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Highly stable glycosylated serine protease from the medicinal plant *Euphorbia milii*

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Abstract

A serine protease, named as ''Milin'' was purified to homogeneity from the latex of *Euphorbia milii*, a medicinal plant of Euphorbiaceae family. The molecular mass (SDS–PAGE), optimum pH and temperature of the enzyme were 51 kDa, pH 8.0 and 60 °C, respectively. Milin retains full proteolytic activity over a wide range of pH (5.5–12) and temperature (up to 65 °C) with casein and azoalbumin as substrates. The activity of milin is inhibited by serine proteases inhibitors like PMSF, APMSF and DFP, but not by any other protease inhibitors such as E-64 and PCMB. Like the other serine proteases from the genus *Euphorbia*, the activity of milin was not inhibited by the proteinaceous inhibitor soyabean trypsin inhibitor (SBTI) even at very high concentrations that is naturally present in plants. The specific extinction coefficient ($\varepsilon^{1\%}_{280\ nm}$), molar extinction coefficient (a_m) and isoelectric point of the enzyme were found to be 29, 152,500 M^{-1} cm^{-1} and pH 7.2, respectively. The enzyme is a glycoprotein with detectable carbohydrate moiety (7–8%) in its constitution, which is essential for the activity. The numbers of tryptophan, tyrosine and cysteine residues in the sequence of milin were estimated chemically and are 23, 14 and 14, respectively. Of the 14-cysteine residues, 12 constituted 6-disulfide linkages while two are free cysteines. The N-terminal sequence (first 12 amino acid residues) was determined and does not match with any sequence of known plant serine proteases. Perturbation studies by temperature, pH and chaotropes of the enzyme also reveal its high stability as seen by CD, fluorescence and proteolytic activity. Thus, this serine protease may have potential applications in food industry.

Keywords: *Euphorbia milii*; Euphorbiaceae; Serine proteases; Plant endopeptidases; Milin; Anti-milin

1. Introduction

A wide range of medicinally important applications have been reported for *Euphorbia milii*, a flowering plant of family Euphorbiaceae (Sauza et al., 1997). A native of Madagascar, this plant is commonly cultivated as an ornamental plant in India. The latex of this plant used to control the mollusks by its embryofeto-toxicity is frequently used in traditional medicine against liver fluke, schistosomiasis in sheep, cattle, and even humans (Sauza et al., 1997; Schall et al., 2001). Also, the latex contains many medicinally valuable alkaloids such as β-sitosterol, euphol, euphorbol, euphorbol hexacosanoate, and a potent antileukaemic macrolide-lasiodiplodin. However, the proteins and other biochemical constituents of the latex have not been investigated in detail.

Abbreviations: NEM, *N*-ethyl maleimide; EDTA, ethylene diammine tetraacetic acid; EGTA, ethylene glycol-bis (α-amino ethyl ether); BAPA, NR-benzoylarginine *p*-nitroanilide; DFP, diisopropylfluorophosphate; DTNB, 5,5μ-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; GuHCl, guanidine hydrochloride; HEPES, N-2-hydroxyeth-ylpiperazine-N-2-ethanesulfonic acid; NEM, *N*-ethyl-maleimide; PCMB, *p*-chloromercurybenzoate; PMSF, phenyl-methanesulfonyl fluoride; SBTI or STI, soybean trypsin inhibitor; TCA, trichloroacetic acid II; TFA, trifluoroacetic acid; TFMS, Trifluoro Methane Sulphonic Acid II; TEMED, *N*,*N*,*N*,*N*-etramethylethylenediamines; βME, β-mercaptoethanol; IAA, Iodoacetic acid.

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A number of intra- and extracellular processes, in higher plants, like seed germination, proteolytic activation of proenzymes, senescence, scavenging of defective metabolites and protection against pests are accomplished by virtue of the presence of proteases (Brady, 1985; Boller, 1986; Berger and Altmann, 2000). Generally, proteases are synthesized as inactive proenzymes to prevent protein degradation at a wrong site or time. Such peptide segments that keep the enzyme inactive are always located at the N-terminal ends of the precursor. These propeptides also function as chaperones in the proper folding and compartmentalization of the enzymes (Kardos et al., 1999). Most of the reported plants protease are cysteine endopeptidases whereas; serine or other endopeptidases are highly rare although abundantly present in microorganisms and mammals.

The latex of several plant families such as Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae, and Euphorbiaceae contains endopeptidases (Caffini et al., 1988). Nearly half of the commercially available enzymes are also proteases, frequently used in food processing, tenderization of meat, brewing cheese elaboration, bread manufacturing, leather and textile industries (Kaneda et al., 1997). Besides, some proteases have also been used as model systems for studies on their structure–function relationship, and in protein folding problem (Sundd et al., 1998; Kundu et al., 2000; Dubey and Jagannadham, 2003; Patel and Jagannadham, 2003). Many of the proteases have been crystallized as well (Kundu et al., 2000). Proteolytic enzymes from plant sources have also received special attention in the pharmaceutical industry and biotechnology due to their property of being active over wide range of temperature and pH. Thus the search for valuable proteases is always on (Nallamsetty et al., 2003). The quest for new potential plant proteases still continues in order to make them industrially applicable and cost effective (Kaneda et al., 1997).

The presence of a number of proteases in the latex of the *Euphorbia* species was reported earlier while working with *Euphorbia lathyris*. Following the description of the Euphorbain C, a cysteine protease from *Euphorbia cerifera*, the product from *Euphorbia lathyris* was named Euphorbain l (Lynn and Clevette-Radford, 1988). Recently another 13 serine proteases were isolated from the latices of six Euphorbia plants as Eu l (*Euphorbia lathyris*), Eu p (*Euphorbia pulcherima*) Eu y_{1-3} (*Euphorbia cyparissias*) Eu t_{1-4} (*Euphorbia tirucalli*) Eu la_{1-3} (*Euphorbia lacteal*) and Eu lc (*Euphorbia lacteacristina*). Likewise, other than genus *Euphorbia*, two serine proteases from the latex of the *Elaeophorbia drupifera* Eu d_{1-2}, and from *Hevea brasiliensis*, Hevain1, were purified. Macluralisin, a serine protease was also purified from the fruits of *Cudrania tricuspidata* (Rudenskaya et al., 1995).

In view of the several medicinal applications of the different parts of *Euphorbia milii*, exploration of various parts of the plant for their biochemical constituents is necessary and attempted in our laboratory. In preliminary screening, the latex of *Euphorbia milii* exhibited high caseinolytic activity, indicative of presence of protease(s). In this paper, isolation, biochemical properties and substrate specificity of a new serine protease from this medicinal plant are reported.

2. Results and discussion

2.1. Purification

A new serine protease 'milin' was purified from the latex of the medicinally important plant *Euphorbia milii* using a single step on cation exchange chromatography on SP-Sepharose fast flow. The simple purification of milin, together with easy availability of large quantity of latex, makes large-scale production of enzyme possible and thereby enables to explore industrial as well as biotechnological applications. Upon loading the processed crude latex on the column, followed by appropriate elution, the resulting profile resolved in to four peaks (Fig. 1), with peak III being the highest in magnitude both in terms of proteolytic activity and homogeneity. Besides, the majority of the total activity loaded on the column was eluted in this peak. Neither protein nor proteolytic activity was detected in the unbound fraction. But some fractions obtained upon buffer wash of the column, before salt gradient elution, exhibited some protein content with measurable proteolytic activity. The fractions of this wash are highly heterogeneous on SDS–PAGE. Preliminary examination reveals that proteins found in the buffer wash may be thiol proteases with molecular mass approximately 27 kDa. A single plant source having higher molecular weight serine as well as lower molecular weight thiol proteases is unusual. This may be unique and interesting feature of the latex of *Euphorbia milii*. Fractions with higher homogeneity(120–160) of peak

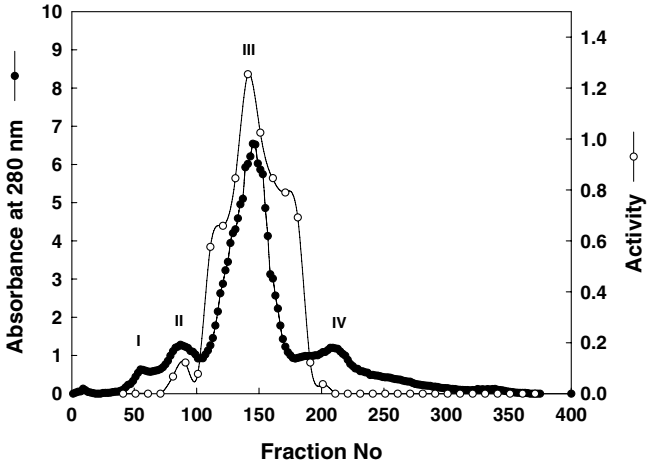

Fig. 1. Elution profile of milin from cation exchanger. SP-Sepharose FF column was pre-equilibrated with 10 mM sodium acetate buffer pH 4.5. The unbound proteins were washed out with the equilibration buffer and the column was eluted with a linear salt gradient of 0.00–0.50 M NaCl at same pH. Fractions of 5 mL volume were collected at a flow rate of 4 ml/min and assayed for protein content (●) and proteolytic activity (○).

III were pooled and dialyzed against 0.01 M Tris–HCl buffer pH 8.0. After the dialysis, the pool was clarified by centrifugation, concentrated by Amicon membrane (10 K) concentrator and stored at 4 °C for further use. The purification results are summarized in Table 1. The specific activity of the purified enzyme was found to be 9.73 U/mg using casein as substrate while the specific activity of crude latex was 4.2 U/mg under similar conditions. Thus, the total enzyme activity recovered after cation exchange chromatography amounted to about 42%. The pure enzyme was named as milin according to the nomenclature of proteases (Table 2).

2.2. Homogeneity and physical properties of the enzyme

The purified enzyme showed a single band on SDS–PAGE under reducing and non-reducing conditions (Fig. 2A). Similarly, a single protein band was also observed on isoelectric focusing with a pI value of 7.2 (Fig. 3) indicating that the protease belongs to the class of neutral proteases (Carpenter and Lovelace, 1943). The p*I* value of milin was compared with the p*I* of other plant proteases in Table 3 and the isoelectric point of other known serine proteases generally lies between pH 4.0 and 8.0. All the reported euphorbian serine proteases are mostly acidic and rarely alkaline (Lynn and Clevette-Radford, 1988). Similarly, some other properties of euphorbian proteases relative to milin are compiled and presented in Table 4, which reveals milin is significantly different from other proteases (Kaneda and Tominaga, 1987; Kaneda et al., 1988; Uchikoba and Kaneda, 1996).

A single protein band on SDS–PAGE and tube gel isoelectric focusing shows the high purity of the enzyme. The approximate molecular mass (Mr) of the purified enzyme was determined as 51 kDa by SDS–PAGE utilizing the plot of relative mobility vs. molecular mass (Fig. 2B) and this value is similar to the molecular mass of other plant serine proteases (Lynn and Clevette-Radford, 1988). The enzyme was stable and active for at least three months at pH 8.0 and 4 °C. The extinction coefficient ($\varepsilon^{1\%}_{280\ nm}$) of the enzyme obtained by dry weight (determined value 29.7) and spectrophotometric (determined value 29.6) methods was averaged to a value of 29.7 and used for all other practical applications. It is worth mentioning that animal serine proteases are comparatively of low molecular weight than the plant serine proteases (Sokolova et al., 1998) as well as less active but exhibit broad substrate specificity (Carter and Wells, 1988). A number of protease inhibitors of animal serine proteases have been isolated from plant sources though these inhibitors are ineffective in inhibiting the activity of plant serine proteases (Uchikoba et al., 1990, 1993). The specific activity of milin is comparatively less to animal or bacterial serine proteases towards natural substrates, e.g. specific activity of milin (9.73 U/mg on casein) is less than the animal trypsin (26.8 U/mg) and bacterial serine proteases (39.6 U/mg) (Yasuda et al., 1999; Dixit and Pant, 2000; Seong-Jun et al., 2003) for natural substrates. However, the protein milin was found to be extremely stable against chemical denaturants. The secondary structure of milin remains intact even in 6 M GuHCl or 8 M urea under neutral conditions (data not shown).

Table 1
Purification of Milin from the latex of *Euphorbia milii*

Step	Total protein (mg)	Total activity (Units[a])	Specific activity (Units/mg)	Recovery (%)
Crude latex	800.00	3360.00	4.20	100.00
SP-Sepharose	145.00	1411.20	9.73	42.00

[a] Definition of one unit: 1 unit of enzyme activity is defined as the amount of enzyme, under the assay conditions described, which gives rise to an increase of unit absorbance at 280 nm/min of digestion. Casein was used as substrate.

Table 2
Characteristic of subtilisin/cucumisin like serine proteases from plant sources

Subtilisin/cucumisin like serine proteases from	Mr (kDa)	pH optima	Temperature optima (°C)	pH stability (at 37 °C)
Euphorbia milii (milin)	51.4	8.0	60	5.0–12.0
Taraxacum officinale (taraxilin)[a]	67	8.0	40	6.0–9.0
Maclura pomifera (macluralisin)[b]	65	8.5	58	7.0–9.0
Cucurbita ficifolia[c]	60	9.2	55	8.0–11.0
Benincasa cerifera[d]	50	9.2	70	4.5–9.5
Cucumis melo (cucumisin)[e]	67	10.5	70	4.0–12.0
Trychosantus cucumeroids A[f]	50	10.0	70	4.0–12.5

[a] Rudenskaya et al. (1998).
[b] Rudenskaya et al. (1995).
[c] Curotto et al. (1989).
[d] Kaneda and Tominaga (1977).
[e] Yamagata et al. (1989).
[f] Kaneda et al. (1986a,b,c).

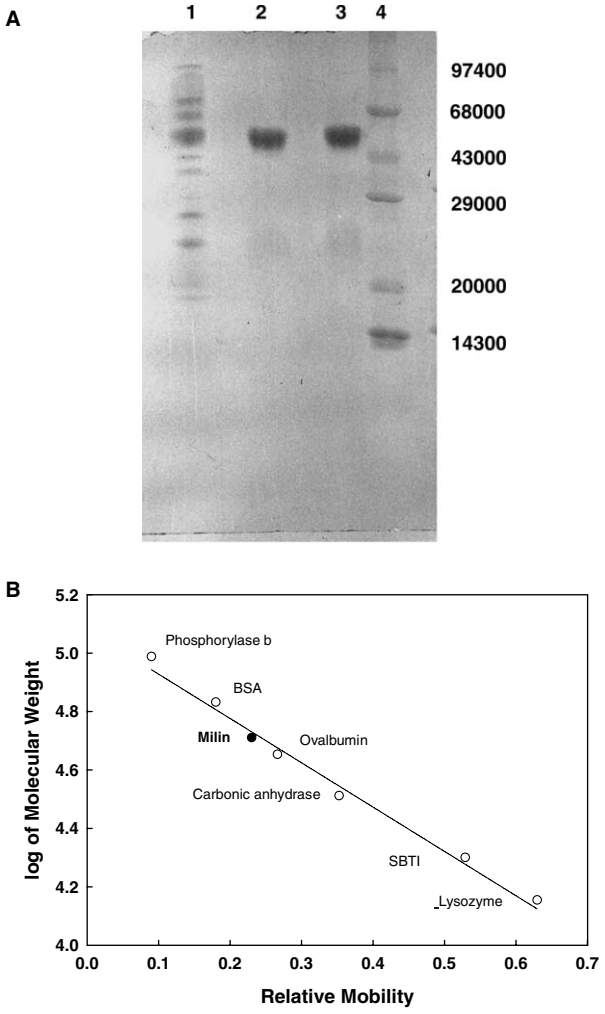

Fig. 2. Assessment of the homogeneity and molecular weight of milin by SDS–PAGE. (A) Milin under non-reduced (Lane 2) and reduced (Lane 3) conditions were electrophoresed on a 15% SDS–PAGE. Lane 1: the starting material (crude latex) under non-reducing conditions. Lane: 4 Molecular weight marker in reducing condition; Phosphorylase b (97.4 kDa), BSA (68.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soybean tyrosine inhibitor (20.0 kDa), and chicken egg white lysozyme (14.3 kDa) were used as molecular weight markers. (B) The molecular mass of milin was estimated by comparing relative mobility of the molecular marker (○) with relative mobility milin (●). The calculated molecular mass of the milin was 51 kDa.

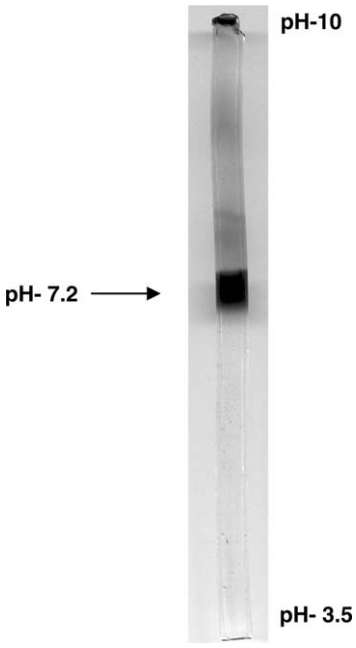

Fig. 3. Isoelectric focusing of milin. Polyacrylamide gels (5%) were prepared in glass tubes, and electrophoretic runs were carried out with ampholine carrier ampholyte, pH 3.5–10.0. The catholyte was 0.01 M ethylenediamine and anolyte was 0.01 M iminodiacetic acids. A sample of milin (100 μg) containing 10% ampholine and 25% glycerol was loaded on the gels and electrophoresed for 3 h at a constant current of 2 mA/rod. The gels were stained with Coomassie G-250.

2.3. Carbohydrate content

The molecular architecture of milin comprises of about 7–8% carbohydrate revealing that the enzyme is a glycoprotein. Further, the protein band in gel electrophoresis can also be stained by Schiff's reagent (generally used to detect glycoprotein) confirming the purified protease is a glycoprotein (data not shown). The carbohydrate moiety may be part of the functional architecture of milin, as in the case of other serine proteases isolated from the genus *Euphorbia*. The carbohydrate moiety in milin may be linked to the protein by N-glycosidic linkage as in the case of some other plant serine proteases (Lynn and Clevette-Radford, 1988). Some times wide smeared bands or two separate bands in close proximity are observed on SDS–PAGE though the protein is isolated from the same source. This may be due to intense differential glycosylation of the proteins similar to the observations reported by Lara et al. (2004) for a different protein. We have performed the PMSF inhibition studies also, on milin. A sample of milin inactivated by PMSF was also subjected to SDS–PAGE resulted multiple bands. This observation rules out the possibility of appearance of multiple bands due to autodigestion as the protein sample is inactive.

Table 3
Effect of different types of inhibitors on the activity of milin

Inhibitor type	Name of inhibitor	[I][a]	Residual activity (%)
Serine protease	PMSF	50 μM	10.0
	APMFS	50 μM	13.0
	SBTI	1 mM	105.0
	DFP	1 mM	5.0
Ser/Cysteine	Leupeptin	25 μM	110.0
Cysteine protease	Iodoacetic acid	50 μM	74.0
	$HgCl_2$	50 μM	80.0
	Sodium tetrathionate	40 μM	92.0
	E-64	10 μM	100.0
	NEM	50 μM	100.0
	PCMB	50 μM	105.0
Metallo-protease	EDTA	5 mM	103.0
	EGTA	5 mM	100.0
	o-phenanthroline	1 mM	100.0

[a] Minimum amount of inhibitor required for maximum inhibition.

Table 4
Summary of some properties of euphorbain proteases[a]

Enzyme	Mr (kDa)	p*I*	pH optima	Glycosylation	Specific amino acid residues		
					Cysteine	Tyrosine	Tryptophan
Milin	51	7.2	8.0	Yes	14	14	23
Euphorbains lc	70	8.0	8.3	Yes	20	27	13
Euphorbains y1	67	5.2	7.01	Yes	9	10	9
Euphorbains l	43	4.9	7.0–7.5	Yes	7	10	7
Euphorbains y3	67	6.3	7.01	Yes	10	14	7
Euphorbains t1	74	5.5	7.5	Yes	18	29	4
Hevain b	58	5.3	6.3	No	10	12	2

[a] Lynn and Clevette-Radford (1988).

Nearly all the Euphorbians and other plant serine proteases such as cucumisin, taraxilin are also glycoproteins though the biological advantage of the presence of such carbohydrate moieties in proteases is not fully understood. Complete loss of proteolytic activity for milin is seen, after deglycosylation with TFMS, suggesting that glycosylation is crucial for the activity of the enzyme (data not shown). Carbohydrate moieties in glycoproteins have been known to impart protein stabilization, protection from degradation, control of protein solubility, and transport inside the cells. In general, the clusters of carbohydrate alter the solubility of the protein. In addition, the carbohydrate moiety attached to a newly synthesized protein in Golgi complex may also influence the sequence of polypeptide folding events that leads to the tertiary structure.

2.4. Specific amino acid residues

The total cysteine content of milin was found to be 14 (measured value 14.2) with 2-free cysteines (measured value 2.3). Thus 12 cysteine form six disulfides bridges. The total number of tryptophan and tyrosine residues in the molecular structure of the enzyme were determined to be 23 and 14, respectively (Table 4). This is one of the striking feature of milin which is unusual for serine proteases from plants though the number of the tyrosine residue in milin is relatively low (14 residues) as compared to other plant serine proteases (Table 3).

2.5. Substrate specificity

Milin hydrolyses denatured natural substrates such as casein, azocasein, azoalbumin and hemoglobin with very high specific activity. The enzyme also showed reasonable amidolytic activity on synthetic substrates, L-Ala-Ala-*p*-nitroanilide, L-Ala-*p*-nitroanilide, and L-Leu-*p*-nitroanilide, while fails to hydrolyze some other synthetic substrates such as *N*-α-Benzoyl-DL-Arginine-*p*-nitroanilide (BAPA), *N*-succinyl-Phe-*p*-nitroanilide and L-γ-Glutamyl-*p*-nitroanilide (Kaneda and Tominaga, 1975; Kaneda et al., 1995; Yonezawa et al., 1997). When the enzyme activity was tested against several peptidyl substrates that are commonly used to assay different group of serine endopeptidases, the highest activity was obtained with L-Ala-pNA. This indicates that the enzyme acted mostly on the non-polar R group amino acids whereas its activity over polar and aromatic amino acid is undetectable (Perona and Craik, 1995).

2.6. Dependence of enzyme activity on pH and temperature

Milin exhibits considerable amount of hydrolyzing activity over broad range of pH (6.0–12.0) as shown in Fig. 4. The enzyme effectively hydrolyzes natural substrates like casein over the broad pH range having an optimum activity at pH 8.0 with a gradual decrease in the activity below pH 5.0. Unlike majority of the euphorbain serine proteases (Lynn and Clevette-Radford, 1988) milin retains about 15% of the activity even at low pH (as low as 3.0). Further, the stability of milin is completely retained in the range of pH 5.0–12.0 with a continuous decrease in activity below pH 5.0. Similarly, substrate hydrolysis can be accomplished by milin in the temperature range of 25–65 °C with a maximum activity around 60 °C (Fig. 5). The enzyme shows its half maximum activity at 48 °C.

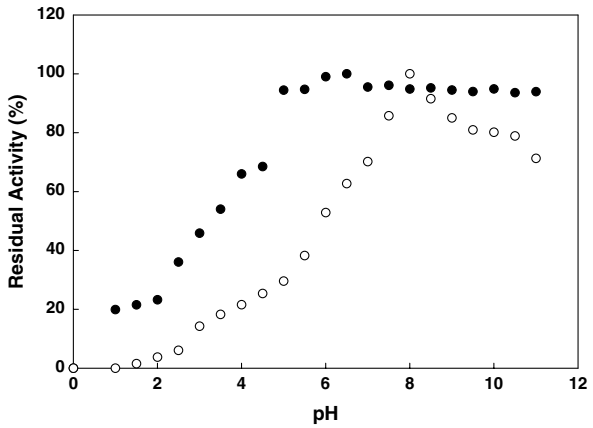

Fig. 4. Effect of pH on the activity and stability of milin. Effect of pH on the activity of the enzyme was studied by carrying out the activity measurements at different pH in the range 0.5–12.0. The enzyme was equilibrated at a given pH and assayed the same pH. Enzyme assays at and above pH 4.0 were done with casein (○) as substrate whereas, below pH 4.0, denatured hemoglobin was used as a substrate. For stability measurements of milin (●) at different pH, the enzyme was incubated at the given pH for 24 h and its activity was determined at pH 8.0 as discussed in Section 4.2.

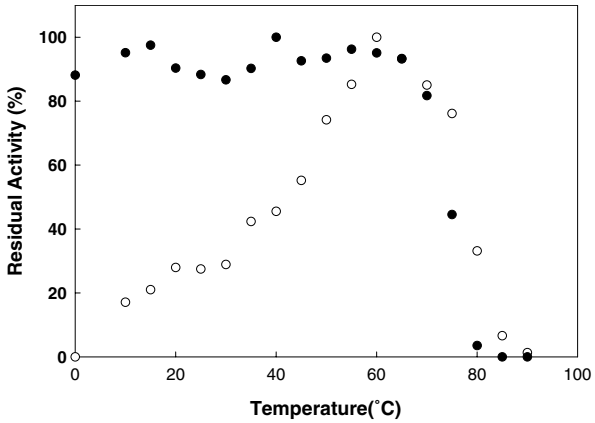

Fig. 5. Determination of temperature optimum and stability of milin. For determining the temperature optimum of milin (○), the enzyme was equilibrated at particular temperature for 15 min followed by activity measurement at same temperature using azoalbumin as substrate. For studies on the temperature stability (●) of milin, the enzyme was incubated for 15 min at a given temperature. An aliquot was taken out to carry out the enzyme assays at 37 °C. The residual activity of the enzyme was deduced from a control assay of the enzyme.

On either side of the optimum temperature, the activity as well as thermal stability of milin decreases very rapidly. Such a high temperature optimum for milin is an indication of high thermal stability of the enzyme, which in turn can make it an excellent enzyme for food industry.

2.7. Effect of various inhibitors on the protease activity of milin

Relative activity of milin in the presence of various inhibitors was carried out as a function of increasing concentration of the compound to establish the nature of the protease and its other characteristics. The minimum amount of each inhibitors required for the maximum effect is given in Table 3. The inhibitors studied include serine protease inhibitors such as PMSF, APMSF and DFP; cysteine protease inhibitors such as E-64, PCMB, NEM, sodium tetrathionate (STT) and metallo protease inhibitors such as EDTA, EGTA, *o*-phenanthroline, etc. Inhibition of enzyme activity by PMSF, APMSF, and DFP was found to be greater than 90%, indicating that the enzyme is a serine protease. Proteinaceous inhibitors such as SBTI and ovomucoid did not alter the proteolytic activity of milin. Generally, these inhibitors successfully inhibit the activity of bacterial or animal serine proteases while fail to do so in the case of plant serine proteases such as cucumisin, bamboo sprout proteases, etc. (Uchikoba et al., 1995; Arima et al., 2000). Over all, the proteolytic activity of milin was not inhibited by PCMB, NEM, E-64, *O*-phenanthroline, antipain, leupeptin, and STT, ruling out that milin is a thiol protease, as most proteases seen in plant latex are thiol proteases in general. Some enhancement of activity of milin as well as a little decrease in activity was observed in the presence of β-mercaptoethanol and IAA, respectively, indicating the involvement of a cysteine residue close to the active site. This is further evidenced by the inhibition of activity seen in the presence of mercuric chloride. Similar observations are reported in the case of cucumisin a serine protease from the prince melon fruits (Uchikoba et al., 1995). EDTA, EGTA, *O*-phenanthroline and other metal chelators did not alter the activity of milin suggesting that no metal is involved in the proteolytic activity of milin ruling out the possibility that the purified enzyme belongs to metalloproteases.

2.8. Effect of substrate concentration

The enzyme obeyed Michaelis–Menten kinetics with synthetic substrates (data not shown) as well as denatured natural substrates. The effect of increasing substrate concentration on the reaction velocity follows typical Michaelis–Menten equation with casein as a substrate (Fig. 6). The nature of the kinetics with respect to the substrates is typically hyperbolic, and at higher concentrations of the substrate, the enzyme activity attains saturation. The value of K_m from the Lineweaver–Burk plot was 33.3 μM for casein as substrate.

2.9. Autocatalysis

Most proteases undergo autodigestion, which is dependent on protein concentration, temperature and other experimental conditions. The autocatalytic nature of milin was monitored in the protein concentration range of 0.01–1.0 mg/ml at 37 °C under neutral buffer conditions. Enzyme sample was incubated under the above specific conditions for 36 h and an aliquot of the sample was used to measure the proteolytic activity. The extent of

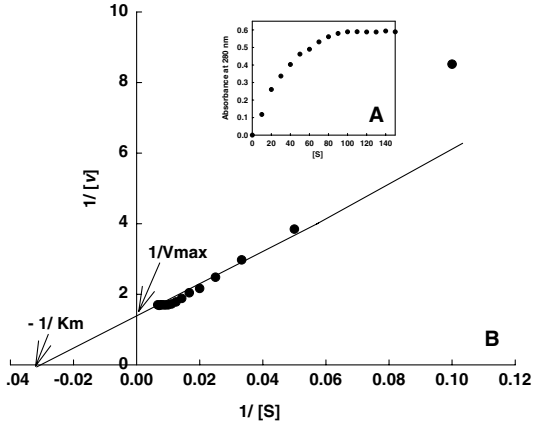

Fig. 6. Effect of substrate concentration on the activity of milin. (A) Effect of substrate concentration on milin activity. Activity of the milin undergoes saturation at the higher substrate concentrations in accordance with Michaelis–Menten equation. Different concentration of substrates (0.0–150 mM) was taken with equal amount of enzyme (25 μg) and activity was measured as given in the text (●). (B) A Lineweaver–Burk plot between 1/[S] and 1/V has been used for the determination of the value of K_m (●). The K_m value of the milin was found to be 33.3 μM using casein as substrates.

autodigestion was expressed in terms of remaining activity as shown in Fig. 7. The magnitude of autodigestion of the protein is concentration dependent and inversely proportional. At very low protein concentrations (0.01–0.08 mg/ml), autolysis is prominent while a marked reduction in autolysis was observed at higher protein concentrations (0.08 mg/ml and above). It is apparent that the protein is less susceptible to autodigestion at concentrations above 0.1 mg/ml. In addition, milin at different concentrations (0.01–0.1 mg/ml) after 36 h of the incubation was subjected to SDS–PAGE (data not shown). The gel pattern shows many fragments with molecular mass lower to the native enzyme at lower concentrations. The intensity of the bands at the corresponding position increases with increase in protein concentration also confirming that the protein is less susceptible to autodigestion at higher concentration of the protein. Similar results were reported for the analysis of autolysis of procerain, a thiol protease from *Calotropis procera* (Dubey and Jagannadham, 2003). Generally, many proteases exhibit autolysis at low concentration (say <1.0 mg/ml) where as milin fails to autodigest even at very low protein concentration such as <0.06 mg/ml. This observation confirms the resistance of the enzyme to autodigestion and must be useful for various industrial application.

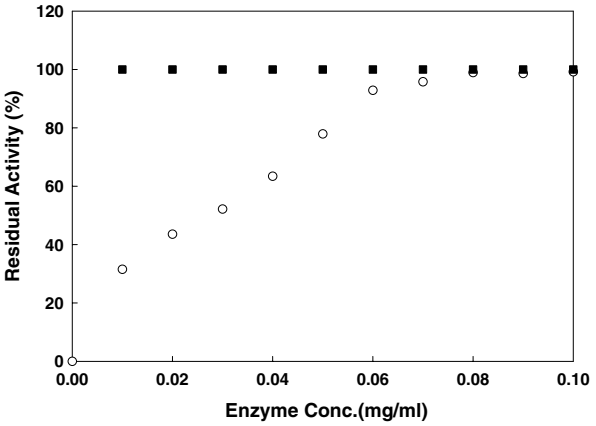

Fig. 7. Autodigestion of milin. Autodigestion of milin, as a function of increasing protein concentration was depicted. Enzyme at different protein concentrations (0.01–0.1 mg/ml) was incubated at 37 °C for 36 h and an aliquot of the enzyme was assayed. The residual activity (○) was deduced from the activity of a control enzyme sample incubated under similar conditions for 30 min(■).

2.10. Stability

Milin exhibits remarkable stability under various conditions. The enzyme retains activity over a broad range of pH of 5–12 (Fig. 4) and temperatures up to 65 °C (Fig. 5). Complete retention of proteolytic activity of the enzyme is also observed in 40% methanol, and 60% acetonitrile. Beyond the concentration of 40% methanol and 60% acetonitrile the enzyme looses the activity slowly. 70% of the proteolytic activity of milin was retained even in the 8.0 M of the urea as well as up to 3 M GuHCl. These are the exceptional features of milin among all the reported serine protease. Stability of milin under different conditions is presented in Table 5. Such high stability against pH,

Table 5
Stability of milin under different conditions

Condition	Milin activity
Milin (glycosylated)	100%
Milin (TFMS deglycosylated)	0%
pH (5–12)	100%
Methanol (40%)	100%
Acetonitrile (60%)	100%
Temperature (60 °C)	100%
Urea (8 M)	70%
GuHCl (3 M)	70%
GuHCl (6 M)	0%

Table 6
Amino terminal sequence of milin as compared to other serine proteases

Enzyme	Amino terminal sequence (first 12 residues)											
Milin	D	V	S	Y	V	G	L	I	L	E	T	D
Euphorbia protease B[a]	T	T	R	T	P	N	F	L	G	L	V	D
Euphorbia pseuchamaesyce[b]	T	T	R	T	P	N	F	L	G	L	V	D
Cucumisin[c]	T	T	R	S	W	D	F	L	G	F	P	L
White gourd protease[d]	T	T	R	S	W	D	F	L	N	F	P	Q
Lily LIM9[e]	T	T	H	T	P	D	Y	L	G	I	Q	T
Arabidopsis ARA12	T	T	R	T	P	L	F	L	G	L	D	E
Alnus ag12[f]	T	T	H	T	P	R	F	L	S	L	N	P
Tomato PR-69.(P69A)[g]	T	T	H	T	S	S	F	L	G	L	Q	Q
Tomato P69 b[h]	T	T	R	S	P	T	F	L	G	L	E	G

[a] Shimada et al. (2000).
[b] Shimada et al. (2000).
[c] Yagmata et al. (1994).
[d] Uchikoba et al. (1998).
[e] Taylor et al. (1998).
[f] Ribeiro et al. (1995).
[g] Tornaro et al. (1996).
[h] Tornaro et al. (1997).

temperature and various organic solvents, enable to explore milin for its industrial as well as biotechnological applications.

2.11. N-Terminal protein sequence determination

The amino terminal sequence of the first 12 residues of *Euphorbia milii* serine protease milin was determined by Edman degradation as Asp-Val-Ser-Tyr-Val-Gly-Leu-Ile-Leu-Glu-Thr-Asp. This N-terminal sequence of milin was aligned with some other serine proteases from plants and presented in Table 6. The sources are: *Euphorbia pseudochamaesyce* latex (Shimada et al., 2000), prince melon fruit serine protease cucumisin (Yagmata et al., 1994), white gourd serine protease (Uchikoba et al., 1998), lily protease LIM9 induce in meiotic prophase (Taylor et al., 1998), *Arabidiopsis* ARA129 (EMBL accession number X85974), Alnus ag12, Tomato PR–P 69A (Tornaro et al., 1996), Tomato P69B (Tornaro et al., 1997) proteases. The N-terminal sequence of the serine protease milin reported in this paper does not resemble with the sequences of any known plant serine protease. Similarly no homology with the sequences of similar proteases from the latex of the plants of other euphorbia family was found, making milin a unique protein. The N-terminal amino acid sequence as well as MALDI-MS tryptic digested peptide fragments mass data were searched for match in SWISS-PROT protein data bank. The absence of any positive match confirms the uniqueness of the milin (data not shown).

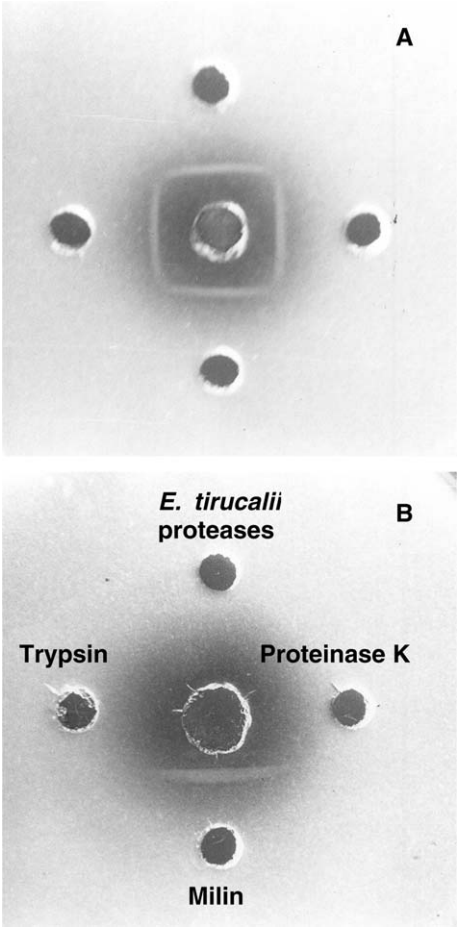

Fig. 8. Ouchterlony's double immunodiffusion immunoassays for cross-reactivity of anti-milin serum with other known serine proteases. (A) Ouchterlony's double immunodiffusion was carried out in (1%) agarose in phosphate buffered saline containing 0.02% sodium azide. Anti-milin serum (100 μl) was added in the central well and the milin (50 μg) was added in all the peripheral wells. The appearance of precipitin bands was observed after 24 h of incubation. The complete continuous arc of precipitin line shows the same antigenic identity of the milin with anti-milin. (B) Cross-reactivity of the anti-milin serum was also assessed by Ouchterlony's double immunodiffusion with milin, proteinase K, crude latex of *Euphorbia tirucalli* and trypsin. The cross-reactivity was observed only with milin among all used serine proteases. This observation identifies the unique antigenic determinants of milin among other serine proteases.

2.12. Antigenic properties

Immunological method have been used to distinguish antigenic determinants of proteases from the same source as well as similar sources. Polyclonal antibodies specific to milin were successfully raised in rabbit. The Oüchterlony's double immunodiffusion with milin (peripheral wells) and its antiserum(center wells) is shown in Fig. 8A. Precipitin lines were distinctly visible after 28–32 h of incubation. The observed precipitin line was devoid of spur formation indicating the complete cross-reactivity and purity of milin. Control experiments with pre-immune serum did not show any cross-reactivity with milin. The anti-milin serum did not cross-react with proteins in the crude latex of *Euphorbia tirucalli*, trypsin, and proteinase K (Fig. 8B) indicating an exclusive antigenic determinant. At the same time proteinase K from different sources was also used in similar experiment and found no cross-reactivity (data not shown).

3. Conclusion

The presence of proteolytic activity in the latex of the medicinal plant *Euphorbia milii* is reported for the first time in the present investigation. During the course of the work a serine protease was purified from the latex using simple purification procedures. The enzyme exhibits activity and high stability over a broad range of pH, temperature as well as at higher concentrations of chemical denaturants. Hence the enzyme may have potential for use in biotechnology application. Besides stability and activity, low susceptibility to autodigestion at ambient temperature also offers promises for the usefulness of the enzyme in the food and textile industries. The protein was not inhibited by the proteinaceous serine protease inhibitor such as SBTI. This property of the enzyme facilitates the frequent use of plant proteins for nutritional purpose. N-terminal amino acid sequence of this plant serine protease milin does not match with any other known plant serine protease. The MALDI-MS tryptic digestion profile and no cross-reactivity of

anti-milin with other serine proteases also suggest the exclusive nature of the enzyme.

4. Materials and methods

4.1. Materials

Superficial incisions on the young stems of the plant *Euphorbia milii* provided milk like latex, which was received fresh. SP-Sepharose FF was purchased from Pharmacia. BSA, RNase A, Hen egg white lysozyme, azocasein, azoalbumin, hemoglobin, DTNB, DTT, GuHCl, urea, *o*-phenanthroline, EDTA, EGTA, leupeptin, SBTI, NEM, β-mercaptoethanol, PMSF, acrylamide, *N*,*N*-methylene bisacrylamide, Coomassie brilliant blue R-250, l-*trans*-epoxysuccinylleucylamide (4-guanidino) butane-*N*-[*N*-(L-3-*trans*-carboxyirane-2-carbonyl)-L-leucyl] agimatine (E-64), hemoglobin, Coomassie brilliant blue R 250, all synthetic substrates, triethanolamine, Agarose, Tween-20, *o*-phenylenediamine, horseradish peroxidase conjugate and other standard proteins were obtained from Sigma Chemical Co., USA. Coomassie brilliant blue G250 was from Eastman Kodak. TFA was obtained from Applied Biosystems. Ampholine carrier ampholytes were from LKB. Acetonitrile was of HPLC grade. Sodium tetrathionate ($Na_2S_4O_6 \cdot 2H_2O$) was synthesized chemically. Hemoglobin was denatured with urea before the assay when used as substrate in proteolytic activity measurements. All other chemicals were of highest purity.

4.2. Methods

4.2.1. Purification

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

4.2.1.1. Step 1 – Removal of gum. Latex was collected in to 0.01 M acetate buffer, pH 4.5 containing 0.01 M STT (sodium tetrathionate) and frozen at −20 °C for 36 h (Sundd et al., 1998). Subsequently, the frozen latex thawed to room temperature and centrifuged at 24000*g* for 15 min to remove insoluble gum and other debris. The clear supernatant was dialyzed against 0.01 M sodium acetate buffer, pH 4.5 and centrifuged as above again to remove any precipitant that resulted during dialysis. The clear supernatant thus obtained was loaded on a SP-Sepharose fast flow column.

4.2.1.2. Step 2 – Ion exchange chromatography on SP-Sepharose. Chromatography was performed at 10 °C. The clear protein solution obtained in the above step was loaded on to SP-Sepharose fast flow column (175 ml bed volume) at a flow rate of 4 ml/min. The column was washed with 0.01 M sodium acetate buffer, pH 4.5 till no proteins were detected in the eluent. The bound proteins were eluted with a linear salt gradient of 0.0–0.50 M NaCl (1 l each) in the same buffer at a flow rate of 4 ml/min and fractions of 5.0 ml volume were collected. The absorbance at 280 nm as well as caseinolytic activity of all the fractions were measured. The elution profile showed at least four peaks (Fig. 1). Out of these, fractions of peak III exhibited higher activity as well as a greater homogeneity. Therefore, the fractions with maximum activity and highest homogeneity of peak III were pooled and the pool was dialyzed against 0.01 M Tris–HCl buffer, pH 8.0. Further the protein solution was concentrated by membrane filtration using Amicon concentration with YM10 membrane. The clear protein solution was stored at 4 °C for further use.

It should be noted that the best resolution of the desired protein was obtained at the pH 4.5. This is why buffer pH 4.5 was used to collect and purify the enzyme. Even though there is some decrease in activity at this pH, upon dialysis to pH 8.0 the activity is recovered.

4.2.2. Protein concentration

Protein concentrations were determined spectrophotometrically (absorbance at 280 nm) as well as by the Bradford assay (Bradford, 1976). BSA was used as standard to generate the calibration curve in Bradford assay.

4.2.3. Protease activity

The hydrolyzing activity of the protease was determined using denatured natural substrates like casein, hemoglobin, and azoalbumin (Sarath et al., 1989). Enzyme solution (25 μg) was incubated in a final volume of 500 μl of 50 mM Tris–HCl buffer pH 8.0 at 37 °C for 30 min. Casein solution (1%) was prepared in 50 mM of Tris–HCl buffer pH 8.0 and added to the enzyme solution making the final reaction volume to 1 ml and the reaction mixture was incubated for the 30 min at 37 °C. The reaction was stopped by adding 0.5 ml of 10% TCA, and incubated for 10 min at room temperature and centrifuged (10,000 rpm for 10 min). The absorbance of the soluble peptides in the supernatant was measured by absorbance at 280 nm. In the case of azoalbumin, or hemoglobin as substrate, 0.5 ml of the supernatant after TCA precipitation was mixed with an equal volume of 0.5 M NaOH and incubated for 15 min. The development of color was measured spectrophotometrically by absorbance at 440 nm. A control assay, without the enzyme was done and used as blank in all spectrophotometric measurements. One unit of the enzyme activity was defined as the amount of enzyme, under given assay conditions that give rise to an increase of one unit of absorbance 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.

4.2.4. Electrophoresis

Homogeneity and intactness monitoring of the enzyme during purification as well as the molecular mass determination of purified enzyme were achieved by using SDS–PAGE (Laemmli, 1970; Schagger and Jagow, 1987) with little modification. On SDS–PAGE, samples of crude latex

(applied to cation exchange chromatography), purified enzyme under reducing and non-reducing conditions; PMSF inactivated enzyme under reducing and non-reducing conditions along with molecular weight markers were applied. The gel was stained with 0.1% Coomassie brilliant blue R-250. Molecular markers used were phosphorylase *b* (97.4 kDa), BSA (68.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.0 kDa), and chicken egg white lysozyme (14.3 kDa). A plot of log (molecular weight) vs. mobility was generated to extrapolate the molecular weight of the purified enzyme.

4.2.5. Isoelectricfocussing

Isoelectric point of the purified enzyme was determined by isoelectric focusing in tube gels as described for procerain (Dubey and Jagannadham, 2003). Ampholines, in the pH range 3.5–10.0 were used to generate the pH gradient. A 5% polyacrylamide gels containing 2% desired ampholine were casted in tube gels. Anodic and cathodic chamber buffers were 0.01 M iminodiacetic acid and 0.01 M ethylenediamine, respectively. The chamber buffers were flushed with nitrogen gas before electrophoresis. The gels were subjected to a pre-run at a constant current of 1 mA per rod for 2 h to develop the pH gradient. Protein sample (100 μg) containing 10% (v/v) ampholine and 25% glycerol was loaded on each gel and electrophoresed at constant current of 2 mA per rod for 4 h. Protein bands were stained with 0.04% (w/v) Coomassie brilliant G-250 dissolved in 6%(w/v) perchloric acid.

4.2.6. Carbohydrate content

Different amounts of the purified enzyme ranging from 1 to 10 μg were taken in a final volume of 50 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 sulfuric acid was added to each well and the increase in absorbance was measured at 492 nm using the Emax Precision microtiterplate reader (Molecular Devices, USA) (Hounsell et al., 1997). Carbohydrate content of milin was extrapolated from the calibration curve generated under similar conditions with galactose as standard.

4.2.7. Extinction coefficient

The extinction coefficient of milin was determined using dry weight (Glazer and Smith, 1961) as well as spectrophotometric (Pace et al., 1995; Aitken and Learmoth, 1997) methods. In dry weight method, different solutions of milin, in distilled water, were prepared by serial dilution and the absorbance of each sample at 280 nm was recorded on Beckman DU640B spectrophotometer. These samples were dried thoroughly in an oven and the dry weight of each sample was 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 centimeter. In spectrophotometric method, the extinction coefficient was determined using the formula, $\varepsilon^{1\%}_{280\ \mathrm{nm}} = 10(5690n_{\mathrm{w}} + 1280n_{\mathrm{y}} + 120n_{\mathrm{c}})/M$, where n_{w}, n_{y}, n_{c} are the number of tryptophan, tyrosine, and cysteine residues in the protein; M is the molecular mass of the protein and 5690, 1280, 120 are the respective extinction coefficients of tryptophan, tyrosine and cysteine residues. The total numbers of tryptophan, tyrosine and cysteine residues in the protein are determined as described below.

4.2.8. Tryptophan and tyrosine content

Total numbers of tryptophan and tyrosine residues in the purified protein was determined by the method of Goodwin and Morton (1946). Absorbance spectra of the purified enzyme in 0.1 M NaOH were recorded between 300 and 220 nm using a Beckman DU 640B 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 is the estimated tryptophan content in moles per liter; A_{280} is the absorbance at 280 nm from the protein spectra; εw and εy are molar extinction coefficients of tryptophan and tyrosine in 0.1 M NaOH at 280 nm ($\varepsilon w = 5225$ and $\varepsilon y = 1576$), respectively. 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.

4.2.9. Measurement of free and total sulfhydryl content

Free and total cysteine residues of milin were determined using DTNB method of Ellman (1959). For free cysteine content determination, the enzyme was incubated in the presence of 0.05 M β-ME in 0.05 M Tris–HCl, pH 8.0 buffer at 37 °C for 30 min and dialyzed extensively for 24 h against 500 ml of 0.1 M acetic acid with four changes of the dialyzate. 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 to attain the pH. Subsequently, 50 μl of 5 mM DTNB solution was added to the reaction mixture and mixed thoroughly. The liberated TNB anion after reaction of sulfhydryl group with DTNB was monitored spectrophotometrically by the measurement of absorbance at 412 nm. The numbers of free cysteine residues are calculated using extinction coefficient of TNB of 14,150 $\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ at 412 nm (Creighton, 1989). Similarly for the estimation of total number of cysteine residues, the enzyme was denatured in 6 M GuHCl and reduced with 0.05 M DTT in 0.05 M Tris–HCl, pH 8.0. The excess DTT in the reaction mixture was removed by dialysis against 500 ml of 0.1 M acetic acid with four changes of the dialyzate (Riddles et al., 1983). The liberated thiol groups were estimated as described in the case of free cysteine estimation. The numbers of disulfide bonds, in the protein, were deduced by comparison of the number of free and total cysteine residues (Goodlett et al., 2000). To validate the measurements, similar contents of papain, ribonuclease A, and lysozyme were also determined.

4.2.10. Assay for amidolytic activity toward synthetic substrate

The enzymatic hydrolysis of different synthetic peptidyl-pNA (peptidyl *p*-nitroanilide) substrates by the purified protease was studied by spectrophotometry method (Arnon, 1970). Substrates used in this study were *N*-α-Benzoyl-DL-Arginine-*p*-nitroanilide (BAPA), L-Ala-Ala-*p*-nitroanilide, *N*-succinyl-Phe-*p*-nitroanilide, L-Glu-*p*-nitroanilide, L-Ala-*p*-nitroanilide, and L-Leu-*p*-nitroanilde. In each case, a stock of solution of the synthetic substrate in the concentration range of 1–12 mM was prepared in DMSO by dissolving the required amount of substrate in minimum volume of DMSO and made up to the required volume with 0.01 M Tris–HCl buffer pH 8.0 at 30 °C. The reaction mixture contained approximately 15–20 μg of enzyme in 0.5 ml synthetic substrates in the same buffer. After 30 min of incubation at 37 °C, the reaction was terminated by addition of 0.2 ml of 30% acetic acid. The liberated paranitroanilide was determined by absorbance measurement at 410 nm using the extinction coefficient of 8800 M^{-1} cm^{-1} for *p*-nitroanilide as a measure of hydrolysis (Erlanger et al., 1961).

4.2.11. pH and temperature optima

The activity of the purified enzyme is measured as function of varying pH to determine the pH optima of the enzyme activity using natural as well as synthetic 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); 0.05 M Tris–HCl (pH 8.0–10.0) and 0.05 M sodium carbonate (pH 10.5–12.0). Substrate solution of azoalbumin or hemoglobin was prepared in the respective buffers. Milin was equilibrated in 0.5 ml of the buffer at a given pH for 15 min and added to the substrate solution incubated at the same pH. The assay procedure is same as described above. Due to insolubility of azoalbumin below pH 4.0, hemoglobin was used as substrate for activity measurements similarly. Hemoglobin was denatured with urea before making the substrate solution.

Similarly the effect of temperature on the activity of milin was also studied using azoalbumin as substrate to determine the temperature optima for the biological activity of the enzyme. The enzyme was incubated at desired temperature, in the range of 10–80 °C, for 15 min in 0.05 M Tris–HCl buffer, pH 8.0 and an aliquot was used for the activity measurement at the same temperature. Prior to the assays, substrate solution was also equilibrated at the corresponding temperature. At each temperature, a control assay was carried out without the enzyme was used as a blank.

4.2.12. Effect of various inhibitors on the protease activity of milin

Effect of various compounds, such as protease inhibitors, on the hydrolysis of a substrate by the enzyme was monitored. The inhibitors used were sodium tetrathionate (STT), iodoacetic acid (IAA), PMSF, EDTA, EGTA, E-64, *p*-chloromercuric benzoate, *o*-phenanthroline, NEM, mercuric chloride, Aprotinin, Leupeptin, and SBTI. A control assay was done without the added compound and the activity was taken as 100% to calculate the change in the activity due to the presence of different compounds. In this study, the enzyme was incubated with each compound for 30 min under experimental conditions and an aliquot was used for the activity measurement. The assay was similar as described above.

4.2.13. Effect of substrate concentration on the reaction velocity

The effect of increasing substrate concentration on the reaction velocity of the enzyme hydrolysis was studied using natural substrates at pH 8.0 and 37 °C. In the case of natural substrate, 6 μg of the enzyme was used, and the concentration of substrate, casein was in the range of 1–150 μM. Assays were performed as already described under proteolytic activity measurements. A blank was used at the specific substrate concentrations without the enzyme. In each case, a Lineweaver–Burk plot was plotted and the value of Michaelis–Menten constant (K_m) was calculated.

4.2.14. Autocatalysis

Proteases, in general, are prone to autolysis under activating conditions and the extent of autodigestion is dependent on the enzyme concentration, pH, temperature as well as type of the activator, if any. The purified enzyme, at different concentrations in the range of 0.01–1.0 mg/ml, was incubated in 0.05 M Tris–HCl, buffer pH 8.0 at room temperature. An aliquot of the enzyme (25 μg) was used for the residual proteolytic activity measurement using azoalbumin as substrate. Activity of the enzyme at same concentration after 30 min of incubation under similar conditions was taken as 100% for the calculation of the residual activity.

4.2.15. N-terminal amino acid sequence

The N-terminal sequence of milin was determined to realize its homology and evolutionary relationship with other plant serine proteases. The purified milin was dialyzed against distilled water and subjected to HPLC (LKB Sweden) on C18 column using 0–100% acetonitrile in the presence of 0.1% TFA. The peak fractions from HPLC elution profile were pooled and freeze-dried using Virtis lyophilizer. The freeze-dried milin was used for the sequence determination on an Applied Biosystem 477A protein sequencer.

4.2.16. Antigenic properties

Antibodies to the purified enzyme were raised in a male albino rabbit (weight about 1.5 kg). The pure enzyme in Tris–HCl pH 8.0 buffers was emulsified with an equal volume of Freund's complete adjuvant and injected (500 μg of enzyme) subcutaneously at multiple sites. After one week a booster dose of 500 μg of enzyme was administered as an

emulsion formed with Freund's incomplete adjuvant. Two more similar doses were administered at intervals of 7 and 15 days after the first booster. After 7 days of the last dose the rabbit was bleed 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 immuno-double diffusion assays.

Oüchterlony's double diffusion was performed as described by Oüchterlony and Nilsson (1986). Two percent agarose in phosphate-buffer saline containing 0.02% sodium azide was solidified in Petri-dishes and appropriate holes were punched into it. Antigens (40 μg) having 1 mg/ml 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. Polyclonal antibodies were raised against milin to study its immunological homology with partially processed crude latex of *Euphorbia tirucalli* (Lynn and Clevette-Radford, 1988), proteinase K of different sources, and trypsin.

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