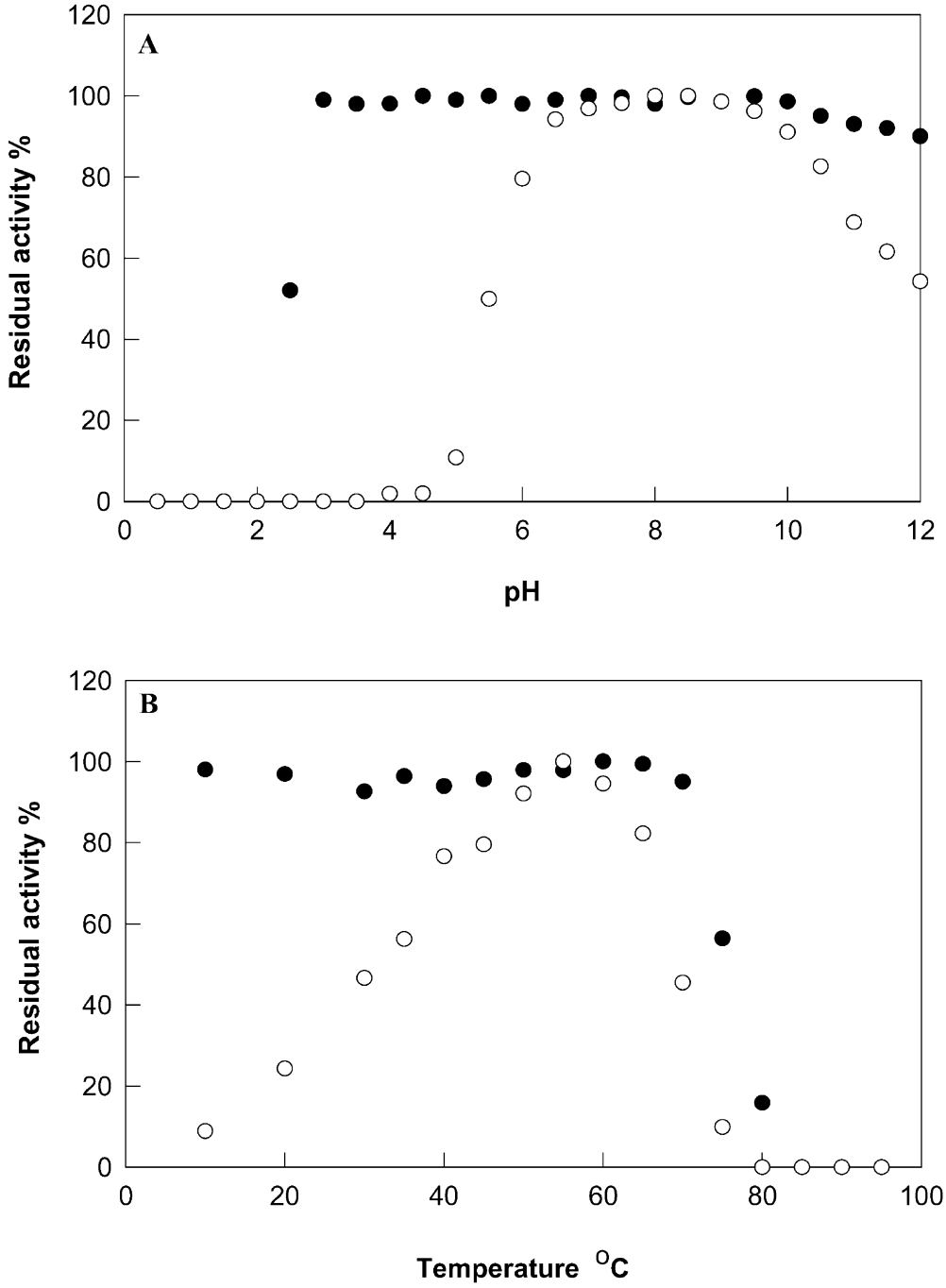

Fig. 3. Effects of (A) pH and temperature (B) on activity (○) and stability (●) of procerain: assay solution consisted of 5 μg of activated procerain in 0.5 ml of buffer of required pH, and 0.5 ml of substrate dissolved in the same pH buffer was added. Procerain was incubated in a solution of required pH for 30 min at room temperature, and activity was measured at the same pH. In the case of stability studies purified procerain was incubated at 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 Material and Methods. In both the cases 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). To see effect of temperature activity (○) and stability (●) of procerain 5 μg of procerain was activated at required temperature for 15 min, 0.5 ml of substrate is added to it and activity was measured at the same temperature. For stability experiments procerain was incubated at required temperature for 15 min, and activity was measured at 37 °C and pH 8.0.

specific activity of procerain on azoalbumin and casein are 6.47±0.1 and 5.12±0.2, respectively at pH 7.5. Specific activity of procerain towards azoalbumin is somewhat lower than ervatamin B (Kundu et al., 2000) but it showed higher caseinolytic activity than papain and ficin.

2.5. Assay for hydrolytic activity toward synthetic substrates

In addition to proteolytic activity procerain showed amidolytic activity towards N-succinyl-Ala-Ala-Ala-*p*-nitroanilide whereas, some other synthetic substrates such as L-Ala-Ala-*p*-nitroanilide, L-Alanine-*p*-nitroanilide, N-α-Benzoyl-DL-Arginine-*p*-nitroanilide, L-Leucine-*p*-nitroanilide, L-γ-Glutamyl-*p*-nitroanilide, and N-succinyl-L-Phenylalanine-*p*-nitroanilide could not be acted upon by the enzyme. Procerain cleaves N-succinyl-Ala-Ala-Ala-*p*-nitroanilide but not L- Ala-Ala-*p*-nitroanilide and L-Alanine-*p*-nitroanilide indicating that the enzyme has some specificity for peptide length. Procerain does not hydrolyze N-α-Benzoyl-DL-Arginine-*p*-nitroanilide, an ideal substrate for papain, ficin and some other plant proteases. Thus, the substrate specificity of procerain seems to be different. Precise reason for substrate specificity of cysteine proteases is not known but it has been speculated to depend on specific amino acid residues in their substrate-binding site (Lee et al., 1997).

2.6. pH and temperature optima

Hydrolysis of azoalbumin by the enzyme was optimum over a broad pH range 7.0–9.0 (Fig. 3A). This broad pH optimum makes it an excellent enzyme for food and pharmaceutical industry. Similar broad pH optimum is reported in actinidin (Mc Dowall, 1970) where as papain, Chymopapain and asclepain are optimally active at neutral pH. The decrease in activity below pH 6.0 could result from the formation of improper ionic forms of the procerain active site or substrate or from a combination of these two. Procerain showed no detectable activity below pH 4.5. The temperature optima of procerain is 55–60 °C (Fig. 3B) as is also in the case of most of the plant proteases. The high temperature optimum of procerain also shows thermal stability of enzyme, which makes it an excellent enzyme for food industry. The knowledge of optimum temperature is of use to explore the usefulness of the enzyme either directly or after modifications.

2.7. Effect of various compounds on the protease activity of procerain

Effect of various compounds on the activity of procerain was carried out to classify the protease using thiol specific activators, inhibitors, and non-specific compounds. The extent of activation of procerain by thiol specific activators such as DTT, L-cysteine, reduced glutathione and β-mercaptoethanol is studied as a function of increasing concentration of the activator (data not shown). Overall an average concentration of 10–15 mM reducing agent is necessary for maximum activation except DTT (only 2–3 mM). At very high concentrations, DTT under similar conditions resulted some drop in the activity, which may be due to reduction of the disulfides in the enzyme. Such activation of procerain by reducing agents confirms that the protein belongs to cysteine protease class.

Inactivation of the enzyme occurs in the presence of thiol specific inhibitors such as E-64, iodoacetic acid and PCMB whereas, non-specific inhibitors such as PMSF, SBTI do not have any marked inhibitory effect as summarized in Table 2.

Lack of inhibition of the enzyme by metal chelators such as EDTA, excludes the possibility of a metal ion being involved in the catalysis. Proteinous inhibitors such as SBTI, which are present in protein rich foods like soybeans, fail to inhibit the enzyme thus paving the way for its industrial usage. The earlier observations with thiol specific activators and inhibitors confirm that the enzyme is a cysteine protease.

2.8. Effect of substrate concentration

The enzyme obeyed Michaelis–Menten equation with denatured natural substrate as well as synthetic substrate (Fig. 4). At higher concentrations of the substrate, enzyme activity was found to reach saturation. Besides, the solubility of the synthetic substrate at the specified pH is also limited and not possible to go beyond 12 mM. The values of K_m, as obtained from Lineweaver–Burk plots were 22.0±0.5 μM for azoalbumin (inset of Fig. 4A) and 4.1±0.25 mM for N-succinyl-Ala-Ala-

Table 2
Effect of various compounds on the proteolytic activity of procerain

Type of inhibitor	Inhibitors	Concentration	Residual activity (%)[a]
Cysteine protease	Iodoacetic acid	40 μM	12
	E-64	3 μM	0
	Mercuric chloride	5 μM	8
	NEM	30 μM	21
	Sodium tetrathionate	30 μM	15
	PCMB	5 μM	10
Serine/cysteine	Leupeptin	10 μM	10
Serine	DFP	1 mM	95
	PMSF	50 μM	85
	SBTI	1 mM	100
Metalloproteases	EDTA	1 mM	100
	EGTA	1 mM	100
	o-phenanthroline	1 mM	100

[a] Enzyme activity was measured as described in Experimental Section and an average of 10 measurements are reported.

Ala-*p*-nitroanilide as substrates (inset of Fig. 4B). This value is almost half of the value of the Km of ervatamin B using the same synthetic substrate (Kundu et al., 2000) and K_m of papain with BAPA as substrate at pH 8.28 (Mole and Horton, 1973).

2.9. Autocatalysis

Under neutral conditions procerain undergoes autolysis in the presence of activators. A typical result of loss of activity of procerain in concentration range 0.05–0.6

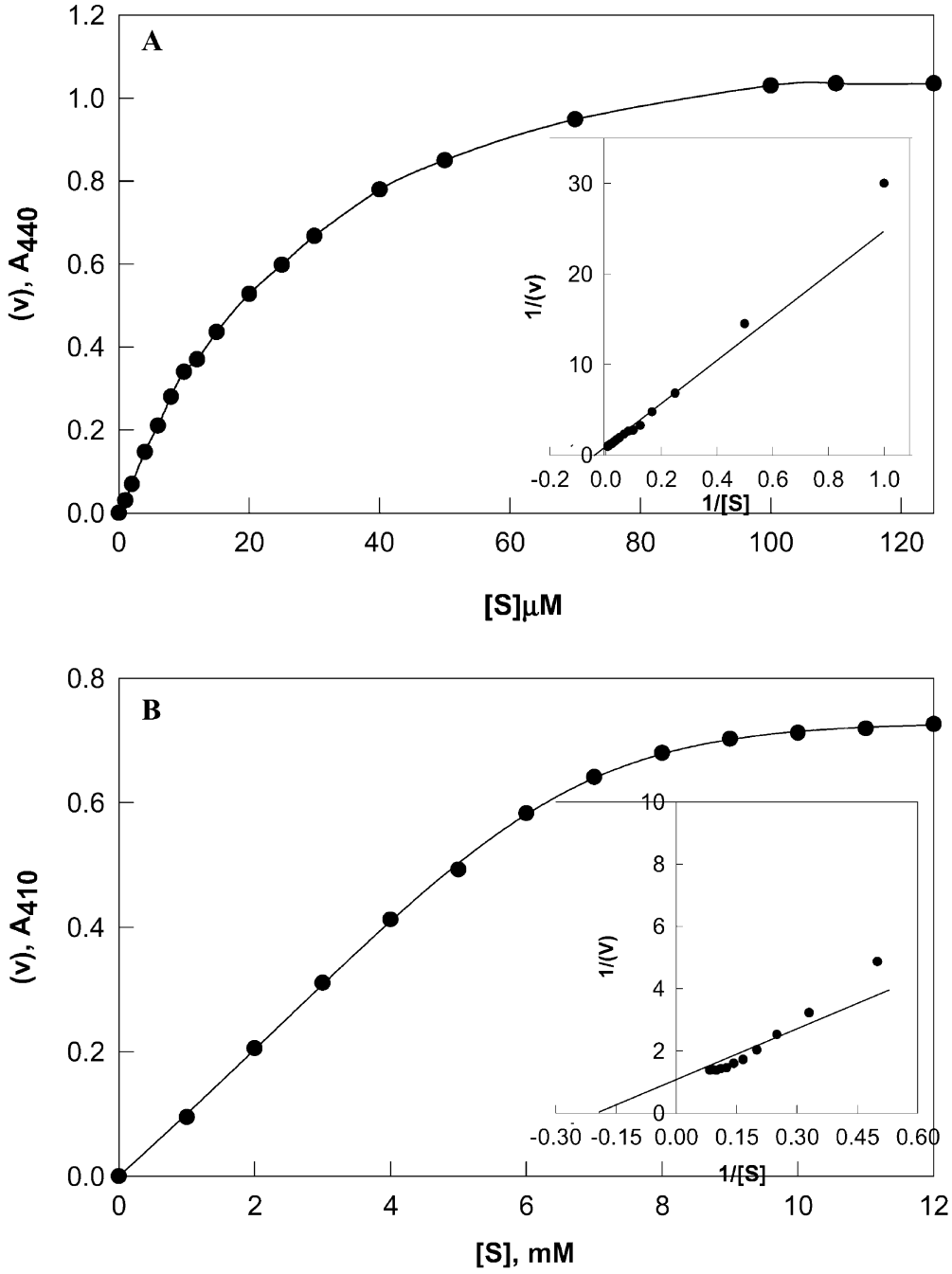

Fig. 4. Effect of substrate concentration on reaction velocity of procerain: (A) to 5 μg of the enzyme in 0.5 ml of 0.1 M Tris–HCl, pH 8.0 containing 0.05 M β-mercaptoethanol was added to 0.5 ml of azoalbumin in concentration range 1–125 μ μM. After 30 min reaction was terminated by addition of 0.5 ml of 10% TCA. The supernatant in each case was mixed with equal volume of 0.5 M NaOH, and the absorbance at 440 nm was considered as reaction velocity. Inset: Lineweaver–Burk plot. K_m was calculated according to Michaelis–Menten equation. (**B**) To 22 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 synthetic substrate in concentration range 1–12 Mm. The digestion was allowed to proceed for 30 min at 37 °C, and reaction was terminated by addition of 0.2 ml of 30% acetic acid. The liberation of *p*-nitroaniline was measured spectrophotometrically at 410 nm. An extinction coefficient of 8800 M^{-1} cm^{-1} was used to calculate initial reaction velocity. Inset: Lineweaver–Burk plot. K_m was calculated according to Michaelis–Menten equation.

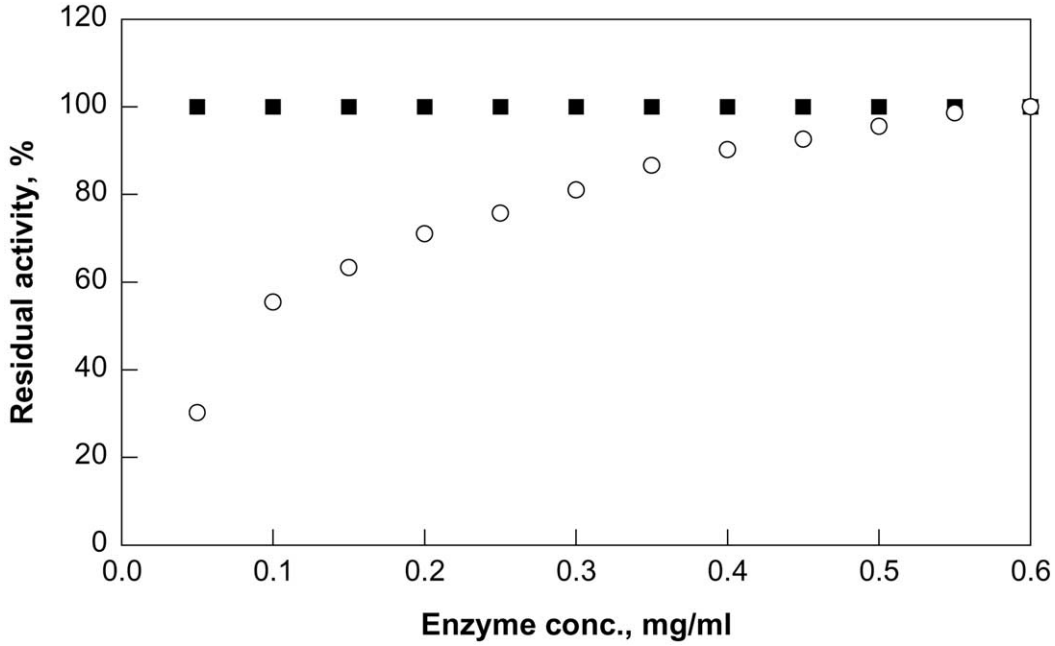

Fig. 5. Auto digestion of procerain under activation conditions: Procerain at different concentration was incubated in 0.05 M Tris–HCl buffer, at pH 8.0, in presence of 0.5 M β-mercaptoethanol at 37 °C. An aliquot containing 5 μg of the enzyme was assayed for the remaining protease activity toward azoalbumin as substrate. Typical results after 48 h of incubation are shown in the figure. The activity of enzyme after 5 min of incubation was taken as 100% activity. Similarly, reversibly blocked procerain was also incubated in the absence of activator and activated before the assay. Reversibly blocked enzyme (■) in absence of activators; (○) reversibly blocked enzyme in presence of activators.

mg/ml after 48 h of incubation is shown in Fig. 5. The extent of loss of activity decreases with increase in concentration, no significant loss of activity was observed above 0.55 mg/ml, while maximum reduction in activity at lowest concentration of the procerain studied (0.05 mg/ml). Further, the protein at different concentrations (0.05–0.6 mg/ml), after six hours of incubation in the presence of activator was subjected to SDS–PAGE (data not shown) and found that the intensity of the band at the position of the native protein increases with increase in protein concentration suggesting that the protein is less susceptible to autodigestion at higher concentrations. At the same time, reversibly inactivated enzyme incubated for the same period of time, in absence of activator, did not show any loss in activity. This loss in proteolytic activity can be attributed to the autocatalytic properties of the enzyme. In the first 6 h there is no significant loss in activity even at the lowest concentration studied where there is maximum autodigestion. However, in the same experimental conditions ervatamin C, papain, calotropin DI and calotropin DII retain only 35, 10, 20 and 40% residual activities respectively after 6 h of incubation (Shapira and Arnon, 1969; Sengupta et al., 1984; Sundd et al., 1998). These observations suggest that procerain is more resistant to autodigestion compare to papain and ervatamin C and other plant cysteine proteases. The autolysis resistant property of procerain may make it a valuable enzyme in various food and biotechnology industries.

2.10. Stability

The stability of proteins and enzymes is usually the factor that limits their usefulness. The most striking property of procerain is its high stability with respect to pH, temperature, denaturants and organic solvents. Procerain retains its full activity over a broad range of pH 3.0–12.0 as well as temperatures up to 70 °C for 15 min. Retention of complete activity in 50% methanol, 90% dioxan, and 80% acetonitrile are reflections of high stability of procerain in organic solvents. However, unlike papain, ficin, stem bromelain and several other cysteine proteases, procerain is stable in 4 M GuHCl even after prolonged periods of exposure. Such high stability towards GuHCl is reported in ervatamin C, a cysteine protease, from latex of *Ervatamia Coronaria* (Sundd et al., 1998). Procerain is stable in 8 M urea and retains all its characteristics even after prolonged exposure. Such stability in the presence of urea is reported in the case of many other cysteine proteases like papain, ficin, stem bromelain (Glazer and Smith, 1971), ervatamin C (Sundd et al., 1998) and ervatamin B (Kundu et al., 2000). This observed high stability of procerain could make it an excellent system for biophysical studies to elucidate structure–function relationship of the enzyme, which in turn could provide a rationale for its extraordinary stability. The broad pH and temperature stability of the enzyme may make it a valuable tool for food industries and biotechnology,

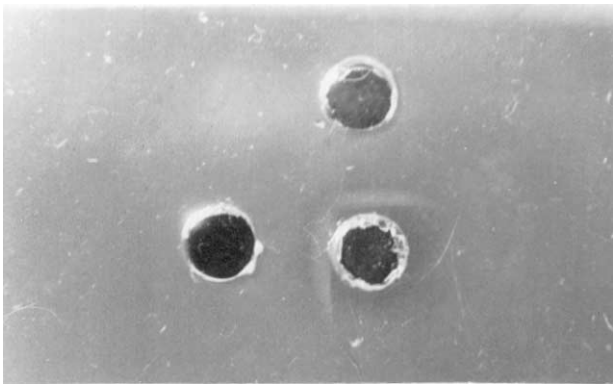

Fig. 6. Ouchterlony's double immunodiffusion to check cross-reactivity of antiprocerain serum with procerain: 1% agarose in phosphate-buffered saline was poured in to petri dish and allow to gel. Wells were punched in the plate as above pattern. In lower right well 100 μl of anti-procerain serum was loaded. Procerain (40 μg) was loaded in upper and lower left wells.

where other proteases fail to work in these extreme conditions.

2.11. Polyclonal antibodies and immunoassays

Polyclonal antibodies specific to procerain can be used to purify the enzyme and as a probe for various conformational studies. The presence of polyclonal antibody in immunized rabbit serum was checked by immunodiffusion. Precipitin lines are distinctly visible by about 24–30 h. One merging precipitin line devoid of spur formation was obtained when anti-procerain serum was loaded in the lower right well and procerain antigen in the upper and lower left wells (Fig. 6) indicating purity of procerain and specificity of anti-procerain. Control experiments with pre immune serum did not show any cross-reactivity.

Typical colour development in indirect ELISA due to procerain anti-procerain complex formation also indicates the presence of antibodies to procerain in the serum of immunized rabbit (Fig. 7). Insignificant colour development in the case of papain, ervatamin C and ervatamin B shows absence of cross reactivity of these related proteases with anti procerain serum. These observations suggest that the antigenic determinants of the later cysteine proteases differ from those of procerain.

2.12. Amino acid sequence

No free N-terminal amino acid was detected in reduced and S-carboxymethyled derivative of procerain by dinitrofluorobenzene, dansyl chloride or phenylisothiocyanate reagents. Thus, it appears that procerain, unlike most of the plant cysteine protease, has its amino terminal blocked. Therefore, N-terminal sequence of the enzyme could not be determined. Blocking of N-terminal of proteins may confer upon them resistance against non-specific proteolytic digestion. Blocked N-terminal residue has also been reported in calotropin DI and DII (Sengupta et al., 1984). However, procerain differs from calotropin DI and DII in isoelectric point, molecular weight as well as in synthetic substrate specificity, as they do not hydrolyze N-succinyl-Ala-Ala-Ala-*p*-nitroanilide, a good substrate for procerain.

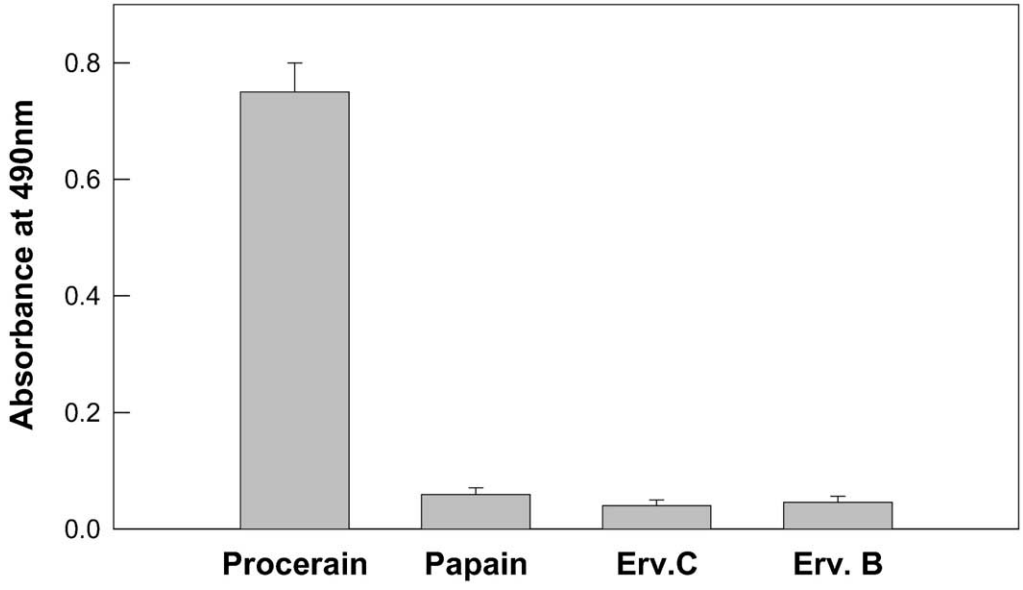

Fig. 7. Bar diagram for an ELISA to check cross-reactivity of antisera to procerain with other related proteases: ELISA was performed as described in Experimental section. At a concentration of 1 μg/ml and antisera to procerain diluted 10,000 times was added 100 μl/well. The goat anti-rabbit IgG, linked to horse radish peroxidase, was diluted 5000 times in PBS-T. H_2O_2 was used as a substrate and *O*-phenylenediamine as a colour indicator. The colour developed was measured at 490 nm. Background contribution has been deducted.

3. Experimental

3.1. Methods

3.1.1. Purification of the procerain

All purification steps were carried out at 4 °C unless stated otherwise.

Step 1. Removal of gum: Latex was collected from the young stems of the plant in 0.01 M acetate buffer pH 4.0 containing 0.01 M sodium tetrathionate and was stored at −20 °C. Sodium tetrathionate (synthesized in the laboratory by the method of Gilman et al. (1946) reversibly blocks the cysteine proteases present in the latex of *Calotropis procera* and prevents any complication due to autodigestion. Throughout the purification procedure 0.01 M sodium tetrathionate was maintained. Frozen latex was thawed to room temperature and centrifuged at 24,000×*g* for 10 min to remove any insoluble materials. The supernatant was used in the next step.

Step 2. Ammonium sulphate fractionation: The supernatant from the above step was brought to 50% saturation with solid ammonium sulphate and allowed to stand in cold for overnight. The resulting precipitate was removed by centrifugation at 20,000×*g* for 10 min and the supernatant was dialyzed against 0.01 M acetate buffer pH 4.0 for 24h with frequent changes of buffer and used in the next step.

Step 3. Ion exchange chromatography on CM-Sepharose: Chromatography was performed at room temperature. Supernatant from the previous step was loaded on a CM-Sepharose (Pharmacia Biotech, Sweden) fast flow ion exchange column pre-equilibrated with 0.01 M acetate buffer pH 4.0. The column was washed with the same buffer until no protein was detected in elute and the bound proteins were eluted with a linear gradient of 0–0.80 M NaCl at a flow rate of 6 ml/min. Fractions of 6 ml were collected; the absorbance at 280 nm as well as caseinolytic activities of the protein in all fractions was checked using casein as a substrate. The bound proteins were resolved in two peaks with considerable activity and designated as peak A and B as shown in Fig. 1A. There was no major difference in terms of pH and temperature optima among the proteins of the two peaks. However, some difference towards synthetic substrate could be detected. The protein pool from peak A was found to be more homogeneous to the protein pool B, and was chosen for further purification. It is worth mentioning here that, if the crude latex after ammonium precipitation loaded directly to SP-Sepahrose could not yield purified protease, i.e. step 3 is necessary.

Step 4. Ion exchange chromatography on SP-Sepharose: Fractions from peak A of the previous step were pooled and dialyzed against 0.01 M acetate buffer pH 4.0 for 24 h with frequent changes of buffer and subjected to cation exchange chromatography on a SP-Sepharose fast flow ion exchange column. The column was eluted isocratically with 0.2 M NaCl initially and followed by a linear gradient of 0.2–0.6 M NaCl at a flow rate of 6 ml/min and the fractions were assayed for protein content as well as protease activity as in the previous step. The bound proteins were resolved in two active peaks designated as peak I and II (Fig. 1B). Ascending limb of the first peak (Fractions 235–262) was found to be homogeneous and exhibited good protease activity thus; the fractions were pooled, concentrated by 80% ammonium sulphate saturation. The resulting precipitate was dissolved in minimum amount of 0.01 M phosphate buffer pH 7.0 and dialyzed extensively against the same buffer pH 7.0 and stored at 4 °C for further use.

3.1.2. Protein concentration

Protein concentration at different stages of purification was determined by absorbance at 280 nm as well as by method of Bradford (1976) using BSA as a standard.

3.1.3. Electrophoresis

Homogeneity and intactness of the enzyme preparation, at different stages of purification were judged using SDS–PAGE by the method of Laemmli (1970). After obtaining pure enzyme, two types of samples of the purified protease, active and inactive (irreversibly blocked by carboxymethylation) were assessed under non-reducing and reducing conditions, respectively. Under the reducing conditions, the protein sample was boiled in a sample buffer containing 50mM β-mercaptoethanol whereas, β-mercaptoethanol was absent under the non-reducing condition. Phosphorylase *b* (97.4 kDa), BSA (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase 29.0 kDa), soybean tyrosine inhibitor (20.1 kDa), and chicken egg white lysozyme (14.3 kDa) were used for calibration. The proteins on the gel were stained using Coomassie brilliant blue R-250 (all the molecular weight markers and Coommassie R-250 were purchased from Sigma Chemical Co. USA).

3.1.4. Isoelectric point

The isoelectric point of the purified enzyme was determined by isoelectric focussing on polyacrylamide gel as described (Kundu et al., 2000) with some modifications. Electrophoretic runs were carried out with ampholine, carrier ampholyte of pH range 9.0–11.0. A 5% polyacrylamide gel containing 2% desired ampholine was cast in tube gels. Anodic and cathodic chambers, flushed with nitrogen gas before electrophoresis, contained 0.01 M HEPES and 0.01 M triethanolamine, respectively. The gel was subjected to a pre-run at a

constant voltage of 300 V for 30 min to develop the pH gradient. The protein sample (100 μg) containing 10% v/v ampholine and 25% glycerol was loaded on gel and electrophoresed for 3 h at the same voltage. Protein bands were stained with 0.04% (w/v) Coomassie G-250 (Eastman Kodak) dye dissolved in 6% (w/v) perchloric acid (Merril, 1990).

3.1.5. Determination of extinction coefficient

The extinction coefficient of procerain was determined using dry weight (Glazer and Smith, 1961) and spectrophotometric (Aitken and Learmonth, 1997) methods. In dry weight method, different solutions of procerain were prepared by serial dilutions and their absorbance at 280 nm was recorded. The samples were dried thoroughly in an oven and the dry weights of each sample were determined using an analytical balance. The extinction coefficient was calculated using Beer Lambert's law; $\varepsilon^{1\%}{}_{280\ \mathrm{nm}} = Acl$, where A, absorbance at 280 nm; c, concentration of protein in mg/ml and l path length of the cuvette in cm.

Spectrophotometric determinations were done using the formula, $\varepsilon^{1\%}_{280\ \mathrm{nm}} = 10(5690n_w + 1280n_y + 120n_c)/\mathrm{M}$, where n_w, n_y, n_c were the number of tryptophan, tyrosine, and cysteine residues in the protein, M was the molecular mass of the protein and 5690, 1280, 120 were the respective extinction coefficients of tryptophan, tyrosine and cysteine residues. The numbers of these amino acid residues were determined as described later.

3.1.6. Tryptophan and tyrosine content

Total numbers of tryptophan and tyrosine residues in the purified protein were determined by the method of Goodwin and Morton (1946). Absorbance spectra of the enzyme in 0.1 M NaOH were recorded between 300–220 nm using a Beckman DU 640 B spectrophotometer. Absorbance values at 280 nm and 294.4 nm were obtained from the spectra. For calculations, the formula, $w = (A_{280} - x\varepsilon_y)/(\varepsilon_w - \varepsilon_y)$, was used where, w was the estimated tryptophan content in moles per litre; A_{280} was the absorbance at 280 nm from the protein spectra; ε_w and ε_y were respective molar extinction coefficients of tryptophan and tyrosine in 0.1 M NaOH at 280 nm ($\varepsilon_y = 1576$ and $\varepsilon_w = 5225$). The total tyrosine and tryptophan content in the protein, x was calculated using $\varepsilon_{294.4} = 2375$. The number of a particular amino acid residue per molecule of the protein was calculated from the ratio of the molar concentrations of the amino acid residues to that of the total protein. To validate the measurements, similar determinations for papain, ribonuclease A, and chicken egg white lysozyme were also carried out.

3.1.7. Free and total cysteine content

Free and total cysteine residues of procerain were carried out using the method of Ellman (1959). For free cysteine measurement, the enzyme was activated with 0.01 M β-mercaptoethanol in 0.05 M Tris–HCl, pH 8.0 for 15 min and then dialyzed for 24 h against 500 ml of 0.1 M acetic acid with three changes of the dialysate. After dialysis, 50 μl of the dialyzed enzyme sample was taken in 700 μl of 0.1 M Tris–HCl, pH 7.3 and the sample was allowed to stand for 10 min for the equilibration of the pH. Subsequently, 50 μl of 5 mM DTNB solution was added and the reaction mixture was thoroughly mixed. The liberated TNB anion after reaction of sulfhydryl group with DTNB was monitored spectrophotometrically. The number of free cysteine residues was assessed using its extinction coefficient of 14,150 M^{-1} cm^{-1} at 412 nm (Creighton, 1989). For estimation of the total number of cysteine residues, the enzyme was denatured in 6 M GuHCl and then reduced with 0.05 M DTT. The DTT in the reaction mixture was removed by dialysis against 500 ml of 0.1 M acetic acid with three changes of the dialysate (Riddles et al., 1983). The liberated thiol groups were estimated as described in the case of free cysteine estimation. The number of disulfide bonds, in the protein, was deduced by comparison of the number for free and total cysteine residues. To validate the results, similar estimations for papain, ribonuclease A and lysozyme were also carried out simultaneously.

3.1.8. Carbohydrate content

Carbohydrate content of procerain was determined by phenol sulfuric acid method (Honsell et al., 1997) as some plant cysteine proteases as calotropin FI, FII (Abraham and Joshi, 1979) and bromelain (Takahasi et al., 1973) are glycoproteins. Different amount of procerain ranging from 1 to 10 μg was taken in a volume of 10 μl in different wells of a microtiter plate and 25 μl of 4% aqueous phenol was added to each well. After 5 min, 200 μl of concentrated H_2SO_4 was added and the increase in absorbance was measured at 492 nm using a Molecular Devices, E_{max} Precision microplate reader. Carbohydrate content of procerain was extrapolated from the calibration curve generated under similar conditions with galactose as standard.

3.1.9. Protease activity

The hydrolyzing activity of the protease was monitored using denatured natural substrates like casein, haemoglobin, azoalbumin and azocasein using the method of Arnon (1970). For assay, 5–10 μg of the tetrathionate-inactivated enzyme was activated at 37 °C by 0.05 M β-mercaptoethanol in 0.05 M Tris–HCl pH 7.5 containing 0.02 M EDTA in a total volume of 0.5 ml. After 15 min 0.5 ml of 0.6% (w/v) substrate was added and the reaction was allowed to proceed for 30 min at 37 °C. The reaction was terminated by an addition of 0.5 ml of 10% TCA and allowed to stand for 10 min. The resulted precipitate was removed by cen-

trifugation at 20,000×*g* and the absorbance of TCA soluble peptides in the supernatant was measured at 280 nm, where casein was the substrate. While azoalbumin, azocasein or haemoglobin were substrates, 0.5 ml of supernatant after TCA precipitation was mixed with an equal volume of 0.5 M NaOH and the colour developed was measured by absorbance at 440 nm after 15 min. A control assay, without the enzyme was done and used as a reference. One unit of enzyme activity was defined as the amount of enzyme under given assay conditions that gave rise to an increase of one unit of absorbancy at 280 nm or 440 nm per minute of digestion. Number of units of activity per milligram of protein was taken as the specific activity of the enzyme.

3.1.10. Hydrolytic activity towards synthetic substrates

The enzymatic hydrolysis of different peptidyl-*p*NA substrates (Sigma Chemical Co, USA) by the purified protease was measured using spectrophotometric method (Arnon, 1970). In each case, 1–12 mM solution of the synthetic substrate was prepared by dissolving the required amount of substrate in minimum volume of DMSO and making up the final volume with 0.01 M Tris–HCl pH 8.0 at 30 °C. The reaction mixture contained 15–20 μg of activated enzyme in 0.5 ml Tris–HCl pH 8.0 and 0.5 ml of peptidyl *p*-NA. Before assay the enzyme was activated as described earlier. After 30 min of incubation at 37 °C, the reaction was terminated by an addition of 0.2 ml of 30% acetic acid. The liberated *p*-nitroaniline was monitored spectrophotometrically by measuring absorbance at 410 nm against a blank with no enzyme. An extinction coefficient of 8800 M^{-1} cm^{-1} for *p*-nitroaniline (Erlanger et al., 1961) was used for calculations of specific activity.

3.1.11. pH and temperature optima

Effect of pH on the activity of purified enzyme was investigated using natural substrates. The buffers used were 0.05 M KCl–HCl (pH 1.0–1.5); 0.05 M Glycine–HCl (pH 2.0–3.5); 0.05 M Na–acetate (pH 4.0–5.5); 0.05 M Na–phosphate (pH 6.0–7.5); 0.05M Tris–HCl (pH 8.0–10.0) and 0.05 M carbonate (pH 10.5–12.0). Substrate solution of azoalbumin or haemoglobin was prepared in the earlier buffers. Procerain was equilibrated in 0.5 ml of the buffer at a given pH for 15 min and added to the substrate solutions at the same pH and assayed as described earlier. Due to insolubility of azoalbumin below pH 4.0, haemoglobin was used as substrate for activity measurements (Sarath et al., 1989).

Similarly, the effect of temperature on the activity of procerain was studied using azoalbumin as substrate. The enzyme was activated at desired temperatures in the range of 10–80 °C for 15 min at pH 8.0 and an aliquot was used for the activity measurements at the same temperature. Prior to the assays, substrate solution was equilibrated at the respective temperatures at pH 8.0. At each temperature, a control assay without enzyme was used as a blank.

3.1.12. Effect of various compounds on the protease activity of procerain

Effect of various compounds on the activity of procerain was carried out using thiol specific inhibitors, activators and non-specific compounds. Procerain was activated by β-mercaptoethanol as described earlier and the activator was removed subsequently by dialysis against 0.1 M acetic acid in cold, 1 μM enzyme was incubated in the presence of increasing concentrations of inhibitor in 0.5ml final volume of 0.05 M Tris–HCl buffer, pH 8.0 for 20 min at 37 °C and assayed with azoalbumin as substrate. The inhibitors used were sodium tetrathionate, iodoacetic acid, E-64, leupeptin, mercuric chloride, pepstatin, PMSF, PCMB, EDTA, EGTA, SBTI, DFP, *o*-phenanthroline, and NEM (all the inhibitors were purchased from Sigma Chemical Co. USA). A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100%.

Various thiol specific reagents such as β-mercaptoethanol, DTT, L-cysteine, and glutathione were use to see activation of the procerain if any. The enzyme was incubated with an activator for 15 min in the concentration range of 1–50 mM and an aliquot was used for activity assay. The assay was done as per the method described earlier.

3.1.13. Effect of substrate concentration on reaction velocity

The effect of substrate concentration on the reaction velocity of enzyme hydrolysis at pH 8.0 and 37 °C was studied using both natural as well as synthetic substrates. In case of natural substrate, 6 μg of the enzyme was used and the concentration of azoalbumin was in the range of 1–125 μM. Similarly for synthetic substrate, 22 μg of enzyme was used in the assayed using N-succinyl-Ala-Ala-Ala-*p*-nitroanilide in concentration range of 1–12 mM. Assays were performed as described above. Blank determinations were carried out simultaneously at the specific substrate concentrations with out the enzyme. In each case a Lineweaver–Burk plot was plotted and value of Michaelis–Menten constant (K_m) were calculated.

3.1.14. Autocatalysis

Proteases, in general, are prone to autolysis under activating conditions and the extent of autodigestion is dependent of enzyme concentration, pH, temperature and type of the activator. Autodigestion of the procerain was followed in the presence of activator β-mercaptoethanol at 37 °C. The enzyme at different concentrations in the range of 0.05–0.6 mg/ml was incubated with 0.05 M β-mercaptoethanol in 0.05 M Tris–HCl pH 8.0

containing 0.002 M EDTA. An aliquot containing 5 μg of the enzyme was used for the determination of remaining proteolytic activity using azoalbumin as substrate. Enzyme incubated without any activator was used as a control. Activity of the enzyme after the first 15 min of activation was taken to be 100% for the calculation of the residual activities.

3.1.15. Stability

Proteases undergo various changes both in function and structure under varying conditions of pH, temperature, denaturants as well as in presence of organic solvents and the extent of changes may vary from protein to protein. Thus the effects of pH, temperature, denaturants and organic solvents, on the activity of the enzyme were studied. The enzyme was incubated at different pH in the range 0.5–12.0 for 24 h and the residual activity was measured at pH 8.0 using azoalbumin as substrate. Similarly, the enzyme samples were incubated at temperatures from 10 to 80 °C for 15 min and assayed for residual activity. On the other hand, enzyme samples were incubated for 24 h, under neutral conditions in the presence of chemical denaturants such as GuHCl, urea or different organic solvents such as methanol, acetonitrile and dioxan. In each case enzyme assays were done as described earlier.

3.1.16. Polyclonal antibodies and immunoassays

Antibodies to the purified enzyme were raised in a male albino rabbit (about 1.5 kg). The enzyme in 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. Pre immune serum was obtained from the rabbit before first injection of antigen. All sera were stored at −20 °C. The presence of antibodies was confirmed by immunoassays.

Ouchterlony's double diffusion was performed as described by Ouchterlony and Nilsson (1986), 1% agarose in phosphate-buffer saline containing 0.02% sodium azide was solidified in petri dishes and appropriate holes were punched in to it. Antigens (40 μg) and 100 μl of antiserum were loaded in the wells and left at room temperature for 24–30 h. A control assay was performed with pre immune serum.

An indirect ELISA was performed (Friguet et al., 1989) to check cross reactivity of various antigens with anti-procerain serum. Wells of microtitre plate were coated with 1 μg/ml of procerain, papain, ervatamin C and ervatamin B (50 μl/well) in sodium carbonate pH 9.6, and incubated overnight at 4 °C. The wells were washed thoroughly with phosphate-buffer 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 procerain appropriately diluted in PBS-T was added to 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, diluted 5000 times in PBS-T was 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 100 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 colour developed was measured in a microplate reader at 490 nm. Pre immune serum was used as negative control.

3.1.17. Amino acid sequence

The purified enzyme was put on a C18 reverse phase Toypearl HPLC column (7.5×300 mm) and eluted with a linear gradient of acetonitrile (0–70%) in 0.1% TFA (v/v) in water for 1 h at flow rate of 1 ml/min using a Shimadzu HPLC system. One ml fractions were collected while monitoring the elution at 280 nm using a variable wavelength detector. The concentration and homogeneity of the fractions were determined spectrophotometrically and by SDS–PAGE, respectively. Peak fractions were pooled, dried by speed vac, and attempts to determine N-terminal sequence were made using an applied Biosystems 477A proteins sequencer using the method of Matsudaira (1987).

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