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ORIGINAL PAPER

Complete conformational stability of kinetically stable dimeric serine protease milin against pH, temperature, urea, and proteolysis

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Abstract Spectroscopic, calorimetric, and proteolytic methods were utilized to evaluate the stability of the kinetically stable, differentially glycosylated, dimeric serine protease milin as a function of pH (1.0–11.0), temperature, urea, and GuHCl denaturation in presence of 8 M urea at pH 2.0. The stability of milin remains equivalent to that of native at pH 1.0–11.0. However, negligible and reversible alteration in structure upon temperature transition has been observed at pH 2.0 and with 1.6 M GuHCl. Irreversible and incomplete calorimetric transition with apparent $T_m > 100$°C was observed at basic pH (9.0 and 10.0). Urea-induced unfolding at pH 4.0, and at pH 2.0 with GuHCl, in presence of 8 M urea also reveals incomplete unfolding. Milin has been found to exhibit proteolytic resistant in either native or denatured state against various commercial proteases. These results imply that the high conformational stability of milin against various denaturating conditions enable its potential use in protease-based industries.

Keywords Milin · Kinetically stable protein · Stability · Proteolysis · Proteolytically stable

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Introduction

Various evolutionary strategies have been devised to modulate conformational stability of protein in order to enhance functionality and survival of proteins even under adverse condition. Among several other natural strategies, the poorly reported kinetic stability of some proteins is one of the most effective. These proteins are trapped in their native conformation by an energy barrier and become resistant to unfolding. The high kinetic barrier separating the folded and unfolded state is intended to allow proteins to maintain their activity and stability in the extreme conditions they may encounter in vivo (Cunningham et al. 1999). All kinetically stable proteins exhibit kinetic adaptation of proteins that must retain enzymatic function in conditions where degradation might easily take place. This kinetic stability is required to protect the structure of proteins against the conformational side-effects of proteins that are susceptible to irreversible denaturation and aggregation.

Various strategies have been used to improve the kinetic stability of proteins, such as the addition of hydrophobic residue on protein surface (Jaenicke and Bohm 1998), disulfide bond engineering (Ladenstein and Antranikian 1998), introduction of metal binding sites (Li et al. 2005), oligomerization (Atomi 2005), and formation of ion pairs (Berezovsky and Shakhnovich 2005; Egorova and Antranikian 2005). To date, no common structural features have been reported to explain kinetic stability in general. Although the kinetic stability of different proteins can be achieved by different means depending upon the nature of the individual depending on the individual protein (Manning and Colon 2004). Kinetically stable proteins have limited access to partially and globally unfolded conformations, which imparts strong proteolytic resistance

by reducing the occurrence of accessible conformations susceptible to proteolytic action. Most of these proteins reveal complete resistance to sodium dodecyl sulfate (SDS) denaturation, with different mobility of heated and unheated samples on SDS-polyacrylamide gel electrophoresis (PAGE). The SDS and proteolytic resistance of proteins have strong correlation with their structural rigidity and kinetic stability (Manning and Colon 2004). Conformational stability of proteins is of great interest because it can provide the basis to attain protein stability (Jaenicke and Bohm 1998; Ladenstein and Antranikian 1998; Li et al. 2005) and for many applications in biotechnological and pharmaceutical industries (Atomi 2005; Egorova and Antranikian 2005). The greater stability of kinetically stable proteins cannot be confered by a single factor. The determination of thermodynamic parameters for the structural stability of kinetically stable proteins is difficult due to the irreversible nature of their unfolding (Manning and Colon 2004). In addition, the scientific community has very limited understanding about the unfolding kinetics of kinetically stable proteins (Xia et al. 2007).

Kinetically stable proteins are unusual in their resistance towards high temperatures and chemical denaturants (Jaswal et al. 2002). We analyzed the conformational stability and proteolytic inertness of the kinetically stable, dimeric, glycosylated serine protease milin to determine the role of kinetic stability in the improvement of protein survival under extreme denaturating conditions (Yadav et al. 2009). Most proteins from xerophytes plants have evolved to remain stable and functional under extraordinarily harsh conditions, including extremely hot, acidic or proteolytic environments (Jaenicke 1991b; Scandurra et al. 2000). Milin was found to be exceptionally resistant against urea, temperature, acid, and mixed (urea and GuHCl) destabilization. The present study illustrates the biophysical and proteolytic characterization of milin to understand this greater stability against various denaturants.

Materials and methods

Material

Latex from *Euphorbia milii* was used to purify milin, as described previously (Yadav et al. 2006). Protein concentration was determined using an extinction coefficient of $\varepsilon_{280}^{1\%} = 29$ as well as by Bradford assay calibrated with bovine serum albumin (BSA) (Yadav et al. 2006). Urea, GuHCl, trypsin, α-chymotrypsin, and protease K were procured from Sigma Chemical Company, USA. All other chemicals were of highest commercially available purity. Samples were prepared in Millipore water and filtered through 0.45 μM filters.

Methods

Circular dichroism (CD) spectroscopy

Circular dichroism (CD) measurements were taken on a precalibrated (with 0.1% d-10-camphorsulfonic acid solution) Jasco-715 spectropolarimeter. Circular dichroism spectra in the range between 190 and 250 nm are typically acquired to obtain the percentage of protein secondary structures in the solution, namely in terms of α-helix, β-sheet, and random coil, with very high sensitivity and resolution (Nicolini et al. 1993). The scanning speed was set as 100 nm/min with a 1.0 s response time. The reported near-ultraviolet (UV) ellipticity curves are the average of three measurements collected from 260 to 320 nm over a 3 min period. The measurements were carried out at 25°C, using a cuvette with 0.1 cm (far-UV CD) or 1 cm (near-UV CD) light path. The protein concentration was 1 and 0.1 mg/ml for near-UV and far-UV CD, respectively. Results are expressed as mean residual ellipticity $[\theta]_{MRW}$, using the equation: $[\theta]_{MRW} = [\theta]_{obs} \cdot MRW/10.c.l$, where $[\theta]_{obs}$, c, and l represent the observed ellipticity in degrees, the protein concentration in mg/ml, and the path length of the light in centimeters, respectively. Mean weight of amino acid residues (MRW) was taken as 110. The content of secondary structures (α-helix, β-sheets) of the protein was calculated using software provided by Jasco.

Tryptophan emission fluorescence measurement

Fluorescence spectra were collected on an LS50B fluorescence spectrophotometer (Perkin Elmer) with a cuvette with 1 cm path length. Tryptophan emission fluorescence measurements were carried out with excitation at 292 nm. Emission fluorescence spectra were collected between 300 and 400 nm, using response time of 1 s and scan speed of 120 nm/min. Slit widths for excitation and emission were 5 and 10 nm, respectively. The protein concentration for each experiment was 0.01 mg/ml. Temperature was controlled by circulating water bath, and accurate measurement of sample temperature was recorded by a thermocouple directly attached to the cell.

Biophysical stability of milin against pH

Effect of pH on the stability of milin was studied as a function of pH. Protein samples were incubated at given pH at room temperature for 24 h in the pH range 0.5–11.5. The buffers used were: KCl–HCl (pH 0.5–1.5), Gly–HCl (pH 2.0.0–3.5), sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.5–10.5), and Gly–NaOH (pH 11.0–12.5). Concentrations of all buffers used were 25 mM. A stock solution of protein was added to the appropriate buffer. The final pH and concentration of the

protein in each sample were measured before and after all experiments.

Thermal-induced unfolding of milin

Thermal-induced unfolding of milin was recorded at 5°C intervals from 20°C to 95°C at acidic pH 2.0, and in presence of 1.6 M GuHCl at pH 2.0. The protein samples were equilibrated for 20 min at the desired temperature prior to far-UV CD and intrinsic fluorescence measurements. The 20 min incubation at desired temperature was found to be standard for the maximum conformational alteration in milin. The temperature was controlled using a Julabo F 25 circulating water bath attached directly to the cell holder for CD and fluorescence measurements. The temperature of the sample in the cuvette was measured using a thermocouple connected to a digital multimeter. The condition for thermal-induced unfolding was 25 mM glycine–HCl at pH 2.0. The pH of each sample was checked before acquiring thermal unfolding equilibrium. The temperature stability of milin at neutral pH was already reported (Yadav et al. 2009).

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 Na-acetate (pH 4.0–5.0), Na-phosphate (pH 6.0–7.0), and Tris–HCl (pH 8.0–10.0). The protein concentration and pH of the samples were rechecked and adjusted to desired concentration. All solutions were degassed under vacuum before being loaded into the calorimeter cells. The calorimetric experiments were conducted at scan rate of 60°C/h. Buffer baselines were obtained under the same conditions and subtracted from the sample curves.

Urea-induced unfolding of milin at pH 2.0 and 4.0

Urea-induced destabilization was performed by incubating the protein sample at a desired denaturant concentration for approximately 24 h at 25°C to establish equilibrium. A desired amount of milin stock solution was added to freshly prepared urea of different concentrations to make up the final protein concentration of 0.1 mg/ml for CD and 0.01 mg/ml for fluorescence measurements. The buffer used to maintain the desired pH was 25 mM glycine–HCl and Na-acetate for pH 2.0 and pH 4.0, respectively. The effect of urea on milin stability at neutral pH was negligible, as reported (Yadav et al. 2009). The final concentrations of the protein and denaturants in each sample were determined by absorption at 280 nm and refractive index, respectively. After 24 h incubation at 25°C, tryptophan fluorescence emission and CD measurements were carried out.

Unfolding of milin at pH 2.0 by GuHCl in presence of 8 M urea

The GuHCl was added in presence of 8 M urea to destabilize the milin, as urea-induced unfolding of milin at pH 2.0 was incomplete. The unfolding was monitored by circular dichroism (CD) and fluorescence measurements as described earlier. The highest molarity/saturated solution of urea (up to 12 M) and GuHCl (8.5 M) were prepared in pH 2.0 buffer separately and mixed to obtain the desired concentrations of denaturants. The volume of final solution was kept constant at 0.5 ml and the concentration of protein was maintained at 0.10 mg/ml for CD and 0.01 mg/ml for fluorescence measurements.

Proteolytic cleavage and SDS-PAGE of milin

Trypsin, chymotrypsin, and protease K were used for proteolytic digestion of milin at 37°C in 25 mM Tris–HCl and 1 mM $CaCl_2$ at pH 8.0. Stock solutions of different protease (100 μM) were prepared in 25 mM Tris–HCl and 1 mM $CaCl_2$ at pH 8.0. The milin (denatured as well as native) and each protease at 10:1 molar ratio were incubated for time periods of 1, 5 or 24 h at 37°C. Harsh denaturing conditions (140°C for 30 min) were used to denature milin at high temperature (Yadav et al. 2009). The proteolytic reactions were terminated by heating the aliquot (50 μg) of digestion reaction with SDS. Electrophoresis was carried out under standard denaturing SDS-PAGE conditions in which samples were heated at 95°C for 5 min in 4% β-mercaptoethanol and 2% SDS. Similar amounts of all proteolytic digest including protease were loaded onto denaturing 20% SDS-PAGE. Fresh inactivated proteolytic enzyme for qualitative analysis as control was loaded onto the same SDS-PAGE gel.

Results

The kinetically stable nature, primary spectroscopic analysis, dimerization, partial sequence, and thermal as well as chemical denaturation at neutral pH of milin have been reported previously (Yadav et al. 2009). In classical denaturation experiments, milin showed complete stability against thermal and chemical denaturation. However, in-depth denaturation study of milin has established the kinetically stable nature of the protein.

pH stability of milin

The insignificant reduction of the near-UV CD ellipticity of the 278 and 288 nm peaks at pH 1.0–10.0 reflects the high stability of milin, but at pH <2.0 a single peak at 282 nm

with similar ellipticity was observed. The positive peaks were shifted to 292 nm at pH ≥11.0, with increased negative ellipticity at 300 nm (Fig. 1a). The far-UV CD spectra of milin remained similar over the pH range 1.0–11.0, with insignificant changes in terms of magnitude and shape (Fig. 1b). The intrinsic fluorescence properties of milin were identical at all pH, and the wavelength maximum remained unchanged at 333 nm over the entire pH range of 1.0–11.0. However, fluorescence intensity decreased gradually at basic pH (9.0–10.0) but remained constant for acidic pH 1.0–7.0 (Fig. 1c).

Thermal stability of milin

Differential scanning calorimetry

The thermal unfolding of milin was apparently irreversible at any pH (data not shown). However, the complete spectroscopic properties as well as the functional activity of milin were recovered below 80°C (data not shown). This phenomenon of milin is similar to that of kinetically stable protein. The accurate thermal melting temperature (T_m) could not be established due to the incomplete thermal transition. However, the apparent T_m seems to be ≥100°C at pH 9.0 and 10.0 (Fig. 2). Milin aggregated at high protein concentration (1.25 mg/ml) at pH 4.0–6.0 (Leite et al. 2002). This feature was also observed in the spectroscopic assay at low protein concentration. The thermal unfolding transitions were initiated at 85°C and 80°C at pH 9.0 and 10.0, respectively. The highest single-scan ΔC_p was 60 and 80 kcal/mole at pH 9.0 and 10.0, respectively (Fig. 2), whereas it was only 40 kcal/mole at pH 7.0 and 8.0 (Yadav et al. 2009).

Effect of temperature on stability of milin

Temperature-induced unfolding of milin was found to be incomplete and irreversible at all pH, similar to in the differential scanning calorimetric experiment (data not shown). Light-scattering experiments confirmed aggregation of milin above 85°C at neutral pH. It was also precipitated above 60°C at pH 4.0–6.0. However, in extreme acidic condition (pH ≤2.0) such aggregation of milin was not observed. Due to this the temperature transitions could not be acquired for the pH range 4.0–6.0. This observation was similar to that of differential scanning calorimetry. The temperature transition was incomplete under all the investigated conditions, but unfolding of milin initiated beyond 70°C at neutral pH (Yadav et al. 2009), 65°C at pH 2.0 (Fig. 3), and 60°C at pH 2.0 in presence of the 1.6 M GuHCl (Fig. 4). The 1.6 M GuHCl was the maximum concentration where milin retained its full spectroscopic as

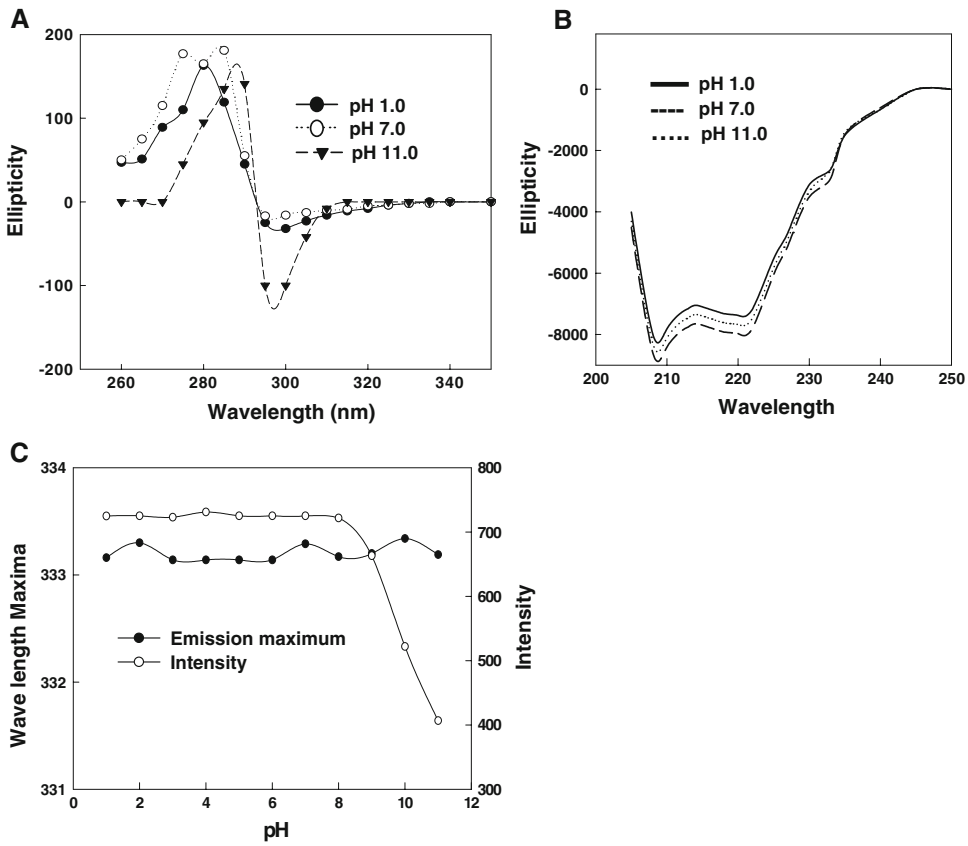


Fig. 1 pH stability of milin. **a** Near-UV CD at pH 1.0, 7.0, and 11.0, **b** far-UV CD at pH 1.0, 7.0, and 11.0, **c** fluorescence transition at pH 1.0–10.0. For near-UV CD, far-UV CD, and fluorescence measurements, 1.0, 0.1, and 0.01 mg/ml milin solution was used, respectively. The path length for near-UV CD, far-UV CD, and fluorescence measurements was 1.0, 0.1, and 1.0 cm, respectively. Samples were incubated under given condition for 24 h at room temperature before measurement

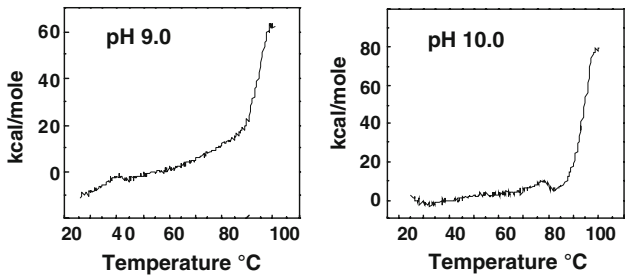


Fig. 2 Differential scanning calorimetry of milin. The calorimetric scans were performed with protein concentration of 1.25 mg/ml at scanning rate of 60°C/h in each buffer at pH 4.0–10.0. The enzyme was extensively dialyzed against respective 0.05 M buffers before scanning. Milin precipitated at pH 4.0–6.0, thus calorimetric scan could not be performed. At pH 7.0 incomplete transitions occurred with much less light scattering (Yadav et al. 2009). Similar scans resulted at pH 8.0. At basic pH, milin was stabilized to a certain extent

well as functional characteristics (Yadav et al. 2006). This critical concentration was used to obtain the next level of thermal unfolding. The far-UV CD and fluorescence wavelength emission maximum change were similar under all the thermal denaturation conditions. However, the fluorescence intensity transition in presence of 1.6 M Gu-HCl at pH 2.0 was sigmoidal (Fig. 4) with 30% reduction in the content of far-UV CD (Yadav et al. 2006). The maximum shift in emission wavelength gradually increases to 347, 348, and 349 nm, at pH 7.0, 2.0, and 2.0 in presence of 1.6 M GuHCl at 98°C respectively (Figs. 3, 4) (Yadav et al. 2009). The shifts of apparent transition midpoint (T_m) with respect to temperature were found to be gradually reduced from pH 7.0 (Yadav et al. 2009), to pH 2.0, to pH 2.0 with 1.6 M GuHCl, respectively (Figs. 3, 4). The linear and continuous decrease in fluorescence intensity at pH 7.0 and pH 2.0 are indicative of local changes in the configurational status of the aromatic amino acid residues (Fig. 3b).

Urea-induced unfolding of milin

Milin exhibits strong resistant to chemical denaturation at acidic pH as well. Urea-induced unfolding of milin at pH 2.0 and 4.0 was also incomplete (Figs. 5, 6). Milin does not precipitate on urea-induced transition at pH 4.0, similar to the temperature-induced transition. Similar fluorescence scan (10% reduction in fluorescence intensity at 8 M urea) and far-UV CD results were observed upon urea transition at pH 4.0 (Fig. 5a, b). These findings substantiate only minor local configurational change in the amino acid arrangement. However, the urea-induced transition at pH 2.0 reveals some degree of destabilization. The reduction in near-UV CD ellipticity (data not shown) and fluorescence intensity of milin at pH 2.0 was 25% and 40%, respectively (Fig. 6b). These results were correlated with the similar reductions in activity. The maximum reduction in far-UV CD on urea-induced transition of milin was only 10% at pH 2.0, indicating mild reduction of secondary-structure content. These results reflect a strong correlation between the molecular structures and stability of milin.

Denaturant stability of milin at pH 2.0 by combination of urea and GuHCl

Urea at pH 2.0 destabilized milin to a certain extent but the denaturation was not complete. The mixed destabilization experiment was performed to obtain the next level of unfolding of milin. The destabilization of milin was further increased by addition of GuHCl. GuHCl (4 M) in presence of urea (8 M) at pH 2.0 was found to be a more effective combination of denaturants. This combination reduces the secondary structure of milin upto 50% (Fig. 7a, c). The fluorescence intensity decreased linearly, although the wavelength maxima remained unchanged (Fig. 7b).

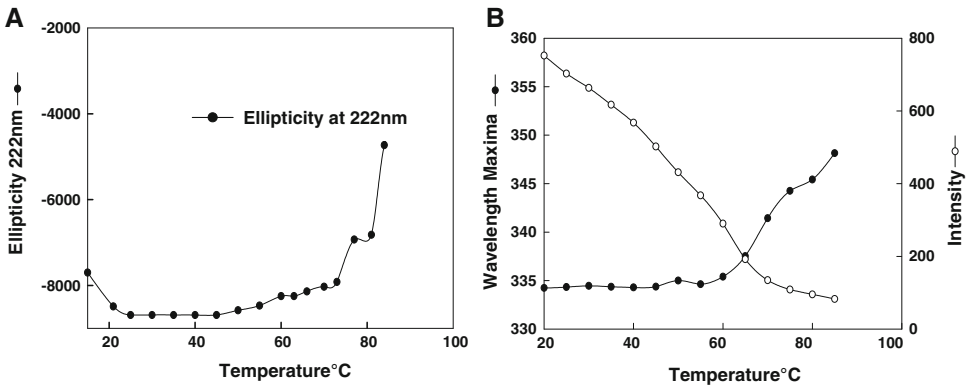


Fig. 3 Thermal stability of milin at pH 2.0. The temperature stability of milin at acidic pH (2.0) was monitored by change in **a** far-UV CD spectra and **b** fluorescence prior to excitation at 292 nm. Protein (0.1 mg/ml) at pH 2.0 was incubated in the cuvette for 20 min at the desired temperature prior to CD measurements. The result is expressed in mean residual ellipticity. The intrinsic fluorescence spectra of milin was excited at 292 nm with emission and excitation slits of 10 and 5 nm, respectively. Protein (0.01 mg/ml) was incubated in the cuvette for 20 min at the desired temperature prior to fluorescence measurements

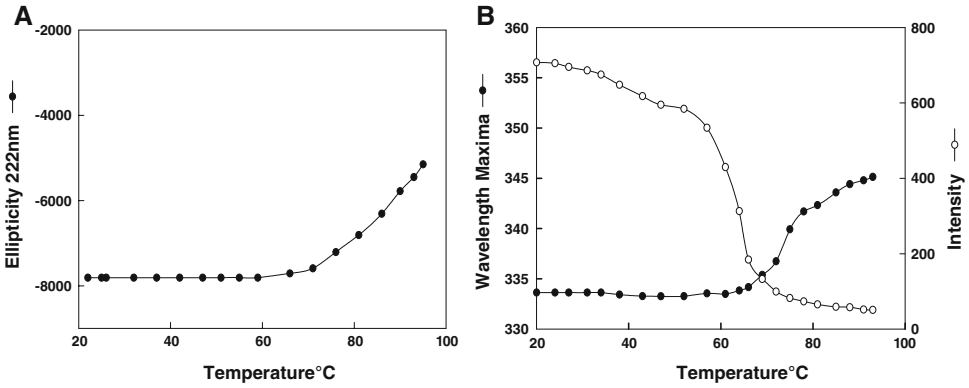


Fig. 4 Thermal unfolding of Milin in presence of GuHCl. The temperature stability of milin in presence of 1.6 M GuHCl was monitored by **a** far-UV CD and **b** fluorescence scan. The 1.6 M GuHCl was used because this was the maximum concentration of GuHCl at which milin retained complete functional stability (Yadav et al. 2009). The remaining experimental conditions are similar to those for Fig. 3

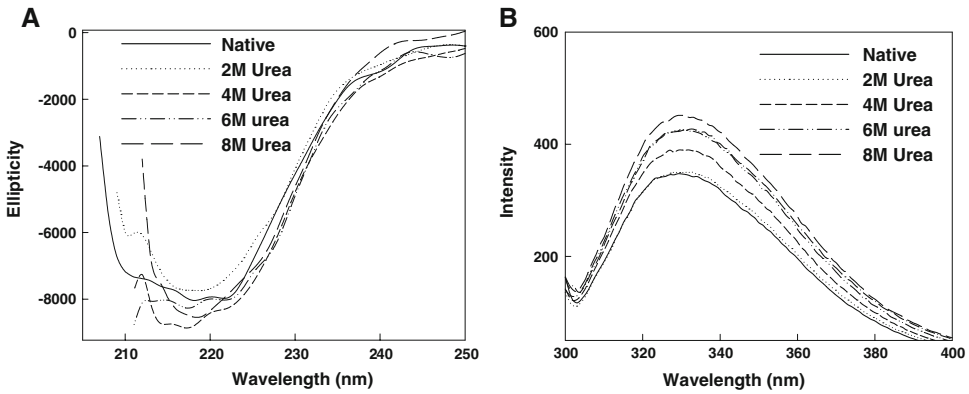
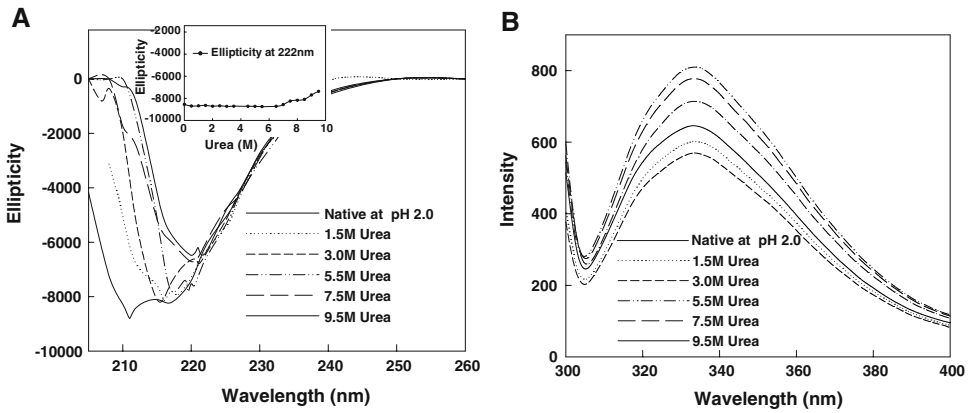


Fig. 5 Stability of milin against urea at pH 4.0. The stability of milin against the chemical denaturant urea was determined by **a** far-UV CD spectra and **b** fluorescence scan. Protein (0.1 mg/ml) was incubated at desired concentration of freshly prepared urea at pH 2.0 for 24 h at 37°C. The spectral measurements were carried out as described in the "Materials and methods" section. The denaturating conditions are given with each figure separately

Fig. 6 Stability of milin to urea at pH 2.0: **a** far-UV CD spectra and **b** fluorescence spectra. The far-UV CD stability transition is shown in the *inset*. Similar experimental procedures were followed as in Fig. 5. The transitions are incomplete with respect to far-UV CD, fluorescence intensity, as well as wavelength maxima. Denaturating conditions are given with each figure



Sequential unraveling of multiple secondary-structure layers of milin could be one possible explanation for this. The data suggest the possibility of highly kinetically stable unfolding barriers in the unfolding pathway of milin.

Proteolytic stability

The proteolytic stability of milin was determined using three mammalian digestive proteins: chymotrypsin, trypsin, and protease K. The proteolytic activities of these enzymes were confirmed by casein hydrolysis assay before the experiments (Yadav et al. 2006). Milin (denatured) exhibits complete proteolytic resistance to digestion by these proteases after 24 h incubation (Fig. 8a). This was confirmed by similar intact bands on SDS-PAGE of milin with and without protease incubation (Fig. 8a). Protease K was fully hydrolyzed even after 1 h of incubation with native milin (Fig. 8b). Low-molecular-weight continuous bands

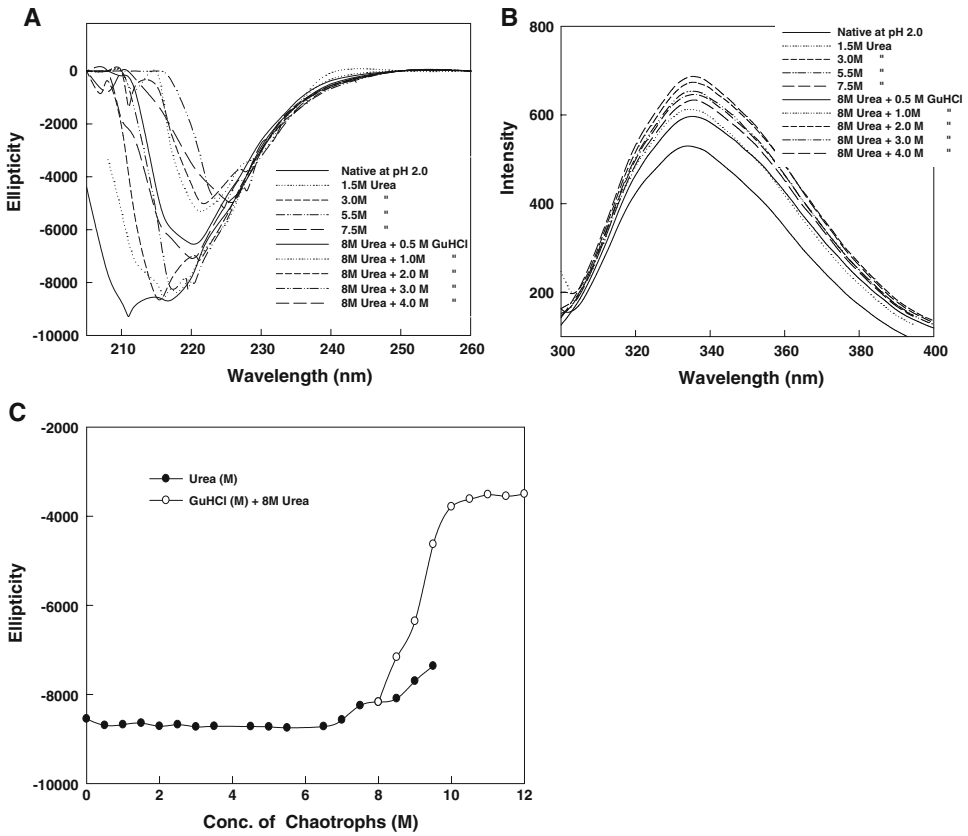


Fig. 7 GuHCl denaturation of milin in presence of 8 M urea at pH 2.0: **a** far-UV CD scan, **b** far-UV CD transition, and **c** fluorescence scan. Above 8 M urea different molarity of GuHCl was used. All parameters and concentration of protein are as described in the "Materials and methods" section

on SDS-PAGE gel were observed, resulting from trypsin and chymotrypsin proteolytic hydrolysis by milin (Fig. 8b). The multiple bands of native milin on SDS-PAGE were due to monomer at 32 kDa (two bands due to differentially glycosylation), kinetically stable nature at 52 kDa (Yadav et al. 2009), and dimer at 64 kDa, respectively. The absence of low-molecular-weight continuous bands for each protease solution as well as for milin excludes the possibility of autodigestion in each case (Fig. 8a, lanes 1, 2, T, C, and P).

Discussion

The high stability of proteins from xerophytes plants, similar to thermopile bacteria, is of great interest because it can provide the basis for understanding alternate routes to attain such stability in plants (Jaenicke and Bohm 1998; Ladenstein and Antranikian 1998; Li et al. 2005). We have established the kinetic stability of differentially glycosylated dimeric serine protease milin originating from xerophytes plant (Yadav et al. 2009). This type of metastability appears to be a consequence of evolutionary pressure to stabilize the protein native state under adverse conditions (Manning and Colon 2004).

Acidic pH induces stable molten globule state

The similar near-UV CD, far-UV CD, and fluorescence spectra of milin at pH 1.0–11.0 along with the loss of activity at acidic pH (pH 2.0) reveals the intermediate molten globule state. The negligible change in the shape of the near-UV CD scan with similar intensity signifies negligible perturbation of the aromatic amino acid π-electron delocalization. This reflects the lack of reduction in the rigidity of milin to a certain extent at acidic pH. For the 300 nm peaks, only tryptophan residue contributes to the CD spectrum of protein, while tyrosine and phenyl alanine residue do not (Strickland 1974). The increase of intensity of the 300 nm peak at pH 11.0 reveals the π-electron delocalization of the tryptophan residues. Near UV CD spectra provide a measure of the level of interaction of the side-chain aromatic ring with other groups such as the side-chain amide carboxylate group and main-chain peptide bond and are applicable in screening the tertiary structure of proteins (Sears and Beychock 1973). The fluorescence emission maximum suffers a redshift when chromophores become exposed to polar solvent, and the quantum yield of fluorescence decreases when the chromophore interacts with quenching agents in either a solvent or in the protein itself, or due to configurational arrangement of amino acid

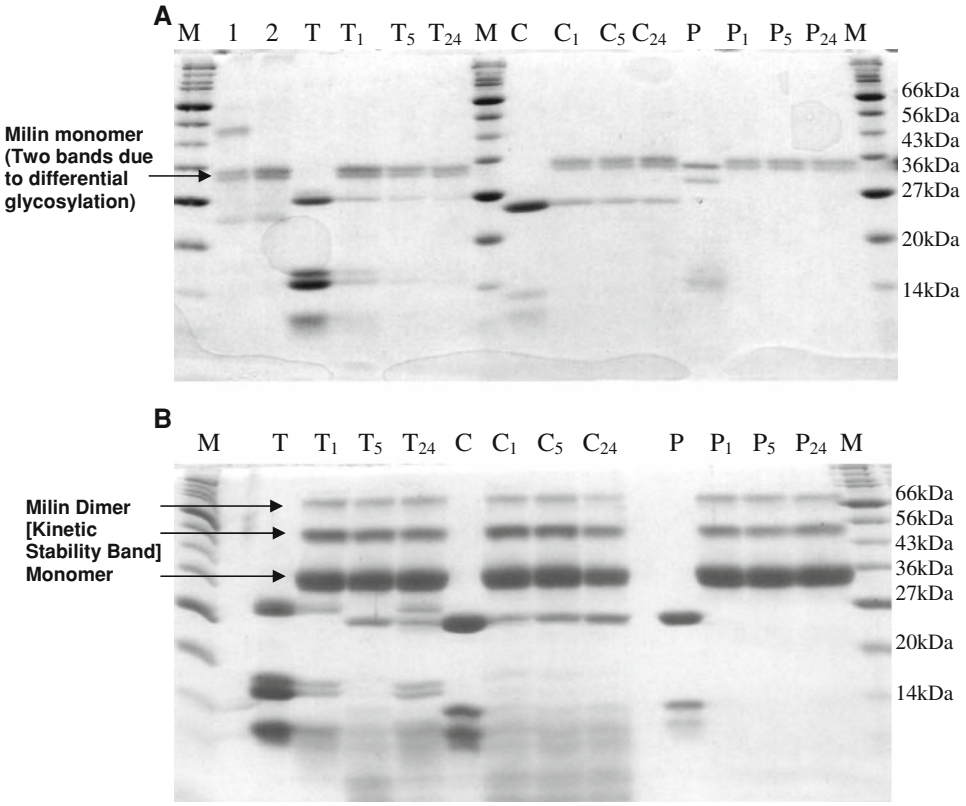


Fig. 8 Proteolytic stability of milin. Protein milin was incubated at 37°C for 1, 5 and 24 h with a 10:1 (molar concentration) ratio of milin [denatured or native:protease (trypsin, chymotrypsin or protease K)], followed by SDS-PAGE (20%) analysis. Protease-digested samples were heated with SDS for 95°C for 5 min prior to loading. **a** Denatured milin. Milin was denatured by heating at 140°C for 30 min (Yadav et al. 2009) and incubated with the proteases for the desired time. Lanes with molecular weight marker of 66, 56, 43, 36, 27, 20, and 14 kDa are labeled *M*. Control lane with no proteases is labeled *1* for native (unheated) and *2* for denatured milin. *Lanes T*, *C*, and *P* contain trypsin, chymotrypsin, and protease K, respectively. Subscript numbers after protease initial letters indicate incubation time in hours with the corresponding protease. Twenty micrograms of protease and protease digest were loaded onto the SDS-PAGE gel. The two bands for milin at 31 and 32 kDa are due to differential glycosylation (Yadav et al. 2009). **b** Native milin. A similar experiment as for denatured milin was carried out with active milin to explore the proteolytic action of milin. The initials used have similar meanings as in Fig. 8a. Twenty-five micrograms of protease and protease digest were loaded onto the SDS-PAGE gel. The lower continuous bands of protease digest confirm the proteolytic action of milin on these proteases. The multiple bands for milin at 32.0, 52.0, and 68 kDa are due to monomer, kinetically stable nature of monomers, and dimer, respectively (Yadav et al. 2009)

side-chain (Cairoli et al. 1994; Halfman and Nishida 1971; Lametti et al. 1995; Ptitsyn 2002). The similar fluorescence spectrum over the entire pH range reveals the lack of exposure of tryptophan residue to the polar solvent. The similar far-UV CD spectra of milin in the pH range 1.0–11.0 are indicative of a highly compact structural organization.

Milin is highly stable against thermal denaturation like kinetically stable proteins

The glycosylated dimeric plant serine protease milin retains its full conformational and functional stability against temperature-induced transition at neutral pH (Yadav et al. 2009). However, the stability of milin is slightly reduced against temperature at partial molten globule state at pH 2.0. Molten globule states of proteins are known to be more sensitive to thermal unfolding, and most thermodynamically stable proteins are unfolded completely. However, the kinetically stable protein milin retains its full conformational stability under such conditions. The minor reduction of secondary structure on the temperature transition of the molten globule state of milin is due to perturbation of the compact globular structure. Exposure of proteins to low concentration of denaturants such as GuHCl resulted in folding destabilization. We have found that 1.6 M GuHCl is the maximum concentration at which milin has similar molten globule state like spectroscopic characteristic at pH 2.0 (data not shown). The temperature-induced unfolding under such conditions resulted in a 50% reduction of secondary-structure contents. This is due to the cumulative effect of acidic pH and mild denaturant on the unfolding of milin. The greater resistance to unfolding reveals the high stability of intermediate GuHCl-triggered acid-induced molten globule state of milin. The decrease of fluorescence

intensity is due to local configurational rearrangement of the amino acids in the protein (Yadav et al. 2009). The gradual decrease of the transition start point as a function of pH/chaotrops is indicative of the highly compact structural integrity of the protein molecule (Figs. 3, 4). The unfolding of milin has been found to be reversible below 70°C under all the above-mentioned experimental conditions. This incomplete denaturation of milin and full peptidase activity suggest the conformational elasticity of the molecule below 70°C (Yadav et al. 2006). The effective light scattering above 80°C at pH 4.0–7.0 reveals the formation of soluble aggregates. Thus it is confirmed that the structural and functional integrity of the kinetically stable protein milin, like other proteins, is largely dependent on ionic networks, packing efficiency through van der Waal interactions, loop stabilization, and oligomerization (Berezovsky and Shakhnovich 2005; Jaenicke 1991a; Karshikoff and Ladenstein 2001; Szilagyi and Zavodszky 2000; Yadav et al. 2009). This is because heat increases the intrinsic rotational and vibrational energy of the proteins, thus disrupting the delicate balance among the weak interactions that stabilize the folded conformation of the protein (Parthasarathy and Murthy 2000).

The calorimetric method provides direct energetic description of protein thermal stability (Privalov 1979). Most kinetically stable proteins show irreversible denaturation reaction (Xia et al. 2007). Heat-induced unfolding of milin follows a similar reaction mechanism, consisting of a destabilization equilibrium followed by irreversible denaturation reaction. We observed high thermal stability of milin at basic pH in comparison with neutral and acidic pH (Yadav et al. 2009). The incomplete transitions at pH 7.0–10.0 reveal higher thermostability and free energy of milin than at acidic pH. The exact melting temperature could not be determined due to incomplete transition, but the apparent T_m seems to be $\geq$100°C. However, the gradual decrease of apparent T_m towards more basic pH reveals the greater destabilization of milin. These results also support the incomplete thermal transition, as probed by CD and fluorescence (data not shown). The high structural and molecular integrity and compactness with protective glycosylation helps to maintain the high free-energy kinetic barriers to destabilization of the protein milin. Interestingly, the enthalpy at 100°C gradually increases from pH 7.0 to 10.0. This could be due to ionic stabilization, glycosylation, as well as greater solubility of the milin at more basic pH than at neutral pH. The solubility of the protein milin decreases drastically upon heating at high concentration (1 mg/ml), resulting in intensive aggregation. Moreover, high concentration of protein as well as pH near the isoelectric point may also lead to difficulties arising from aggregation of the denatured protein or possibly self-association of native state. This could be another reason for the precipitation of protein milin in the pH range 4.0–6.0 (Yadav et al. 2009).

Stability against urea illustrates the kinetic stability of milin

The high rigidity of proteins is due to the absence of bulk water, which reduces the energetic driving force for partial and global unfolding (Partridge et al. 1999). Under normal conditions, milin has a compact surface through which bulk water cannot penetrate to induce local and global destabilization (Machius et al. 2003). Total 30% reduction in activity with 8 M urea at neutral pH represents an intermediate state of exposed hydrophobic surface (Yadav et al. 2006). This small reduction in activity is probably due to the solvent effect of urea at such a high concentration as well as due to minor perturbations in surface structure. The strong resistance to unfolding of milin by urea at acidic pH is due to high rigidity and the lack of a weak point on its surface to enter the molecule (Manning and Colon 2004). However, mildly acidic pH (4.0) affects the global perturbation only by inducing configurational arrangement of amino acid side-chain (Parthasarathy and Murthy 2000). This is also observed as a mild reduction of fluorescence intensity (Fig. 4). Under extremely acidic conditions (pH 2.0), at which milin behaves as a molten globule state, more perturbation of the rigidity of the molecule is exhibited (Kurzban et al. 1991). This is why urea-induced unfolding is more effective at highly acidic pH (Fig. 5).

The unfolding mechanism of GuHCl is different from that of urea. The mixed denaturation experiment was designed to investigate the cumulative effect of increasing concentration of GuHCl in presence of 8 M urea on the acid-induced (pH 2.0) molten globule state of milin. The secondary-structure content of milin is drastically reduced by GuHCl-induced mixed denaturation. However, the negligible shift of fluorescence wavelength maxima reveals the independent secondary-structure layer organization. This experimental observation also confirms the kinetically stable nature of protein, where each structural layer is independent. The stability of milin against urea seems to originate from a large kinetic barrier against unfolding, which likely arises from strategically placed ionic interactions and charge clusters.

Proteolytic resistance of milin reveals kinetic stability

Kinetic stability is a feature used by nature to allow proteins to maintain activity under harsh condition and to preserve the structure of protein that is prone to misfolding (Manning and Colon 2004). The kinetically stable protein milin is proteolytically resistant due to its amino acid composition and structural organization. Proteolytic resistance endows

these enzyme with long lives as well as stability toward intracellular and intercellular proteases (Rupley 1967). These enzymes are also helpful in the defense mechanism as well to reduce the drought effect where the plant *Euphorbia milii* generally grows. There is a very good correlation between protein thermostability and its resistance to proteolysis (Daniel et al. 1982). This would imply that kinetically stable proteins have a sufficiently different exterior surface to confer this resistance to proteolysis (Grzelczak and Lane 1984). Since protein unfolding is known to increase proteolytic susceptibility, it may be that milin has a tighter or more folded structure and thus fewer proteolytically susceptible conformational subsets (Jaswal et al. 2002). Milin also exhibits proteolysis resistance in completely denatured condition. This indicates that the amino acid sequences of milin that provide the resistance are prone to further proteolysis. These results also imply that proteolytic inertness of milin is not only due to its structural rigidity but also depends on the amino acid sequences (Fig. 8a). The glycosylation may have the effective role of protecting milin from proteolysis (which is the subject of a separate paper). Active milin (native, glycosylated, dimeric) has high specific activity (Yadav et al. 2006) and also acts on proteases to digest them (Fig. 8b). This is one of the strong characteristic of milin for its potential use in leather and detergent industries.

Many proteases have evolved kinetic stability to survive under highly proteolytic environment and temperature (Xia et al. 2007). The stability of the extracellular dimeric plant serine protease milin reveals a novel mechanism that appears to lead to longer functional lifetime for the protease. Kinetic stability of milin exhibits a highly cooperative barrier to unfolding and extreme rigidity to make it resistant to proteolytic degradation (Ohnishi and Kameyama 2001). This is likely to be a mechanism conserved among the majority of extracellular pro-proteases and may emerge as a general strategy for intracellular eukaryotic proteases subject to harsh conditions as well (Cunningham et al. 1999).

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