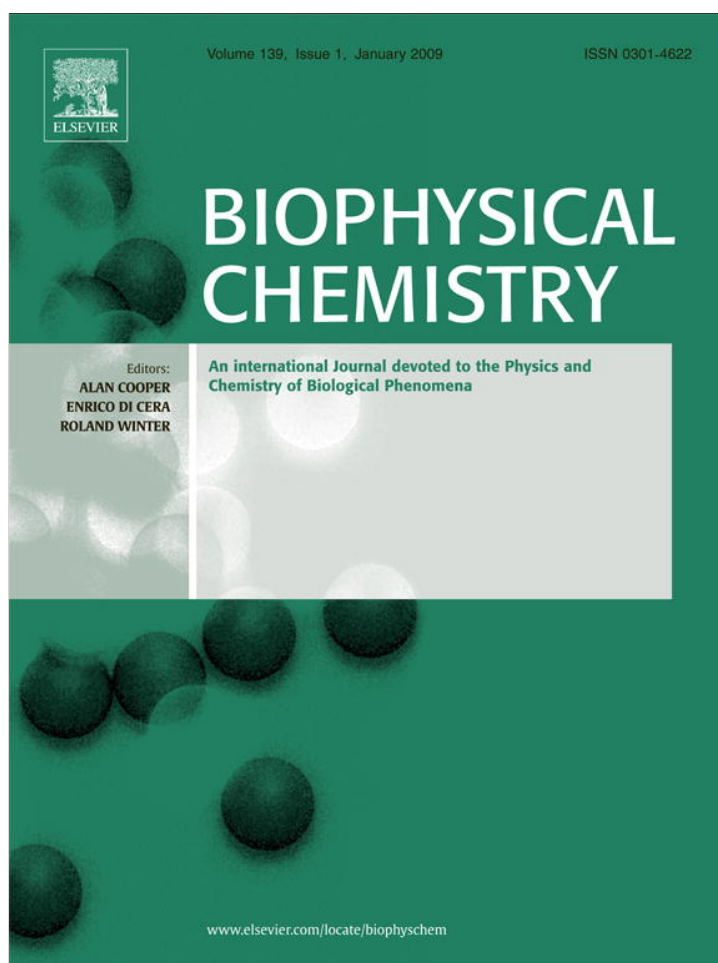


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## A kinetically stable plant subtilase with unique peptide mass fingerprints and dimerization properties

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### ABSTRACT

Milin, a potent molluscicide from the latex of *Euphorbia milii*, holds promise in medicinal biochemistry. Electrophoresis, size exclusion chromatography, mass spectrometry and other biochemical characteristics identify milin as a homodimeric, plant subtilisin-like serine protease, the first of its kind. The subunits of milin are differentially glycosylated affecting dimer association, solubility and proteolytic activity. The dimeric dissociation is SDS-insensitive and strongly temperature dependent but does not appear to be linked by disulfide bridges. N-terminal sequence of acid hydrolyzed peptide fragments shows no homology to known serine protease. Peptide mass fingerprinting and *de novo* sequencing of the tryptic fragments also did not identify putative domains in the protein. Milin seems to be a novel plant enzyme with subunit association partly similar to human herpes virus serine proteases and partly to penicillin binding proteins. Its behaviour on SDS-PAGE gels and other properties is like “kinetically stable” proteins. Such subunit association and properties might play a critical role in its physiological function and in controlling Schistosomiasis.

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### 1. Introduction

Schistosomiasis, a communicable disease of concern especially in developing countries, is still a major cause of morbidity and mortality. The risk of transmission of this disease can be controlled by the use of natural molluscicides, a relatively inexpensive treatment. However, the search for a suitable natural molluscicide with desirable properties has not yet yielded fruitful and confirmatory results. Latexes of plants belonging to the Euphorbiaceae family are potential molluscicides, with latex of *Euphorbia milii* reported to be the most effective. Recently, we have shown that a putative serine protease from the latex of this plant, called milin, can be a potent molluscicide [1].

For milin to be considered as an attractive natural drug for use as an anti-Schistosomiasis [1] agent, it must be thoroughly characterized in several aspects. The finding that though there are reports of 12,000–35,000 species of latex producing plants [2] only those from the Euphorbiaceae family and especially *Euphorbia milii* are the most effective, points to the fact that milin could be a novel protein of medical importance. We have purified the protein to homogeneity

from latex and the preliminary biochemical characteristics have been reported [3]. The protein has catalytic properties of a serine protease; however, its N-terminal sequence (12 amino acids) shows no homology with known serine proteases of either the trypsin or subtilisin or serine-carboxypeptidase family [3]. Typical of secreted proteins, it is glycosylated. It also exhibits relatively high stability against various denaturants.

Contrary to the perception that plant serine proteases are rare, the last decade has seen several reports of such proteases in a wide range of tissues and organs [4]. However, most reports are only preliminary and the enzymes are poorly characterized except cucumisin, the first plant serine protease to be isolated. Their discovery in plants in large number means that they have important biological functions to perform. Yet, their primary structure, three-dimensional structure and biophysical characteristics are largely unknown. The presence of structural domains other than the proteolytic ones, their typical folds and denaturation behavior are yet unexplored. Milin, with its therapeutic (and yet unknown physiological) importance and unique characteristics can very well serve as a model plant serine protease.

The unique N-terminal amino acid sequence of milin necessitates that further peptide sequences of the protein be generated. Such an exercise would establish the novelty of the enzyme in its primary structure compared to known serine proteases. Knowing further sequences would also help identify the presence of conserved domains, if any. The sequence information along with its already studied biochemical characteristics would probably help in placing the new

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protease in a proper family of serine proteases. Finally, it would be useful in ongoing attempts at solving the three-dimensional structure of the protease. Hence, *de novo* sequencing of the enzyme at various positions along its primary structure was accomplished by matrix-assisted laser desorption/ionization time of flight (MALDI TOF TOF).

Various serine proteases from animal and virus sources are well studied [5–7]. Many of them are multimeric and subunit association plays a key role in their functional attributes and stability. Among others, the trypsin-like serine protease Granzyme A, localized in the cytoplasmic granules of activated lymphocytes and natural killer cells, was reported in the homodimeric form [8]. Similarly, the disulphide linked dimeric serine protease Factor IX was reported where the dimerization is essential for its activity [7]. The dimeric forms of proteins are advantageous in secretion machinery as well since large area of protein surfaces are protected in the dimer [9] and increase unusual protease activity [10]. The activation of HIV 1 protease requires dimerization where the dimer interfaces of protease and extra protease domain influence the activation [11]. Even chymotrypsin dimerizes under certain conditions [12]. Yet all plant serine proteases reported so far have been found to be mainly monomeric, in spite of most of them having high molecular weight [4]. Here we suggest the failure of normal sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to detect oligomerization of highly stable plant serine protease like milin. In the present report, the oligomerization properties of milin have been investigated in light of the fact that subunit association is proving to be vital for biological function for a wide range of protein classes, including but not limited to tumor necrosis factor receptor (TNFRs), epidermal growth factor receptors (EGFRs), serpins, GTPase etc. [13–17].

## 2. Materials and methods

### 2.1. Material

Latex from *Euphorbia milii* was used to purify milin as described previously [3]. Protein concentration was determined using an extinction coefficient of  $\epsilon_{280}^{1\%} = 29$  [3]. Guanidine hydrochloride (GuHCl) was procured from Sigma Chemical Company, USA. All other chemicals were of highest purity available commercially. The samples were prepared in Millipore water and filtered through 0.45  $\mu\text{m}$  filters.

### 2.2. SDS-PAGE

Electrophoresis was carried out under standard denaturing SDS-PAGE conditions where samples were heated at 95 °C for 5 min in 4%  $\beta$ -mercaptoethanol and 2% SDS. In addition, other harsh denaturing conditions were also used. For example, the protein (100  $\mu\text{g}$ ) was incubated at increasing concentration of GuHCl (final volume 10  $\mu\text{l}$ ) for 24 h and the corresponding samples were loaded on the gel with heating (for 5 min at 95 °C) and without heating. Heating the protein in presence of SDS at 140 °C for different time periods (2, 5, 8, 10, 12, 15, 20, 25 min) both in absence and presence of reducing agent was used to monitor the effect of temperature on dissociation of dimer and stability of milin. Mineral oil was layered on the protein samples to prevent evaporation.

### 2.3. Native PAGE and gel filtration

For native PAGE, the protein samples were loaded on the gel without heating under non-denaturing and non-reducing conditions. Bovine serum albumin (BSA) and ovalbumin in the native form were used as references. Gel filtration experiments were carried out on Sephacryl S-200 under gravity. The column was equilibrated and protein eluted with 50 mM potassium phosphate buffer, pH 8.0, containing 100 mM NaCl at a flow rate of 0.5 ml/min at 25 °C. The fractions size was 0.5 ml. The elution was monitored by absorbance measurements at 280 nm. The column was pre-calibrated with standard molecular weight markers.

### 2.4. Mass spectrometry

Mass spectrometry (MS) was used to determine the molecular mass of milin. The purity and oligomerization state of the protein were also revealed in the process. The protein solution was extensively dialyzed against Millipore water and 0.8  $\mu\text{l}$  of the sample was spotted on a MALDI plate. Then 1  $\mu\text{l}$  of sinapinic acid was used as matrix after proteins samples were dried. BSA was used as standard. The data were obtained in MALDI-TOF TOF 4800 using linear spectrum.

### 2.5. Deglycosylation of milin by TFMS

Deglycosylation of milin was achieved using Tri fluoro methane sulphonic acid (TFMS), a prominent chemical deglycosylation agent [18]. Lyophilized milin (1 mg) was incubated at –20 °C with 150  $\mu\text{l}$  of pre-chilled working TFMS (–20 °C) solution (having anisole, 1:1 v/v) for 30 min. The reaction was neutralized by adding 6% pre-chilled pyridine. The pyridine solution was diluted using 1:1 v/v methanol: water solution. The reaction solution was desalted by dialysis. Precipitated deglycosylated milin was solubilized in 1.5 M GuHCl at neutral pH and used for further studies. For SDS-PAGE, precipitated deglycosylated milin was heated with sample buffer for 15 min at 95 °C and loaded on 15% polyacrylamide gels.

### 2.6. Proteolytic activity of milin

The ability of the enzyme to hydrolyze peptide bonds was routinely tested as described before using casein as substrate [3]. Active enzyme was indication of functional native protein while specific activity as reported before was indication of the purity of the protein [3]. Deglycosylated milin solubilized in 1.5 M GuHCl, as prepared above, was also assayed against casein. Native, glycosylated enzyme in 1.5 M GuHCl was used as reference for such measurements.

### 2.7. N-terminal sequence and homology search

N-terminal sequence of the subunits of milin was determined as mentioned previously. The protein was subjected to SDS-PAGE as described above. Protein bands on the gel were transferred to PVDF membrane by Western blot method. The PVDF membrane was then briefly stained with Coomassie and the corresponding bands were carefully and neatly excised. Homology of the N-terminal sequence of the enzyme to known proteases was searched using NCBI blast search ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)), MEROPS database ([www.merops.sanger.ac.uk](http://www.merops.sanger.ac.uk)) and manual inspection of serine protease sequences in published literature.

### 2.8. Circular dichroism

The circular dichroism (CD) spectra of milin under various conditions were recorded on a JASCO 715A spectropolarimeter, pre-calibrated with 0.1% d-10-camphorsulfonic acid solution. Secondary structures in the protein were monitored using far-ultraviolet CD (far-UV CD) spectra in the wavelength range 180–260 nm, with a protein concentration of 0.1 mg/ml in a 1 mm path length cuvette. Whereas the changes in the tertiary structure were measured with a 10 mm path length cuvette in the wavelength region of 260–340 nm with a protein concentration of 1 mg/ml.

The results were expressed as mean residue ellipticity  $[\theta]_{MRW}$ , using the equation:

$$[\theta]_{MRW} = \theta_{obs} \times MRW / 10.c.l \quad (1)$$

where  $\theta_{obs}$ , c, and l represent respectively the observed ellipticity in degrees, protein concentration in mg/ml and the path length of the light in cm. Mean weight of amino acid residues (MRW) was taken as 110. The

secondary structural content ( $\alpha$ -helix,  $\beta$ -sheets) of the proteins was calculated using software provided by JASCO.

### 2.9. Thermal and chemical denaturant induced unfolding of milin

The protein samples were incubated in the cuvette at the desired temperature for 20 min prior to spectral measurements. The temperature was controlled using a Julabo F 25 water bath attached directly to the cell holder in CD and fluorescence. The temperature of the sample inside the cuvette was measured using a thermocouple connected to a digital multimeter. The fluorescence measurements were carried out on a Perkin-Elmer LS-50B spectrofluorimeter. The protein concentration was 0.01 mg/ml for all fluorescence measurements. Tryptophan was selectively excited at 292 nm. The emission was recorded from 300 to 400 nm with 10 and 5 nm slit widths for excitation and emission, respectively.

Calorimetric measurements were performed with a Microcal MC-2 differential scanning calorimeter. Protein solutions (1.25 mg/ml) were extensively dialyzed against 0.01 M buffer at the desired pH. The protein concentration and pH of the samples were rechecked and adjusted to desired concentration. All solutions were degassed under vacuum before being loaded in to the calorimeter cells. The calorimetric experiments were conducted at a scan rate of 60 °C/h. Buffer baselines were obtained under the same conditions and subtracted from the sample curves.

Urea-induced denaturation was performed by incubating the protein sample at a desired denaturant concentration for approximately 24 h at 25 °C to attain equilibrium. The final concentrations of the protein and denaturant in each sample were determined by spectrophotometry and refractive index respectively. Measurements were done using both CD and fluorescence.

### 2.10. Preparation of acid hydrolysis fragments

Milin (200  $\mu$ g) was heated in the presence of 0.1 M HCl for 10 min at 80 °C. Mild acid hydrolyzed sample was loaded on SDS-PAGE. Different bands obtained by acid hydrolysis were transferred to the PVDF membrane and sequenced by N-terminal sequencing as described above.

### 2.11. Tryptic digestion of milin for MS analysis

SDS-PAGE gel loaded with milin was extensively washed with water after Coomassie Blue staining/destaining. The bands of interest were excised and cut into  $\sim 1 \times 1$  mm squares with glass capillary. 2–3 excised squares were taken and destained further using destaining solution (100  $\mu$ l of 50 mM ammonium bicarbonate in 50% acetonitrile and 0.1% TFA) for 20 min. The washing was repeated three times with fresh destaining solution each time. The supernatants were discarded. The gel pieces were soaked in 100  $\mu$ l of 100% acetonitrile for 5 min till they turned white and opaque. Acetonitrile was subsequently removed and gel was dried in speed-vac for 30 min. A working solution of trypsin was prepared using sequencing grade trypsin from Promega (20  $\mu$ g vial). Contents of this vial were dissolved in 1.0 ml of 20 mM ammonium bicarbonate and 10 aliquots (100  $\mu$ l) were prepared. These aliquots were frozen at  $-70$  °C for future use. The dried gel pieces were rehydrated in trypsin solution (10  $\mu$ l per gel piece) and incubated at 37 °C for 24 h. The digested peptides were extracted in 50  $\mu$ l of 50% acetonitrile: 0.1% TFA solution in 0.5 ml Eppendorf tubes and agitated for 20 min. The supernatant was removed and the extraction was repeated with fresh extraction solution. The extract was dried in the Speed-Vac and reconstituted in 10  $\mu$ l 0.1% TFA. 1  $\mu$ l of rehydrated tryptic digested sample was spotted on polished stainless steel MALDI target and left for drying. An equal volume of a saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) in 0.1% TFA/50% acetonitrile was spotted on the dried sample.

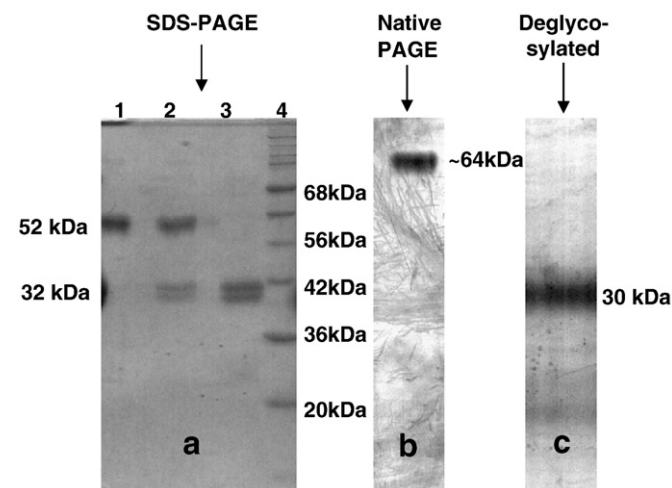
The mixture was allowed to dry at room temperature and used for MALDI MS analysis.

### 2.12. de novo sequencing by MALDI TOF TOF

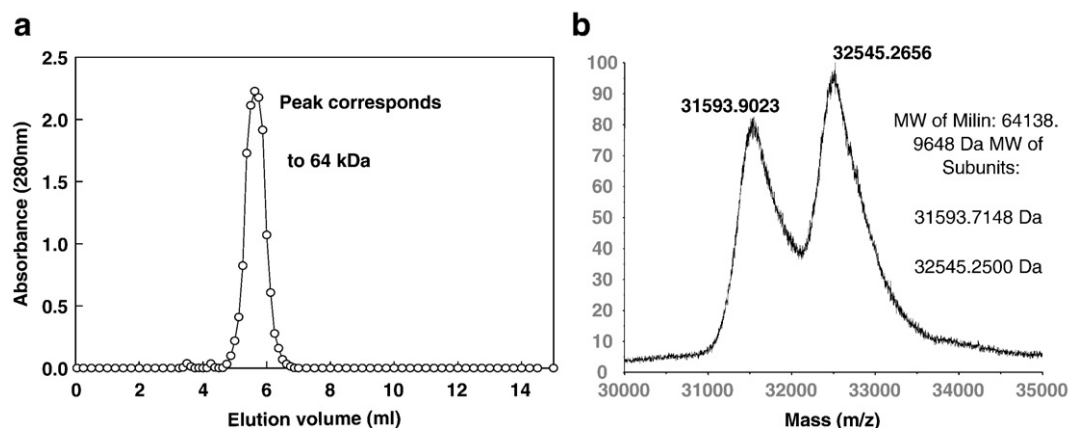
Peptide masses were determined using Matrix Assisted Laser Desorption Ionization -Time of Flight (MALDI-TOF) (4800 MALDI TOF TOF, Applied Biosystems) at Center for Cellular and Molecular Biology (CCMB), Hyderabad, India. Positive-ion mass spectra were recorded in both the linear and reflective modes [19]. MALDI relies on the utilization of a matrix compound capable of absorbing ultraviolet (UV) light. The matrices were of approximately 10,000-fold molar excess of the peptide on final deposition. The solvent was allowed to evaporate and co-crystallized analyte molecules embedded in matrix crystals were acquired. Calibration for protein mass fingerprint (PMF) samples (digests) were performed both externally, using a mixture of nine peptides ranging from  $m/z$  757.40 to 3147.47 and internally by using autolytic tryptic fragments. All samples were analyzed in reflector mode before and after derivatization to obtain PMF spectra. The instrument was switched to PSD mode and ion selector was set to the  $m/z$  values of precursor ions. The sample probe is then placed into the MS at high vacuum [19,20] because MALDI is a competitive process in which the ionization of an analyte may be inhibited dramatically by the presence of others [20]. Thus, in tryptic peptide mixtures, arginine-containing peptides ionize preferentially due to the strong gas phase basicity of this amino acid [21,22].

## 3. Results

The preliminary biochemical characteristics of milin have been reported elsewhere [3]. On classical SDS-PAGE, milin showed a single band at  $\sim 52$  kDa [3]. Since the plant serine proteases reported so far are all monomeric with molecular weight in the range of 20–125 kDa [4], we took milin to be monomeric as well. However, discrepancies were noticed during extensive work with the protein and we sought to investigate the oligomerization properties of the enzyme as well as its other characteristics.



**Fig. 1.** Electrophoretic analysis of molecular weight and oligomerization of milin (a) Milin subjected to SDS-PAGE. Lane 1, Protein sample prepared as per classical SDS-PAGE conditions (SDS,  $\beta$ -mercaptoethanol, 95 °C); Lane 2, The protein was heated to 100 °C for 30 min prior to SDS-PAGE; Lane 3, Milin heated to 140 °C for 30 min prior to SDS-PAGE; Lane 4, Molecular weight marker. Milin shows temperature dependent dissociation. (b) Native PAGE of milin. 15% acrylamide gel was prepared without SDS. Protein (15  $\mu$ g) was loaded on the gel without SDS,  $\beta$ -mercaptoethanol and heat and electrophoresis was performed through cathode to anode. The molecular weight of milin is  $\sim 64$  kDa. (c) Milin was deglycosylated and the precipitated protein was heated gradually for 25 min with sample buffer at increasing temperature from 50 to 95 °C and loaded on the gel. The band at  $\sim 32$  kDa confirms the homodimeric association in milin.



**Fig. 2.** Molecular weight and oligomerization of milin: chromatographic and mass spectrometric analysis (a) Gel filtration of milin. Milin (200  $\mu$ g) was loaded under gravity on Sephacryl S-200 column of bed volume 15 ml, pre-equilibrated with 50 mM phosphate buffer, pH 8 containing 100 mM NaCl. Elution was performed using the same buffer at a flow rate of 0.5 ml/min and 0.5 ml fractions were collected. The absorbance of the fractions is reported against the elution volume. Albumin, ovalbumin, chymotrypsinogen and RNase were used as molecular weight markers under identical conditions. (b) MALDI TOF analysis. MS analysis showed two peaks at 31,593.9 and 32,545.3 Da respectively. The cumulative molecular weight (64 kDa) coincides with that obtained by gel filtration demonstrating the dimeric nature of milin. Mass resolution was obtained in the linear modes utilizing continuous ion extraction at threshold laser irradiance of 25 kV accelerating potential and 50 laser pulses were averaged.

### 3.1. Molecular weight of milin is 64 kDa

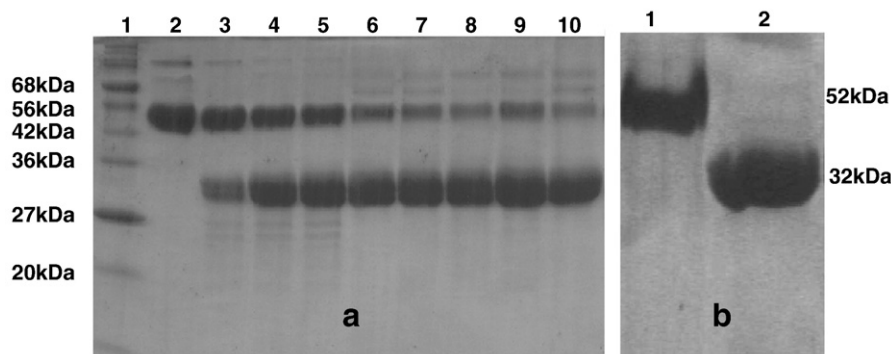
Initially, a molecular weight of  $\sim$ 52 kDa was observed for milin by SDS-PAGE under standard denaturing conditions (lane 1, Fig. 1a) [3]. However, native PAGE analysis revealed that the molecular weight of milin is  $\sim$ 64 kDa and not  $\sim$ 52 kDa (Fig. 1b). Further, the molecular weight by gel filtration was found to be  $\sim$ 64 kDa as well (Fig. 2a), indicating that SDS-PAGE for milin results in anomalous data. The MS data for milin also showed two peaks indicating molecular weights of 31,593 kDa and 32,545 kDa, leading to a cumulative molecular weight of  $\sim$ 64 kDa (Fig. 2b). The peaks probably represent two subunits of milin.

### 3.2. On SDS-PAGE under harsh experimental conditions milin dissociates into subunits

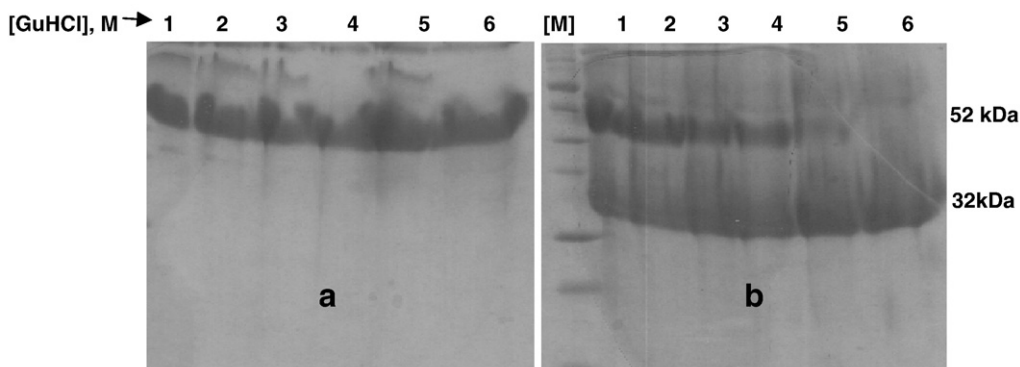
The anomaly in molecular weight and oligomerization observed for milin on SDS-PAGE and MALDI TOF were investigated further. As seen before [3], milin when heated at 95  $^{\circ}$ C showed a single band at  $\sim$ 52 kDa on SDS-PAGE (lane 1, Fig. 1a). Here, the protein was subjected over time to even higher temperature (140  $^{\circ}$ C) under reducing conditions prior to SDS-PAGE. As shown in lane 3, Fig. 3a, on being subjected to the higher temperature for 2 min, a band also appeared at  $\sim$ 32 kDa. The intensity of the lower band increased and that of the upper band decreased progressively with time (lanes 4–10, Fig. 3a), indicating that milin indeed contains subunits of molecular weight  $\sim$ 32 kDa thus corroborating the MS data. In absence of heat, however, the low molecular weight band

was not observed (lane 2, Fig. 3a). The subunit association in milin thus seems to be based on a number of intricate interactions since the combination of lower temperature (95  $^{\circ}$ C) and  $\beta$ -mercaptoethanol (lane 1, Fig. 1a), or  $\beta$ -mercaptoethanol alone (lane 2, Fig. 3a) cannot dissociate the subunits. A combination of reduction ( $\beta$ -mercaptoethanol) and high temperature is needed for complete dissociation unlike most other oligomeric proteins. Further, the negligible contribution of disulfide bonds to dimer association is exemplified in the appearance of similar  $\sim$ 32 kDa band when milin was heated to 140  $^{\circ}$ C for 30 min under non-reducing condition (without  $\beta$ -mercaptoethanol) as well (lane 2, Fig. 3b). Under similar conditions, absence of heat does not result in dissociation (lane 1, Fig. 3b). Such anomaly on SDS-PAGE was observed for a class of proteins known as “kinetically stable” proteins [23,24]. These proteins are resistant to SDS denaturation and hence show anomalous migration on electrophoresis under reducing and non-reducing conditions.

The strong association between milin subunits is further demonstrated in Fig. 4. When incubated with increasing concentrations of GuHCl (1–6 M) and subjected to SDS-PAGE without heating, at all concentrations of the denaturant single bands at  $\sim$ 52 kDa were observed (Fig. 4a). Samples prepared similarly as above but heated to 95  $^{\circ}$ C for 5 min prior to SDS-PAGE showed additional bands at  $\sim$ 32 kDa (Fig. 4b). In 5 M GuHCl and higher, only the low molecular weight protein band was observed indicating complete subunit dissociation at these concentrations of the denaturant when heated (Fig. 4b). The subunit association thus seems to be temperature dependent as seen



**Fig. 3.** Effect of high temperature (140  $^{\circ}$ C) on subunit association of milin (a) Milin (25  $\mu$ g) in SDS-PAGE loading buffer, layered with mineral oil on the top of the sample to prevent evaporation, was heated to 140  $^{\circ}$ C over time. Lane 1. Molecular weight marker. Lanes 2–10. Milin heated for 0, 2, 5, 8, 10, 12, 15, 20, and 25 min, respectively. (b) SDS-PAGE was performed under non-reducing condition (absence of  $\beta$ -mercaptoethanol). Lane 1. Sample loaded without heating; Lane 2. Sample heated for 30 min at 140  $^{\circ}$ C.



**Fig. 4.** Effect of chemical denaturant (GuHCl) on dimer integrity of milin. Milin was incubated in various concentrations of GuHCl (1–6 M) for 24 h prior to SDS-PAGE. (a) GuHCl incubated protein loaded in reducing condition but without heating showed single bands at ~52 kDa. (b) Protein sample as above but heated for 5 min at 95 °C before loading showed subunits as well. The upper bands correspond to nearly dimeric molecular weight and lower bands correspond to monomeric molecular weight.

in both Figs. 3 and 4, and further emphasized in Fig. 1a, where no dissociation was observed at 95 °C (lane 1), partial dissociation at 100 °C (lane 2), and complete dissociation at 140 °C (lane 3).

### 3.3. Milin is a dimeric protein

Milin showed a single band on native PAGE and single peak on gel filtration corresponding to a molecular weight of ~64 kDa (Figs. 1b and 2a). Further, two protein peaks were observed in the mass spectrum, whose individual molecular weights add up to ~64 kDa, indicating that milin contains subunits with monomer molecular weights of ~32 and ~31 kDa (Fig. 2b). The MALDI laser ionization must have separated the two subunits. All these experiments convincingly prove that milin is dimeric. Similarly, milin dissociates into subunits of molecular weight ~32 kDa under harsh treatment on SDS-PAGE (Figs. 3 and 4). Such an oligomerization state is unprecedented for a plant serine protease to the best of our knowledge [4]. Recently characterized serine proteases from plant latex like cryptolepain and carnein have similar molecular weights but show single peaks on mass spectrometric analysis, indicating that they are monomeric proteins [25].

### 3.4. Milin is homodimeric

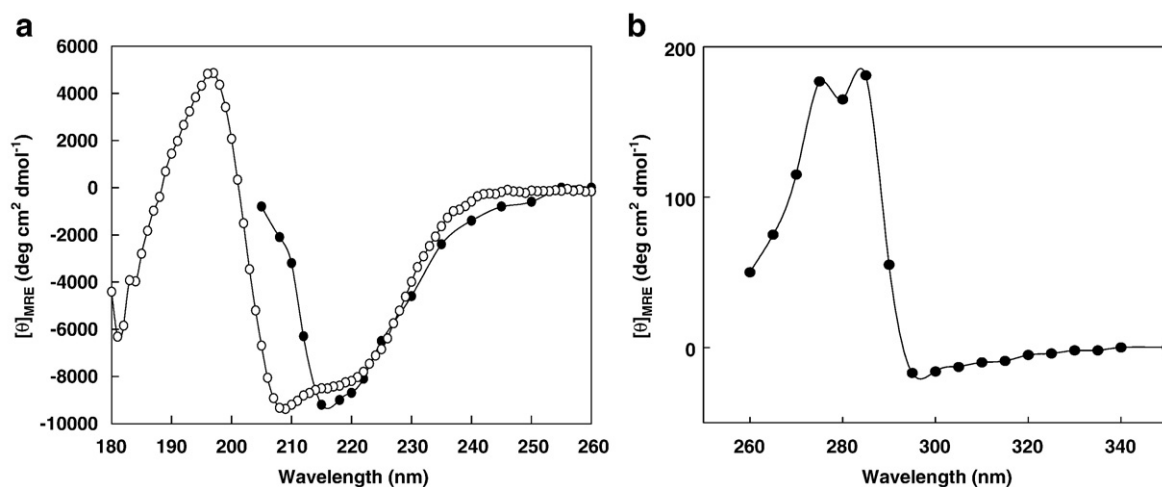
The difference in molecular weights of the monomers as seen in MS plot suggests that milin consists of heterodimers. A closer inspection of the ~32 kDa band observed on SDS polyacrylamide

gels also seem to support such an observation, since two closely spaced bands were actually seen (Fig. 1a, lane 3). The possibility, however, existed for differential glycosylation of the monomers, given the fact that milin was shown to be heavily glycosylated [3]. To verify this possibility, milin was deglycosylated chemically (Materials and Methods), and the protein free of carbohydrate moieties, was subjected to classical SDS-PAGE. The deglycosylated protein dissociates easily into subunits and only a single band was observed at ~32 kDa (Fig. 1c). This shows that milin is homodimeric and that the monomers are differentially glycosylated resulting in slight differences in their molecular weight under native conditions, as observed by MS analysis. Similar difference in molecular weight (~1 kDa) between subunits was observed on SDS-PAGE due to differential glycosylation in case of Scrapie prion protein [26,27].

The N-terminal sequence of milin was previously determined to be DVSYVGLILETD using native pure protein [3]. We repeated the procedure using the two closely spaced (~31.5 and 32.5 kDa) bands observed on SDS-PAGE under harsh conditions (Lane 3, Fig. 1a). Both the protein bands contained the same N-terminal sequence as above, confirming the observation that milin is homodimeric.

### 3.5. Strong subunit association in milin is necessary for stability, solubility and activity

As seen in Fig. 1c, deglycosylated milin easily dissociates into subunits unlike native protease, which requires heating to 140 °C for complete



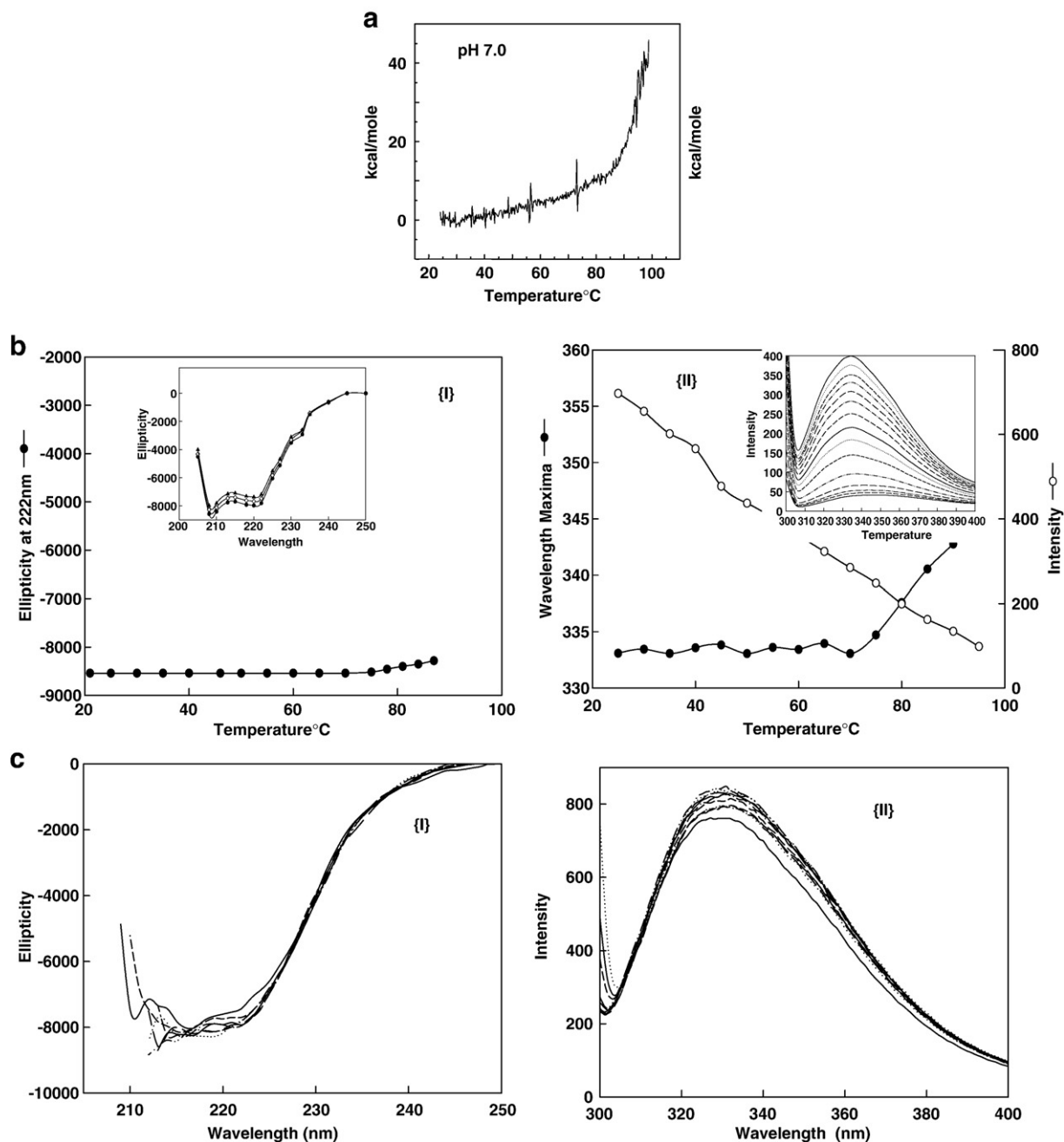
**Fig. 5.** CD spectra of milin. (a) Far UV CD spectra of glycosylated (○) and deglycosylated (●) milin. The samples were incubated at neutral pH for 24 h at room temperature before the measurements. (b) Near UV CD spectra of glycosylated (native) milin. The protein concentrations for near UV and far UV CD measurements were 1 mg/ml and 0.1 mg/ml, respectively.

dissociation. This shows that deglycosylated milin is less stable since the dimers are probably loosely associated. The deglycosylated protein also tends to precipitate indicating that solubility is decreased. The precipitated protein is soluble in 1.5 M GuHCl. Under these conditions, the deglycosylated protease showed no proteolytic activity either. The reference protein, native milin in 1.5 M GuHCl, on the other hand, showed considerable proteolytic activity [3]. It has been shown before that deglycosylation does not necessarily inactivate proteins [18]. Hence the activity loss on deglycosylation must be due to loss of proper dimer association. This represents that dimerization might be necessary for stability, solubility and activity of milin. However, the specific role

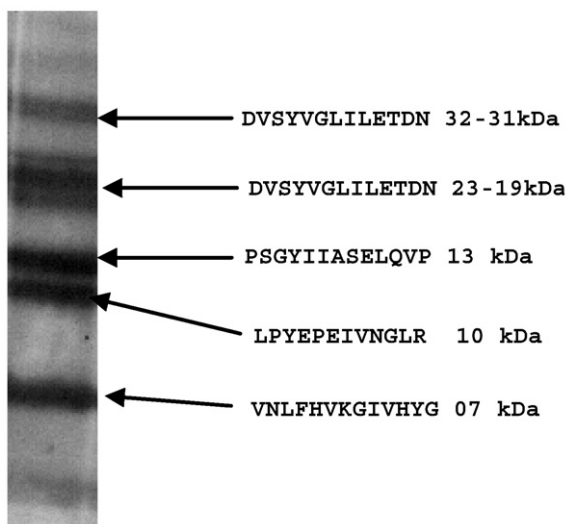
of glycosylation in dimerization of milin can be examined only after the characterization of the sugars bound to this plant serine protease.

### 3.6. Milin belongs to $\alpha/\beta$ class of proteins

Since milin seems to be a novel plant serine protease, circular dichroism was used to get an estimation of its secondary structure and native fold (Fig. 5). The far UV CD spectrum of native milin (Fig. 5a) is typical of  $\alpha/\beta$  class of proteins [28]. The spectrum shows negative peaks at 222 nm and 208 nm, positive peak at 195 nm and goes to negative again above 172 nm (Fig. 5a), all characteristics of an  $\alpha/\beta$



**Fig. 6.** Thermal and chemical denaturation induced unfolding of Milin; (a) Differential Scanning Calorimetry of Milin. The calorimetric scans were performed with a protein concentration of 1.25 mg/ml at a scanning rate of 60 °C/h. The enzyme was extensively dialyzed against 0.01 M phosphate buffer, pH 7.0 before scanning. (b) Temperature induced unfolding of milin at pH 7.0 (I) Far UV CD temperature transition curve. Inset: The entire far UV CD spectra at 40, 60 and 80 °C. (II) Temperature transition fluorescence curve. The inset shows fluorescence spectra at different temperatures. (c) Urea induced equilibrium denaturation of milin at pH 7.0. (I) Far UV CD Scan at different concentrations of urea (II) Fluorescence scan at various concentrations of urea. The protein (0.1 mg/ml) was incubated in the cuvette for 20 min at desired temperature prior to CD measurements. The results were expressed in mean residual ellipticity. The intrinsic fluorescence spectra of milin was obtained by excitation at 292 nm with the emission and excitation slits 10 and 5 nm respectively. The protein (0.01 mg/ml) was incubated in the cuvette for 20 min at desired temperature prior to fluorescence measurements.



**Fig. 7.** N-terminal sequences of various peptides from milin upon acid hydrolysis. The peptides were generated by mild acid hydrolysis in 0.1 M HCl. The molecular weight of the fragments and their corresponding N-terminal Edman sequence are represented in the figure.

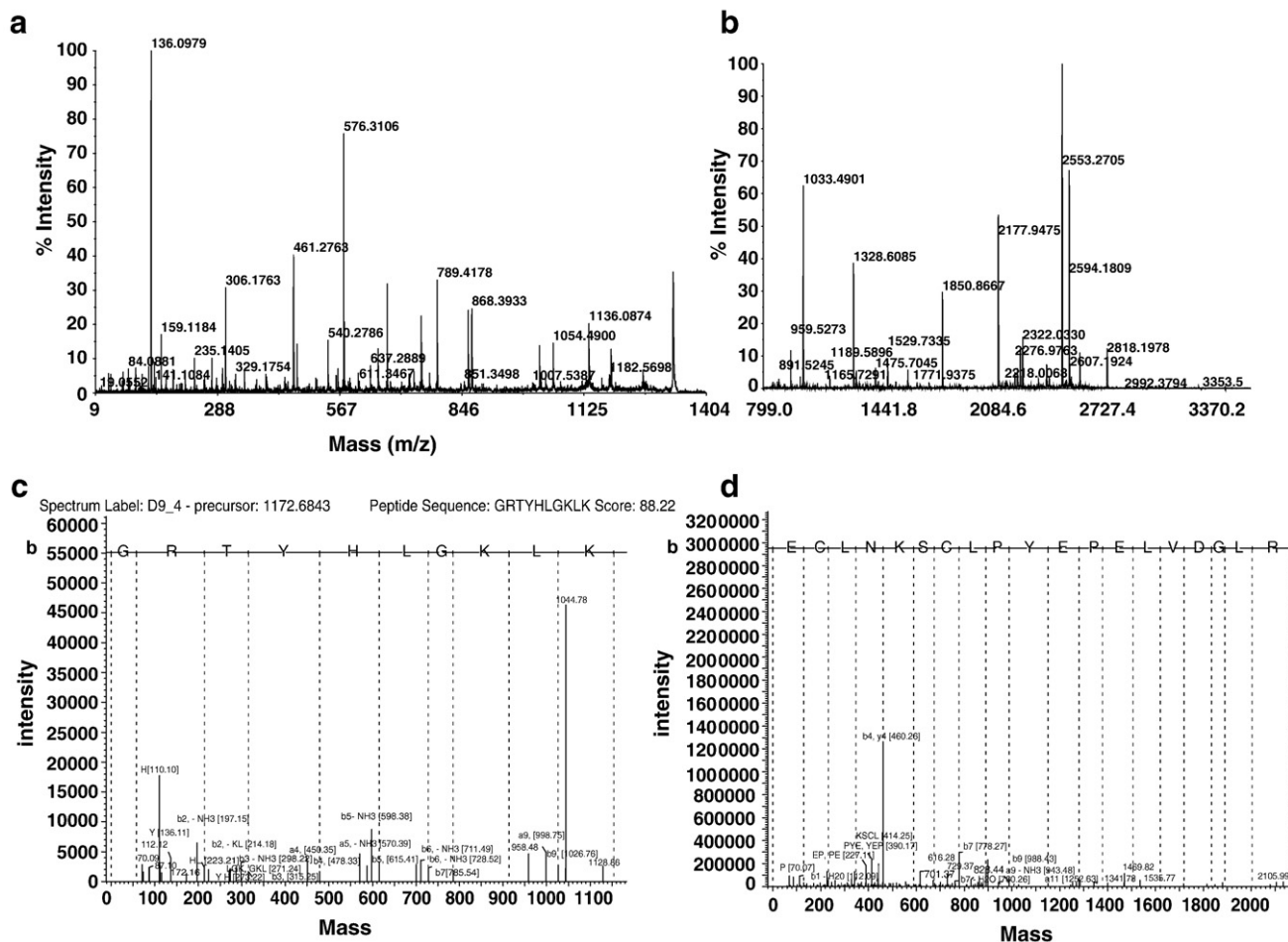
class of protein. However, deglycosylated milin shows spectrum typical of prominently  $\beta$  class of proteins, with a negative peak at 218 nm (Fig. 5a). The mean residue ellipticity for native milin at

222 nm was  $8.5 \pm 0.20 \times 10^3 \text{ cm}^2 \text{ dmol}^{-1}$  indicating 29% of  $\alpha$ -helical content and 38% of  $\beta$ -sheet content. The integrity of the three-dimensional structure of native milin was probed with near UV CD (Fig. 5b). The spectrum revealed three prominent peaks at 278, 288, and 300 nm of mean residue ellipticity 180, 180 and  $-50^\circ \text{ cm}^2 \text{ dmol}^{-1}$  respectively.

### 3.7. Milin is strongly resistant to thermal and chemical denaturation

Milin seems to be strongly resistant to denaturation and subunit dissociation as observed during SDS-PAGE analysis above. This was further confirmed by other biophysical experiments involving temperature and chaotrope induced denaturation of milin. Thus, the transition scan obtained by DSC after correction for buffer-buffer tracing is incomplete at pH 7.0. (Fig. 6a). This does not allow accurate estimation of thermal stability or melting temperature ( $T_m$ ). However, the apparent  $T_m$  seems to be approximately  $90^\circ \text{C}$  indicating that milin is thermostable. Similar result was obtained when the effect of temperature on milin was probed by far UV CD measurements (Fig. 6b(I)). There was no change in ellipticity up to about  $75^\circ \text{C}$  and even beyond that temperature the changes were negligible. The constant fluorescence emission maximum over temperature corroborates this finding (Fig. 6b(II)). The linear decrease in fluorescence intensity probably results from signal quenching and not due to denaturation.

Milin is resistant to chemical denaturation as well. Urea induced unfolding of milin is also incomplete (Fig. 6c). With increasing



**Fig. 8.** MALDI TOF spectra and *de novo* sequencing of milin (a) Peptide mass fingerprints of milin on trypsin digestion. In gel trypsin digestion was performed and MALDI TOF generated peptide finger print data was obtained. The mass of different peaks was searched in NCBI for identification of the molecule. (b) The TOT/TOF of 1456.8484 tryptic fragment contained immonium ion of Tyrosine ( $m/z$  136). This represented high resolution of the *de novo* fragments of milin. Peptides with high resolution of mass were used for *de novo* sequencing. (c) and (d) Peptide sequencing by tandem mass spectrometry. The TOF/TOF spectra of tryptic fragment of mass 1172.6883 and 2177.9521 Da were sequenced by computer based automated program for *de novo* sequencing respectively.

concentration of urea neither the far UV CD spectra (Fig. 6c(I)) nor fluorescence spectra (Fig. 6c(II)) change significantly. Hence  $C_m$  could not be determined.

### 3.8. N-terminal sequences of acid hydrolyzed fragments of milin are unique

Milin was hydrolyzed into seven major fragments with molecular weight ranging from 7 kDa to 32 kDa (Fig. 7). N-terminal sequences of the resulting fragments were determined by Edman's method. These peptides, their molecular weights and corresponding N-terminal sequences were: DVSYVGLILETD for 32.5, 31.5, 23, 21 and 19 kDa fragments; PSGYIIASELQVP for 13 kDa fragment; LPYPEIVNGLR for 10 kDa fragment; and VNLFHVKGIVHYG for 7 kDa fragment. The 31.5 kDa fragment dissociated into 19 and 13 kDa or 21 and 10 kDa bands, while 32.5 kDa bands dissociated into 21 and 13 kDa or 23 and 10 kDa bands. Few other less intense bands that appear upon acid hydrolysis were not sequenced. The differences in the molecular weight of three bands (viz. 19, 21 and 23 kDa) were due to differential glycosylation; the 13 and 10 kDa bands were not glycosylated. The N-terminal sequences determined do not show sequence homology to amino terminus of known serine proteases. Homology search across databases for match at positions other than N-terminus revealed other distant possibilities with very weak scores including transcription regulator, protease domain of an ATP-independent heat shock protease (PDB ID: JLIJ) etc.

### 3.9. Protein mass fingerprint (PMF) and de novo sequence of peptides establish milin as a novel serine protease

Since milin did not show distinct N-terminal sequence homology to known proteins, attempt was made to identify the protein by peptide mass fingerprinting. For this, the protein was enzymatically digested and resolved into a number of peptides (Fig. 8a and b). The masses of the peptides were determined and searched against relevant databases in NCBI. No significant hits were obtained based on peptide masses. Subsequently, the different peptide fragments of milin generated above and with good resolution were subjected to *de novo* sequencing by MALDI TOF/TOF. The TOF/TOF spectra of representative peptides and their *de novo* sequences are shown in Fig. 8c and d. Sequences of several peptides thus obtained are listed in Table 1. These sequences when submitted for BLAST search in NCBI database with different variables show the uniqueness of the protein sequence. No putative domains were obtained with any confidence using these sequences as search query. However, weak scores were obtained with similarities to esterase of the  $\alpha/\beta$  hydrolase superfamily and putative GTP binding protein.

**Table 1**  
*de novo* sequences of some tryptic peptides of milin

<i>m/z</i> PMF	Sequences	Score
842.5258	VTAVSLPR	67.9688
959.5275	TYHLGQLK	100.00
1033.5172	EFAYTKFK	83.4287
1172.6843	GRTYHLGQLK	88.2225
1172.6924	KWYHLVNGR	77.0785
1328.5978	AAYYANVDWKK	93.4696
1456.8484	SFHSYLGSVNAFK	93.8099
1601.3941	RLARDEMESLPER	77.1276
1771.9249	PSGYIIASEIQVPEIR	84.4429
1772.1573	PSGYLLASELQVPLER	94.4161
1850.8716	AVVHYGTSKT(317)DSIR	67.5228
2177.9521	ECLNKSCLPYEPELVDGLR	82.2166
2594.5638	GLNSTANSTEYSHFSYLGSVNAFK	95.3245

*m/z* refers to mass/charge ratio. PMF refers to Peptide mass fingerprint.

## 4. Discussion

The search for stable and active biochemical components from plant sources with novel properties and therapeutic applications has received widespread attention lately. The discovery of milin with unique properties and putative medical applications [1,3] will bolster such effort.

### 4.1. Milin is a plant subtilase with unique amino acid sequence

The proteolytic activity of milin has been described before [3] and observed in the current investigation as well. Typical cysteine protease inhibitors or metal chelators did not inhibit the protease activity of milin thus ruling out the possibility of the peptidase being a cysteine protease or metallic protease [3]. The protein was, however, strongly inhibited by phenylmethylsulfonyl fluoride (PMSF), 4-amidino-phenyl-methane-sulfonyl fluoride (APMSF) and diisopropylfluorophosphate (DFP) indicating that the catalysis is mechanistically dependent on a serine residue. The serine protease family includes various members like trypsin, chymotrypsin, elastases, subtilases, carboxypeptidases etc. [29]. That milin was not inhibited by soybean trypsin inhibitor (SBTI) also indicated that the serine protease activity of milin is not trypsin-like. With synthetic substrates, milin showed maximal hydrolysis at the peptide bond following a small hydrophobic amino acid, viz Ala [3], a property similar to subtilases [30]. Trypsin prefers basic residues (Arg/Lys) and chymotrypsin prefers large hydrophobic residues (Phe/Tyr/Trp) for proteolysis unlike milin [31]. Elastases also prefer Ala/Val, however, they do not generally work on single amino acid synthetic substrates [32]. Interestingly, human herpes virus serine proteases also cleave peptide bond between Ala and Ser [33]. Vast majority of the known plant subtilisins like cucumisin are active over a broad pH range with an optimum at alkaline pH and have relatively high thermal stability [3]. Milin also exhibits similar properties [3] including the fact that it shares a pH optima of 8.0 with varicella-zoster virus protease [33]. Milin is probably not a serine carboxypeptidase either since these enzymes have acidic optimum pH for their activity towards peptide substrates [34]. Characteristic of typical bacterial subtilisins (structure of plant subtilisins not yet solved), milin exhibits secondary structure with  $\alpha/\beta$  fold (Fig. 5) as ascertained by circular dichroism [35]. Three-dimensional structures have shown that subtilisins possess  $\alpha/\beta/\alpha$  sandwich or Rossmann fold, while other serine proteases belonging to the trypsin/chymotrypsin class are predominantly  $\beta$ -sheet protein [32,35–37]. Interestingly, human cytomegalovirus serine protease also belongs to  $\alpha/\beta$  class of proteins [6]. Hence, the biochemical (inhibition, substrate specificity, pH optimum etc.) and structural evidences indicate that milin is a plant subtilase.

However, the N-terminal sequence of milin showed no homology to known plant subtilases, either by *in silico* or manual search. In fact, no significant homology was observed with any member from other classes of serine protease either. Milin also showed absence of the conserved G–X–S/C–G–G sequence for chymotrypsin-like and G–T–S–M/A for subtilisin-like proteases [33] as evidenced from *de novo* sequences. Search across various protein databases reveal that milin has a unique N-terminus. Our added effort in sequencing N-terminal residues of acid hydrolysed fragments or *de novo* sequencing of tryptic digests failed to identify any putative domains in various portions of the protein as well. It has been reported [30] that the most striking feature of members of the subtilisin family is the high degree of the sequence variability within their catalytic triad; apart from the catalytic triad, all other residues may virtually be replaced by one or more distinct residue including variability at the N-terminus. These observations and those above indicate that milin is a plant subtilase with unique amino acid sequence. Interestingly, human herpes virus serine proteases also exhibit sequence variability [16]. Milin is also novel among plant serine proteases in being dimeric, as discussed below.

#### 4.2. Anomalous behaviour of milin on SDS-PAGE

Contrary to our previous report [3], the molecular weight of milin was found to be 64 kDa using a combination of gel filtration, mass spectrometry and SDS-PAGE under extreme conditions (Figs. 1, 2). Unique properties of milin when subjected to standard denaturing condition prior to SDS-PAGE previously resulted in an anomalous molecular weight of 52 kDa and the conclusion that the protein is a monomer. The relatively high mobility of the protein in SDS-PAGE gel probably resulted due to the formation of some kind of compact structure that resists subunit dissociation. The protein was probably partially denatured forming a compact intermediate. The loss of proteolytic activity of milin beyond 75 °C [3] excludes the possibility of limited proteolysis resulting in the 52 kDa band under the denaturing conditions [38]. The pI of the protein was found to be 7.0 [3] and hence the possibility of the anomalous migration due to high negative charge on the protein can also be negated [38]. The present investigation though provides a solution to the observed anomaly in molecular weight and oligomerization by reporting approximately 32 kDa bands on SDS-PAGE under extreme conditions (very high temperature and presence of denaturant like GuHCl) that breaks down the compact structure (Figs. 3, 4). The molecular weight is half of the actual molecular weight indicating the presence of subunits and that milin is a dimer. This conclusion was further confirmed when milin, a glycoprotein, was chemically deglycosylated. Deglycosylated milin loses its dimeric assembly and hence shows molecular weight corresponding to monomers on SDS-PAGE under standard denaturing conditions (Fig. 1c). Most plant serine protease reported so far have been found to be monomeric with the molecular weight in the range of 30–110 kDa [4]. Thus, it is recommended to try extreme conditions of denaturation prior to SDS PAGE before concluding the oligomeric nature of such proteases by SDS-PAGE, specially the ones that are poorly characterized biochemically and biophysically.

#### 4.3. Dimerization of milin is novel

It is evident from the present investigation that milin is a dimeric serine protease. This is the first report of a dimeric plant serine protease to the best of our knowledge. Dimerization of milin is certainly not dependent on disulphide bridges since the dimer can be dissociated both in the presence or absence of reducing agents as investigated by SDS-PAGE. It appears that temperature plays a significant role in the dimer dissociation (Figs. 1–4 and 6). There is a threshold requirement of thermal energy necessary for the dissociation of dimers in SDS-PAGE loading buffer. The threshold is lowered in presence of high concentration of another denaturant like GuHCl, indicating that a combination of non-covalent forces probably keeps the monomers associated. Milin was also shown to be strongly resistant to temperature and chemical denaturation.

Some of the above observations are similar to those reported by Chalut et al. (1999) for penicillin binding protein 1B in *E. coli*. For this protein, the dimer was stable at 60 °C but almost totally dissociated at 80 °C and the same pattern of dissociation was observed with and without  $\beta$ -mercaptoethanol. Milin was also stable to 100 °C but dissociated when heated above this temperature prior to SDS-PAGE (Fig. 3). In the presence of GuHCl, the dissociation temperature was 95 °C under similar conditions (Fig. 4).

Experimental evidences indicate that the aqueous solubility of milin decreases when subunits dissociate, mainly due to deglycosylation. 1.5 M GuHCl is required to solubilize the resulting precipitate. Deglycosylated protein runs normally on SDS-PAGE without extra input of thermal energy or without 6 M GuHCl and shows complete dissociation with a molecular weight equivalent to milin monomer (Fig. 1c). Deglycosylated protein monomers are also proteolytically inactive. Since deglycosylation is known not to inactivate proteins in general [18], the inactivation must be due to loss of dimer integrity. It

is possible that either the active site is close to dimer interface or structural parts from one subunit cross over into the other requiring intact dimer for catalysis. Herpes virus serine proteases form dimers where such crossover between subunits has been observed in crystal structures [33]. However, no report of glycosylation having such effects on serine protease dimers exist and milin is the first report. The importance of this post-translational modification in maintaining oligomerization integrity needs to be examined further for milin and other proteins.

#### 4.4. Milin seems to be a kinetically stable protein

All the general characteristics and anomalies observed for milin on SDS-PAGE is akin to kinetically stable proteins, which have infrequent occurrence in nature [23,24]. Members of this novel class of proteins show differences in migration of their protein band on SDS-PAGE on heating and without heating [23,24]. They seem to be trapped by a transition state energy barrier in their highly stable native conformation. The unfolding transitions are thus not easily overcome so that randomization of such proteins is difficult relative to other proteins and they are resistant to harsh denaturing conditions, especially SDS [24,39,40]. Nature has favored such a unique property to allow these proteins to maintain their activity under hostile environment. Milin seems to be a member of this class as well; especially evident from the SDS-PAGE results in which heating to a certain temperature is required for complete denaturation. It has been shown that majority of the kinetically stable proteins are enzymes, belong to  $\alpha/\beta$  class of proteins and are dimeric in nature [23,24,41]. Interestingly, milin also follows this general trend. It is even more interesting to note that papain, the most widely studied plant cysteine protease from latex, also belongs to the class of kinetically stable proteins [24]. It is not dimeric in nature but contains two domains with the active site cleft situated between them. One obvious question to ponder is whether most proteases from latex are also members of this class, enabling them with enhanced stability.

It has been reported that rigid protein structure is the physical basis of kinetic stability and resistance to SDS. Protection of surface residues from the solvent, increase in rigidity and stabilization of specific region of a protein are potential effects of oligomerization that may increase kinetic stability, as seen for milin. This also supports the evolutionary pressure of forming dimers or bi-domains for proteases from latex [42–49].

The extreme stability of milin is further exemplified by thermal and chemical induced denaturation studies. Temperature induced unfolding of milin was incomplete with an apparent  $T_m$  of 90 °C. Gradual decrease of the transition start-point as a function of chaotrope concentration is also an indication of the highly compact structural integrity of the protein molecule. The similar shape and ellipticity of the far UV CD spectra and little shift in the fluorescence emission maximum on chemical or thermal denaturation is indication of the formation of a new intermediate conformational state of the molecule which resists further denaturation.

#### 4.5. Physiological and therapeutic importance of milin

The unique properties of milin indicate novel physiological role for the plant protein. Latex of several plants has been found to be rich in proteolytic enzymes including mostly cysteine proteases and some serine proteases although their physiological roles are largely unknown. They have often been indicated to play important role in plant defense, especially papain and papain-like cysteine proteases [2]. It is to be noted that cysteine proteases belonging to the papain superfamily all have two domains with the active site situated between them. Milin, on an analogous note, has two subunits that can probably hold the active site in between them. In doing so the active site is shielded from the waxy environment of the latex and large areas

of protein surfaces are best protected in dimers [9]. This would explain why monomers of milin are inactive or less active. Thus, dimerization could be a way of regulating physiological function of milin or enhancing its catalytic activity. Human herpes virus serine proteases and human cytomegalovirus proteases are also inactive in their monomeric form and dimerization activates them [6,14,15,33]. Chymotrypsin is also known to dimerize under certain conditions [12].

*Euphorbia milii* is a wild, tropical, xerophytic plant that has to survive high temperatures in summers and other extreme environmental stress. The fact that milin needs high input of thermal energy for dimer dissociation and is a kinetically stable protein could be a means for the protein to tide over duration of high temperature by forming a compact structure that resists thermal denaturation. The active site is protected under such hostile conditions and any mild denaturation can be reversed at more favourable lower temperatures [50,51]. Glycosylation can help the protein acquire a sheath of aqueous layer for similar purposes. Sugar residues can also help milin in recognizing specific receptors on insects and other pests thus triggering defense action through proteolysis.

The therapeutic importance of milin has already been reported [1]. It seems to be an effective molluscicide that can control Schistosomiasis. It has been noted above that the temperature-dependent dissociation of dimers in milin on SDS-PAGE is akin to that of penicillin binding protein 1b in *E. coli* [52]. The presence of penicillin binding protein  $\beta$ -lactamase with serine protease activity has been reported in *Schistosomiasis japonicum* [53]. This, though a curious coincidence, could be related to the usefulness of milin in treating the disease. There is every possibility that milin is multifunctional and just not a simple serine protease like many other such enzymes reported in plant latex. The oligomerization and other novel properties of milin hint at such possibilities.

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