J. Biochem. **141**, 251–259 (2007)
doi:10.1093/jb/mvm026

Identification and Characterization of Functional Intermediates of Stem Bromelain During Urea and Guanidine Hydrochloride Unfolding

Basir Ahmad, Tabrez Anwer Shamim, Soghra Khatun Haq and Rizwan Hasan Khan*
Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh–202002, India

Received October 11, 2006; accepted December 2, 2006; published online December 15, 2006

By comparing changes in enzyme activity with changes in spectral features for stem bromelain (EC.3.4.22.32) in the absence and presence of urea, Guanidine hydrochloride and ethanol; four intermediate states could be identified: two activity-enhanced state obtained in the presence of 5 M urea and 2 M GnHCl, termed X and X′, respectively, and a third, similarly active state closely resembling the native protein in the presence of 8–9 M urea, termed Y. The enhanced activity of these states is due to local conformational changes accompanied by increased dynamics in the active site. Further, the enzyme does not lose its activity after substantial tertiary structure changes in 8–9 M urea (Y state), suggesting that active site containing domain is more resistant to chemical denaturation than the other structural domain. This makes stem bromelain and in general cysteine proteases an exception to the hypothesis that active site is the most labile part of enzyme.

Key words: circular dichroism, cysteine protease, functional intermediate state, intrinsic fluorescence, proteolytic activity.

Abbreviations: ANS, 1-anilino-8-nephthalenesulphonicacid; CD, circular dichroism; GnHCl, guanidine hydrochloride; PFI, partially folded intermediate; SB, stem bromelain.

The function of a protein depends on its ability to acquire a unique three-dimensional structure. Understanding how this process occurs is one of the great challenges are protein chemists facing world wide. Proteins are known to accumulate different conformational states during their unfolding by various denaturants (*1–3*). In order to understand the phenomenon of protein folding, all conformational states should be described with respect to their structure and function, because such conformational states might resemble the intermediate state along the *in vivo* protein-folding pathway, and thus play an important role in understanding the mechanism of protein folding (*4, 5*).

Like other cysteine proteinases, stem bromelain (SB) belongs to the $\alpha + \beta$ protein class and the highly similar amino acid sequences of papain (*6*), actinidin (*7*), proteinase Ω (*8*), chymopapain (*9*) and SB (*10*) indicate that the polypeptide chains of these proteins may share a common folding pattern. This has been substantiated for the former three proteinases by detailed X-ray diffraction studies (*10–13*). While a detailed study on the folding aspects of papain, a thiol protease, has been made by several workers (*14, 15*), very little information about the general folding aspects of SB a cysteine protease from *Ananas comosus*, is currently available. The process of thermal denaturation of bromelain, as studied by circular dichroism (CD) and differential scanning calorimetry (DSC), is completely irreversible and apparently follows a simple two-state mechanism of the type $N \rightarrow D$ (*16*). SB, when exposed to increasing alkalinity, exhibits conformational response through at least three different stages due to the ionization of tyrosine hydroxyl groups (*17*). Earlier studies from our laboratory have demonstrated the existence of a partially folded intermediate (PFI) for SB at low pH and other intermediates in the presence of salts and alcohols (*18–20*). However, chemical denaturation studies have not been reported for this protein so far.

In the present communication, we have carried out experiments to study the structural and functional properties of SB in the presence of denaturants like GnHCl, urea and ethanol and identified and characterized functional intermediates of the enzyme in its unfolding pathway. The information could be helpful in understanding the structure function relationship of SB in particular and cysteine proteases in general. Understanding the structure–function relationship of an enzyme under different solvent conditions may provide insight into the molecular basis of the stability of the enzyme, which can be used to design protocols and or an enzyme with special properties for biotechnological applications.

MATERIALS AND METHODS

Materials—SB (EC 3.4.22.32) lot no. B4882, ultra pure urea, guanidine hydrochloride (GnHCl) and 1-Anilino 8-naphthalene sulfonic acid (ANS) typeA-3125 were obtained from Sigma Chemical Co., St. Louis, USA. Ethanol was obtained from Merck, Germany. All other chemicals used were of analytical grade.

*To whom correspondence should be addressed. Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh–202 002, India. Tel: +91-571-2720388, Fax: + 91-571-2721776,
E-mail: rizwanhkhan@hotmail.com; rizwanhkhan1@yahoo.com

Methods—Spectrophotometric measurements

Protein concentration was determined spectrophotometrically using specific extinction coefficient $\varepsilon^{1\%}_{1cm}$ of 20.1 by measuring the absorbance of protein solution at 280 nm on a Hitachi U-1500 spectrophotometer (*16, 21*). A stock solution of ANS was prepared and concentration was determined using an extinction coefficient of $\varepsilon_M = 5000 M^{-1} cm^{-1}$ at 350 nm (*22*).

Denaturation Experiments—Solutions for the denaturation experiments of SB were prepared in 60 mM sodium phosphate buffer of pH 7.0. Time-dependent changes in the structural parameter and enzymatic activity of SB at increasing denaturant concentrations were monitored to standardize the incubation time required for achieving equilibrium under given conditions. At 1, 5 and 9 M urea and 0.5, 2, 4 and 6 M GnHCl, both enzyme activity and fluorescence properties become constant within 20 min. This suggests that minimum incubation time of 30–60 min is sufficient for achieving equilibrium. To a 0.5 ml stock protein solution (15 mM) in buffer, different volumes of a stock denaturant solution (10 M urea, 8 M GnHCl or 100% ethanol) were added to get a desired concentration range of denaturant. The final solution mixture (3.0 ml) was incubated for 30–40 min at room temperature before optical and activity measurements.

Enzyme Assay—The proteolytic activity of SB was assayed by the procedure of Demeester *et al.* (*23*). A denatured casein solution (2%) at pH 7.0 was incubated for 10 min at room temperature with SB solution pretreated with increasing concentration of denaturants. The reaction was stopped by adding a protein precipitation reagent (0.11 M TCA, 0.22 M sodium acetate and 0.44 M acetic acid) and the undigested casein moved by centrifugation or filtration. The amount of peptide remaining in solution was determined spectrophotometrically at 275 nm against a blank containing all the reaction mixture except activity. The data were plotted as percent activity versus denaturant concentrations

Determination of Extent of Autolysis—The extent of autolysis was measured by TCA precipitation method (*24*).

CD Measurements—CD measurements were carried out with a Jasco spectropolarimeter, model J-720, equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulfonic acid. All the CD measurements were carried out at 25°C with a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of ± 0.1°C. Far-UV CD spectra were measured at a protein concentration of 2.0 μM and near-UV CD spectra were measured at protein concentration of 20–30 μM. The pathlength was 1 mm and 1 cm respectively. Results were expressed as mean residue ellipticity (MRE) in deg $cm^2 dmol^{-1}$ defined as

$$\mathrm{MRE} = \frac{\theta_{obs}(\mathrm{mdeg})}{10 \times n \times Cp \times l}.$$

Where θ_{obs} is the CD in millidegree; n is the number of amino acid residues; l is the pathlength of the cell in cm and Cp is the molar fraction. Helical content was calculated from the MRE values at 222 nm using the following equation as described by Chen et al. (*25*):

$$\%\ \mathrm{helix} = \left[\frac{\mathrm{MRE}_{222nm} - 2340}{30300}\right] \times 100.$$

Since dynode voltage exceeded 0.6 kV ≤ 215 nm, far-UV CD spectra were recorded in the range of 250–215 nm.

Fluorescence Measurements—Fluorescence measurements were performed on Shimadzu spectrofluorimeter, model RF–540 equipped with a data recorder DR–3. The fluorescence spectra were measured at 25 ± 0.1^0 C with a 1 cm pathlength cell. The excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 295 nm and emission spectra was recorded in the range of 300–400 nm.

For ANS binding fluorescence experiments, the excitation wavelength was set at 380 nm and the emission spectra were taken in the range of 400–600 nm. The molar ratio of protein to ANS was 1:50. ANS fluorescence studies at pH 7.0 in the presence of urea and GnHCl were performed using a fixed protein concentration (2.0 μM).

Acrylamide Quenching Experiments—In the quenching experiments, aliquots of 5 M acrylamide stock solution were added to protein solutions (3 μM) to achieve the desired range of quencher concentration (0.1–0.5 M). Excitation was set at 295 nm and the emission spectrum was recorded in the range 300–400 nm. The decrease in fluorescence intensity at λ_{max} was analysed according to the Stern-Volmer equation (*26*).

$$\frac{F_0}{F} = 1 + K_{sv}[Q]$$

Where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher, respectively, K_{sv} is the Stern-Volmer constant, and $[Q]$ is concentration of the quencher.

RESULTS

SB like other proteolytic enzymes, undergoes autocatalysis (*27,28*). To avoid any complication due to autolysis, active amino acid side chain is modified during folding/unfolding studies e.g. in case of cysteine proteases free sulfhydryl group of active site cysteine is blocked by caboxymethylation (*29*). This inactive enzyme cannot be native even if observed physical parameters are very similar to the active enzyme. To study the effect of various solvents on the properties of active SB, we investigated the time course of the autolysis process and enzymatic activity of SB in the absence and presence of GnHCl, urea and alcohol. No autolysis or decrease in enzyme activity was observed upto 60 min (Fig. 1, data in the presence of denaturant omitted for brevity). But the enzyme shows almost complete auto digestion in 24 h. The SDS-PAGE of stem bromelain incubated for 0–1 h in phosphate buffer of pH 7.0 also shows no digestion of the protein (data not shown).

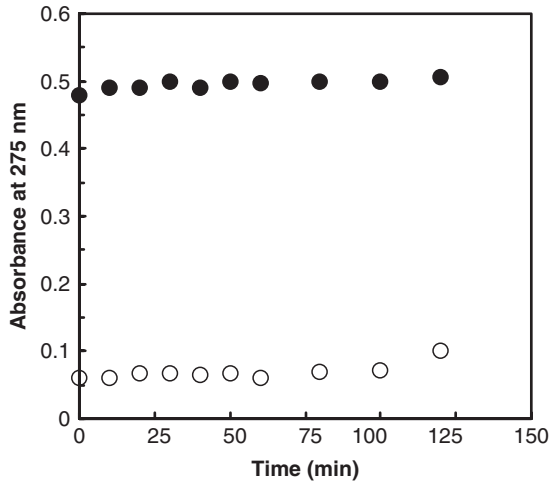

Fig. 1. **Extent of autolysis (○) and caseinolytic activity (●) of SB monitored by the time dependent changes of absorbance at 275 nm of supernatant of TCA precipitated SB.**

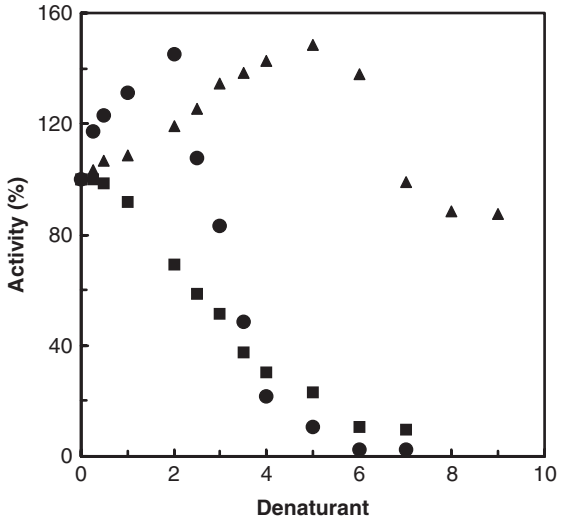

Fig. 2. **Percent caseinolytic activity of SB in the presence of increasing concentration of urea (▲), GnHCl (●) and ethanol (■).** Concentration ranges of denaturants used were 0–9.0 M for urea, 0–7.0 M for GnHCl and 0–70%(V/V) for ethanol. Activity of native SB has been taken as 100%. Each data point is the mean of 3–4 independent observations with standard deviation ±0.3–±5.1% (X-axis scale: 1 unit = 1M, 10%).

Effect of Denaturants on the Enzymic Activity of Stem Bromelain—Determination of enzyme activity along increasing denaturant concentration is a very sensitive probe to detect even small structural changes that an enzyme undergoes. Figure 2 shows the effect of increasing concentrations of urea, GnHCl and alcohol on the enzymatic activity of SB. The denaturation process of SB in the presence of increasing concentration of urea and GnHCl (see profiles in Fig. 2) is a combination of at least two distinct processes. The first transition (upto 2 M GnHCl and 5 M urea) caused an increase in the enzyme activity by ~46%. Further increase in urea concentration upto 9 M resulted in the decrease of enzyme activity by a mere ~13%. No significant change in activity of SB even in denaturing concentration of urea is indicative of its structural stability in concentrated urea solution. On the other hand, a sharp decrease in the enzyme activity above 2 M and finally a complete loss of activity was observed at 6 M GnHCl. As shown in the figure, denaturation profile of SB by ethanol is markedly different from urea and GnHCl. A steady decrease in enzyme activity of SB was observed on treatment with increasing concentration of ethanol. Overall, SB shows a similar change in the activity upto 5.0 M urea and 2.0 M GnHCl which is markedly different from that observed in the presence of ethanol. The most interesting feature is the exceptional retention of activity in 9 M urea.

Table 1. **Kinetic parameters of proteolytic activity of SB in the absence and presence of urea and GnHCl concentration at which enhanced activity was noted.**

Condition	Km(mM)	k_{cat} (min^{-1})
Native SB (pH 7.0)	0.129 ± 0.08	703 ± 21.8
SB + 5.0 M urea	0.217 ± 0.03	1040 ± 42.8
SB + 2.0 M GnHCl	0.221 ± 0.07	1085 ± 31.3

To further characterize the activity-enhanced states (i.e. in 2 M GnHCl and 5 M urea), kinetic constants (Km and k_{cat}) for the enzyme in the presence and absence of denaturants were calculated. As shown in Table 1, Michaelis constant, Km and catalytic constant, k_{cat} were calculated to be 0.129 ± 0.08 mM and 703 ± 34.9 min^{-1}, respectively in the absence of denaturant. In the presence of 5 M urea, Km was 0.217 ± 0.03 mM and k_{cat} was 1040 ± 42.8 min^{-1} and in the presence of 2 M GnHCl, the Km and k_{cat} were 0.221 ± 0.07 and 1085 ± 45.6 min^{-1}, respectively. The results suggest catalytic properties of SB in the presence of 5.0 M urea and 2.0 M GnHCl are almost similar.

Effect of Denaturants on the Conformation of SB—Figure 3 shows the GnHCl, urea and ethanol induced conformational transition of SB as monitored by the measurements of MRE at 222 nm. Urea and GnHCl induced transitions of SB monitored by measurements of MRE at 222 nm are also biphasic i.e. they involve at least two distinct reactions. SB shows a small decrease in the ellipticity at 222 nm in urea concentration range from 0 to 5 M (first transition), indicating a slight increase (~4.6%) of alpha helical content. In the second transition of urea denaturation profile, ellipticity at 222 nm increases and become equal to native protein. This reflects that the enzyme is structurally stable with no loss in proteolytic activity even in 9 M urea. GnHCl denaturation profile of SB is a two-step process with accumulation of an intermediate at around 2–2.5 M GnHCl when monitored by ellipticity measurements at 222 nm. The first transition (0.5–2 M GnHCl) is characterized by small reduction of helical content (~10.3%), while the second transition (2.5–6 M GnHCl) is accompanied by complete loss of secondary structure and activity. Small decrease in the ellipticity at 222 nm was observed in increasing concentration of ethanol. Thus the inactivation of SB in ethanol without affecting secondary

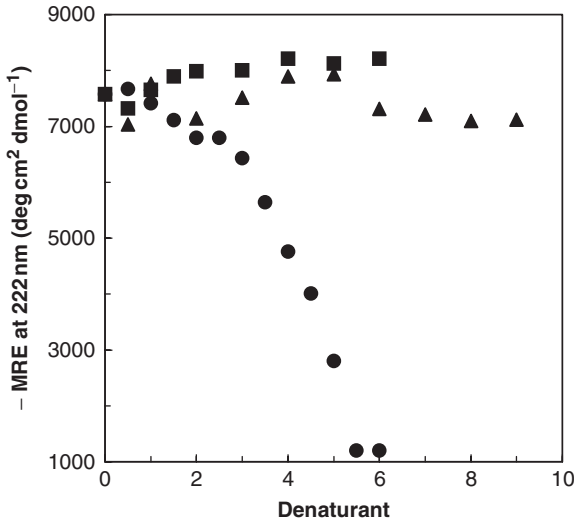

Fig. 3. **Urea (▲), GnHCl (●) and ethanol (■) induced denaturation profiles of SB as monitored by MRE measurements at 222 nm.** Concentration ranges of denaturants used were 0–9 M for urea, 0–7 M for GnHCl and 0–70% (v/v) for ethanol. Each data point is the mean of 3–4 independent observations (X-axis scale: 1 unit = 1 M, 10%).

structure indicates that alcohol denature the SB by different mechanism than urea and GnHCl.

SB contains five tryptophan (Trp) residues distributed in the entire polypeptide chain (*10*). The emission spectra of Trp residues were measured after excitation at 296 nm. This excitation wavelength was selected to minimize radiation less energy-transfer from the tyrosine residues, which can contribute to tryptophan emission when an excitation wavelength of 280 nm is used (*30*). Figures 4A and B exhibit the GnHCl, urea and ethanol induced conformational transitions of SB as measured by intrinsic fluorescence emission intensity at 342 nm and wavelength maximum (λ_{max}) of emission, respectively. As shown in figure, SB showed significant increase in tryptophanyl fluorescence intensity between 0 and 5 M urea and 0–2 M GnHCl with a red shift of the emission maximum from 342 to 346 nm. From 6 to 9 M urea no significant change in fluorescence intensity was observed but the λ_{max} of emission shifted to longer wavelength by 3 nm (346–349 nm). Between 2 and 5 M GnHCl, a transition occurred which resulted in significant decrease in fluorescence intensity and a change of the λ_{max} of emission to 355 nm. These changes in fluorescence properties (from 2 to 6 M GnHCl) together with the observed abrupt decrease in enzymatic activity (Fig. 2) and secondary structure content (Fig. 3) indicated that unfolding of SB occurred in this range of GnHCl concentrations.

As seen in Figs 3, 4A and B, changes in the MRE at 222 nm and fluorescence properties upon addition of urea or GnHCl to give a final concentration of 5 M or 2 M, respectively, was accompanied by a significant increase in enzyme activity. Moreover, changes in structure (changes in mean residue ellipticity at 222 nm) correlates reasonably well with the changes in enzyme activity,

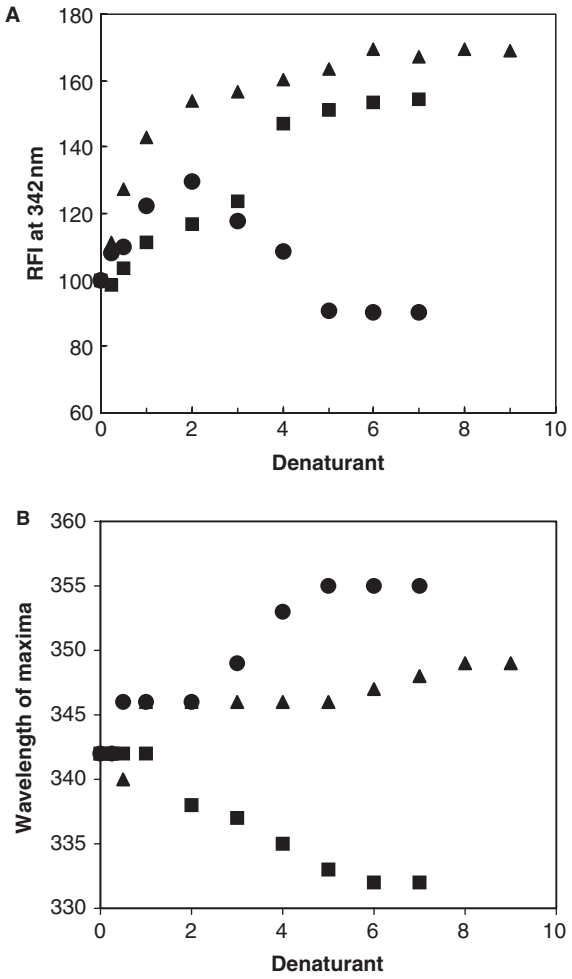

Fig. 4. **Urea (▲), GnHCl (●) and ethanol (■) induced denaturation profiles of stem bromelain as monitored by measurements of tryptophanyl fluorescence intensity (FI) at 296 nm (A) and wavelength of maximum emission (B).**

(Fig. 5A and B) therefore, these results are indicative of a conformational change in SB induced by urea or GnHCl, which is favourable for its proteolytic activity. Alcohols are known to disrupt hydrophobic intractions and hence ethanol was not able to induce this conformation. Therefore, mechanism of conformational alteration of SB by urea, GnHCl and ethanol may be represented as:

$$N \rightleftharpoons X \rightleftharpoons Y \quad \text{(Urea)}$$
$$N \rightleftharpoons X' \rightleftharpoons D \quad \text{(GnHCL)}$$
$$N \rightleftharpoons D' \quad \text{(Ethabol)}$$

Where N and D is the native and denatured states of SB, respectively. X and X′ are the conformational states of the protein with enhanced proteolytic activity. D′ is the denatured state without activity but native-like secondary structure content. To ascertain whether the protein states X, X′ and Y represented intermediate conformational states of SB, we compared the ANS fluorescence spectra, far UV CD spectra, near UV CD spectra,

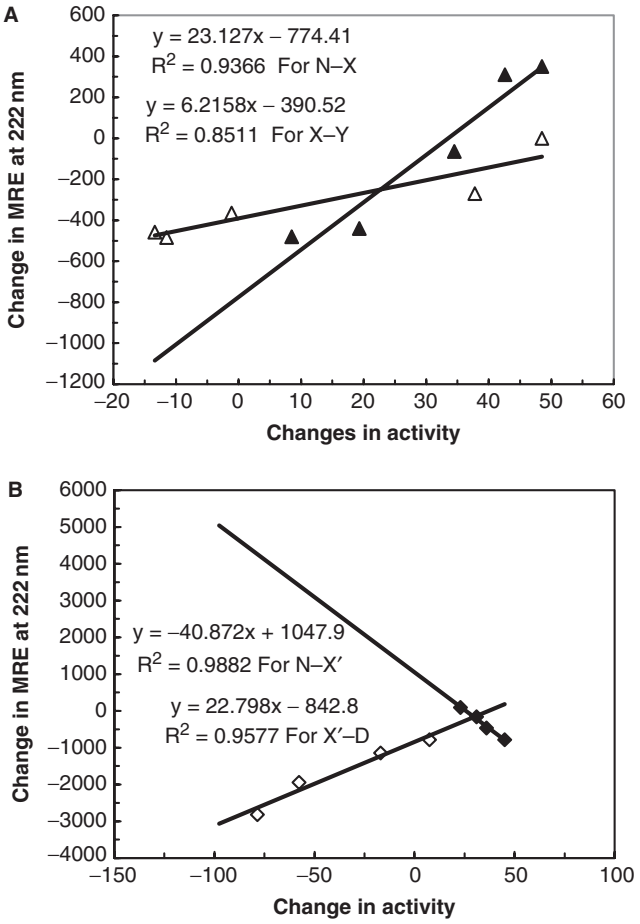

Fig. 5. **Correlation analysis between changes in MRE values and changes in activity for N–X (▲) and X–Y (△) transitions induced by urea (A) and N–X′(■) and X′–D (□) transitions induced by GnHCl (B) of S.**

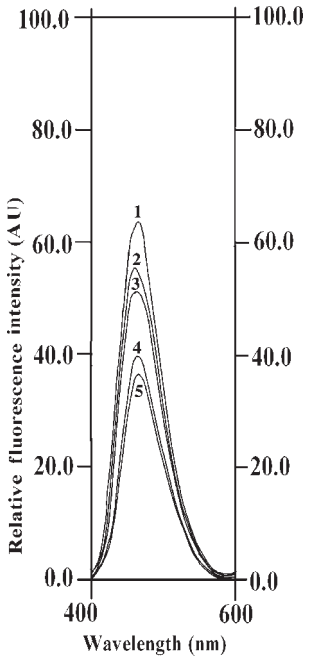

Fig. 6. **Fluorescence emission spectra of ANS bound to Native state at pH 7.0 (curve1), X state in 5.0 M urea (curve 2), X′ state in 2.0 M GnHCl (curve 3), Y state (curve 4), and unfolded state in 7.0 M GnHCl (curve 6).**

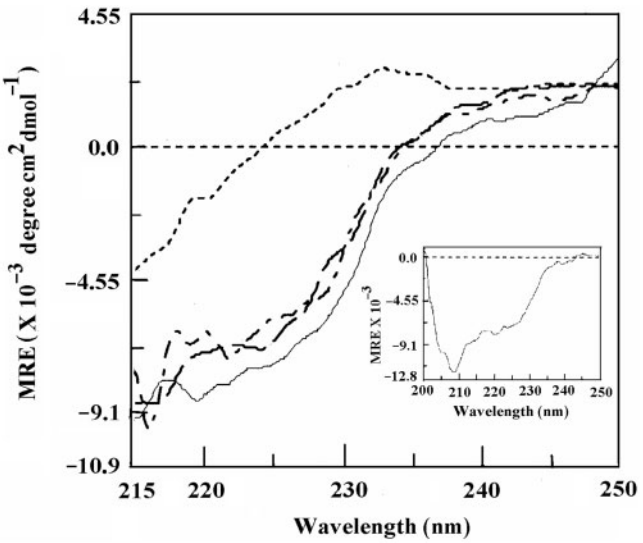

Fig. 7. **Far-UV CD spectra of different states of SB. Native state at pH 7.0 (inset), X state in 5.0 M urea (solid line), X′ state in 2.0 M GnHCl (dashed line), Y state (dashed-dotted) and unfolded state (dotted).**

intrinsic fluorescence spectra and tryptophanyl fluorescence quenching of these states with those obtained at pH 7.0 (native state) and in the GnHCl denatured states

ANS Binding—Changes in the ANS fluorescence are generally used to detect non-native conformation of globular proteins (*31*). The hydrophobic fluorescent dye, ANS was used to probe X, X′ and Y conformations of SB for the exposure of hydrophobic surfaces. Figure 6 showed the ANS fluorescence spectra of N, X, X′, Y and D states. Maximum binding of ANS to N state showed that N state had sizeable amount of exposed hydrophobic region compared with D state. Changes in binding of ANS to X and X′ state were comparable with N state and are equal. This indicates that both states are similar and may have been acquired through similar mechanism. Although binding of ANS to Y state has decreased significantly, it is higher than the completely unfolded state (D). This is indicative of a conformation intermediate to N and D states.

Far UV Circular Dichroism—Figure 7 shows the far-UV CD spectra of functional intermediates of SB obtained by treatment of the protein with 5 M urea (X state, curve 1), 9 M urea (Y state, curve 2) and 2.0 M GnHCl (X′ state, curve 3). Curve 4 shows the spectrum of SB in 7 M GnHCl, a concentration at which the protein is considered to exist in a random coil conformation (*30*). The far-UV CD spectrum of native SB (Fig. 7 inset) is typical of $\alpha+\beta$ proteins, the CD signal of which is more intense at 208 nm than at 222 nm (*32*). The curves for X, Y and X′ states retained the elements

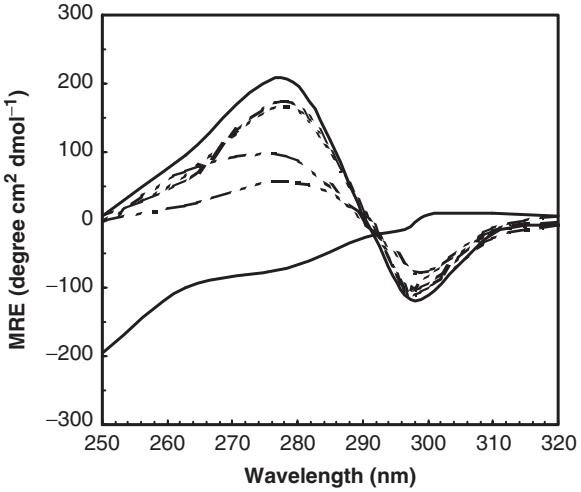

Fig. 8. **Near-UV CD spectra of different states of stem bromelain.** Native state at pH 7.0 (—), X state in 5.0 M urea (– –), X′ state in 2.0M GnHCl (- - -), Y state (– · –), ethanol induced state (–··–) and unfolded state (—). Curves are numbered 1–6 in this order as the arrow indicates.

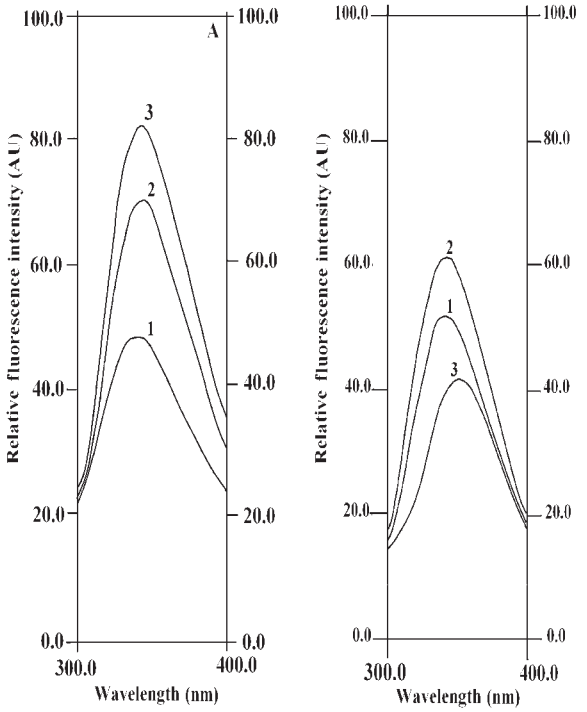

Fig. 9. **Tryptophanyl fluorescence spectra of different states of SB.** (A) Native state at pH 7.0 (curve1), X state in 5.0 M urea (curve 2), Y state in 9.0 M urea (curve 3), (B) Native state at pH 7.0 (curve1) X′ state in 2.0 M GnHCl (curve 2), and unfolded state in 7.0 M GnHCl (curve 3).

of secondary structure, although there was a decrease in the ellipticity at 222 nm of X state, indicating a gain of helical structure (~4.6%) from native SB. On the other hand Y and X′ states showed small loss of helices (~10%) compared with native state. SB in the presence of 7 M GnHCl lost all the elements of secondary structure as well as proteolytic activity and hence was assumed to represent the completely unfolded state of SB.

Near UV Circular Dichroism—Figure 8 shows the comparative near-UV CD spectra in the region 320–250 nm for native (pH 7.0, curve1), X (5 M urea, curve2), Y (9 M urea,curve 3), X′ (2 M GnHCl, curve 4) and D (7 M GnHCl, curve 5) states of SB. These spectra in near-UV region were used to probe the asymmetry of the aromatic amino acid's environments in SB. The spectrum of native SB exhibits a negative band at 298 nm and a positive band at 279 nm. The spectrum is very similar to spectrum reported earlier by Arroyo-Reyna *et al*. (*32*). In the X and X′ states of SB, there was a small loss of signal at 279 nm and 298 nm whereas, in 7 M GnHCl there was complete disappearance of all minima. A comparison of near-UV CD spectra of all the three functional states (X, X′ and Y) with that of native and denatured states show that X and X′ states have lost small amount of tertiary structure while Y state has lost most of the tertiary structure. Moreover Y state has native like activity and secondary structure. These results indicate that loss of tertiary structure in $X \rightleftharpoons Y$ transition has not affected the domain containing active site. This leads us to suggest that perhaps only specific tertiary contacts rather than the entire 3-dimensional conformation is essential for enzyme activity of SB.

Tryptophanyl Fluorescence—The intrinsic fluorescence spectra of SB in the native state at pH 7.0, in the presence of 5 M urea and 9 M urea and in the presence of 2 and 6 M GnHCl are shown in Fig. 9A and B respectively. The emission spectrum of native SB at pH 7.0 showed a maximum at 342 nm. The emission maximum shifted to shorter wavelength compared with the emission spectrum of solvent exposed NATA (356 nm, data not shown). This indicates that some tryptophanyl residues are located in hydrophobic environment. Extensive sequence homology with papain suggested that three of the five Trp in SB may be buried in the hydrophobic core and the remaining two may be located on the surface of the protein molecule. There were significant changes in the intrinsic fluorescence spectra of both urea induced (X) and GnHCl induced (X′) states when compared with the spectra of the native protein and denatured protein. Both states showed increased fluorescence intensity and λ_{max} of emission compared with native state but have smaller λ_{max} of emission than the D state. This indicates that fluorophores are in slightly more polar environment in X and X′ conformations.

Tryptophanyl Fluorescence Quenching by Acrylamide—The exposure of the tryptophanyl residues was further examined by fluorescence quenching using neutral quencher, acrylamide (*26*). Figure 10 shows Stern–Volmer plots for fluorescence quenching of SB by acrylamide at different denaturant concentrations. Values of stern–volmer constant (Ksv) are shown in Table 2. Ksv values of SB at 5 M urea (6.09) or 2 M GnHCl (6.07) were quite similar but higher than that of native state (4.82) and quenching was accompanied by a red shift in λ_{max} of emission. These results indicate that tryptophanyl residues of X and X′ states were more

accessible to quencher than the N state. The Ksv value of the denatured SB (6 M GnHCl) was significantly higher than those for the denaturant induced states, suggesting that in X, Y and X′ states, the tryptophanyl residues were not fully accessible and have intermediate exposure between native and denatured states.

Taken together these results i.e. enzymatic activity, intrinsic fluorescence, ANS binding and fluorescence quenching, far UV CD suggest that both the state X and X′ are similar in tertiary structure but secondary structure differ slightly. This again provides evidence that both the states were achieved through similar mechanism.

DISCUSSION

There is a well-known relationship between protein structure and function. A unique 3-dimensional conformation in a protein is a prerequisite for its desired function. In this study an attempt has been made to gain insight into the structure–function relationship of SB in particular and cysteine proteases in general in view of its behaviour towards urea, GnHCl and ethanol. During unfolding by various denaturants four intermediate conformations have been identified between native SB and completely denatured (7M GnHCl) SB. Two intermediate conformations with enhanced proteolytic activity (X and X′) have been identified at 5 M urea and 2 M GnHCl, respectively and a third retaining native-like activity at 9 M urea. One the other hand, a fourth intermediate (D′) stabilized in 60–70% ethanol was found to be non-functional, but retained all the elements of secondary structure. This intermediate was used for comparison purpose in further discussions.

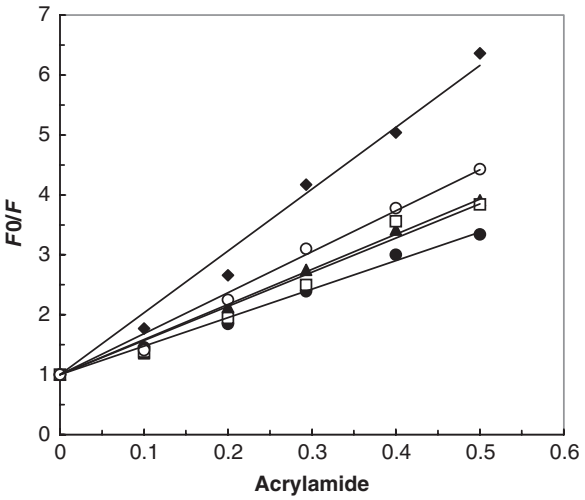

Fig. 10. **Stern–Volmer plots of acrylamide quenching.** Native state at pH 7.0 (●), X state in 5.0 M urea (▲), X′ state in 2.0 M GnHCl (□), Y state (○), and unfolded state in 7.0 M GnHCl (◆).

As shown in Tables I and II, the X and X′ states have been characterized by (i) retention of native-like secondary structure (ii) increased wavelength of maximum emission (λ_{max}) of intrinsic fluorescence (iii) increased acrylamide quenching of Trp fluorescence (iv) changes in near UV CD in aromatic region (v) substantial ANS binding and (vi) increased Km value. These observations indicate that N–X and X′ transitions is accompanied by partial exposure of buried Trp, probably located close to active site of the enzyme. Thus the enhanced activity of these intermediates (X and X′) seems to be due to conformational and dynamic changes in the SB active site. This agrees with previously reported finding that activation of different enzymes by denaturants was associated with conformational changes in the tertiary structure (*33–36*) and secondary structure (*37*) of the enzymes, or the dynamics of enzyme active site (*38*).

Third functional intermediate (Y) formed in 8–9 M urea has interesting properties that will help in understanding the stability and environment of active site of SB. The Y state has been characterized by retention of native like MRE at 222 nm and proteolytic activity but has undergone significant tertiary structure alterations. This marked decrease of tertiary structure without any major effect on the enzyme activity might be ascribed to the conformational changes of SB without affecting local environment of the active site. The pronounced tertiary structure destabilization of SB in 8–9 M urea and the unaffected local active site stability explains the stability shown by other cysteine proteases in concentrated urea solution (*39*). These observations make the cysteine proteases an exception to the hypothesis that the active site is the most labile part of the enzymes (*40*). X-ray crystallography or solution NMR studies of these states would help to determine the essential tertiary contacts required to contain the active site of this enzyme.

Table 2. **Summary of some structural and functional properties of HSA.**

Variables	Different conformational states of stem bromelaine					
	N	X	X′	Y	D	D′
% activity	100	148.5	145	88.5	9.5	2.6
[a]MRE at 222 nm	−7580	−7930	−6794	−7122	−1203	−8211
[a]MRE at 278 nm	207	174	165	93	−70	56
λ_{max} of emission	342	346	346	349	355	332
FI at 342 nm (%)	100	163.6	129.8	169.6	90.2	153.6
ANS fluorescence (relative intensity)	65	58	55	38	37	–
K_{SV}	4.75	5.69	5.85	6.80	10.32	–

N, X, X′,Y, D and D′ have been defined in text.
[a]deg. $cm^2\,dmo^{l-1}$.

CONCLUSION

On the basis of the results obtained in this study it is clear that SB unfolds through functional intermediate states (FIS). The accumulation of the FIS during urea and GnHCl induced unfolding appears to be achieved through similar mechanism, mainly by disruption of tertiary contacts. For the first time, some generalization for the unusual stability of the active site of cysteine proteases of papain superfamily can be drawn. This class of proteins unfolds through FIS, and the domain containing active site is more resistant to denaturants like urea and GnHCl.

Cysteine proteases owing to exceptional stability of their active site could be used as a model to design the enzymes that are resistant to denaturation or engineering the enzyme with enhanced activity.

Facilities provided by Aligarh Muslim University is gratefully acknowledged. This work is submitted for DBT for getting Bioscience award (2005). S.K.H. and B.A. acknowledge Council of Scientific and Industrial Research for financial assistance in the form of Senior Research Fellowships. T.A.S. thank Department of Biotechnology, India for previous studentships.

REFERENCES

1. Fink, A.L., Oberg, K.A., and Seshadri, S. (1997) Discrete intermediates versus molten globule model for protein folding: characterization of partially folded intermediates of apomyoglobin. *Fold. Des.* **3**, 19–25
2. Privalove, P.L. (1996) Intermediate states in protein folding. *Mol. Biol.* **258**, 707–725
3. Baldwin, R.L. (1996) On-pathway versus off-pathway folding intermediates. *Fold. Des.* **1**, 1–8
4. Arai, M. and Kuwajima, K. (2000) Role of the molten globule state in protein folding. *Adv. Protein. Chem.* **53**, 209–271
5. Schartz, G. (1993) The protein import machinery of mitochondria. *Protein Sci.* **2**, 141–146
6. Cohen, L.W., Coghlan, V.M., and Dihel, L.C. (1986) Cloning and sequencing of papain-encoding cDNA. *Gene* **48**, 21–227
7. Carne, A. and Moore, C.H. (1978) The amino acid sequence of the tryptic peptides from actinidin, a proteolytic enzyme from the fruit of Actinidia chinensis. *Biochem. J.* **173**, 73–83
8. Dubois, T., Kleinschmidt, T., Schnek, A.G., Looze, Y., and Braunitzer, G. (1988) The thiol proteinases from the latex of Carica papaya L. III. The primary structure of proteinase omega. *Biol. Chem. Hoppe-Seyler* **369**, 741–754
9. Watson, D.C., Yaguchi, M., and Lynn, K.R. (1990) The amino acid sequence of chymopapain from Carica papaya. *Biochem. J.* **266**, 75–81
10. Ritonja, A., Rowan, A.D., Buttle, D.J., Rawlings, N.D., Turk, V., and Barett, A.J. (1989) Stem bromelain: amino acid sequence and implications for weak binding of cystatin. *FEBS Lett.* **247**, 419–424
11. Kamphuis, I.G., Kalk, K.H., Swarte, M.B.A., and Drenth, J. (1984) Structure of papain refined at 1.5 AÊ resolution. *J. Mol. Biol.* **179**, 233–257
12. Baker, E.N. (1980) Structure of actinidin after refinement at 1.7 AÊ resolution. *J. Mol. Biol.* **141**, 441–484
13. Pickersgill, R.W., Sumner, I.G., and Goodenough, P.W. (1990) Preliminary crystallographic data for protease omega. *Eur. J. Biochem.* **190**, 443–449
14. Mendiola, S.S., Dom_ýnguez, A.R., and Arana, A.H. (1993) Cooperativity in the unfolding transitions of cysteine proteinases. Calorimetric study of the heat denaturation of chymopapain and papain. *Biochim. Biophys. Acta.* **1203**, 121–125
15. Hernandez-Arana, A. and Soriano-Garcia, M. (1988) Detection and characterization by circular dichroism of a stable intermediate state formed in the thermal unfolding of papain. *Biochim. Biophys. Acta.* **954**, 170–175
16. Arroyo-Reyna, A. and Hernandez-Arana, A. (1995) The thermal denaturaton of stem bromelain is consistent with an irreversible two-state model. *Biochem. Biophys. Acta.* **1248**, 123–128
17. Murachi, T. and Yamazaki, M. (1970) Changes in conformation and enzymatic activity of stem bromelain in alkaline media. *Biochemistry* **9**, 1935–1938
18. Haq, S.K., Rasheedi, S., and Khan, R.H. (2002) Characterization of partially folded intermediate of stem bromelaine at low pH. *Eur. J. Biochem.* **269**, 47–52
19. Haq, S.K., Rasheedi, S., Sharma, P., Ahmad, B., and Khan, R.H. (2005) Influence of salts and alcohols on the conformation of partially folded state of stem bromelain at low pH. *Int. J. Biochem. Cell. Biol.* **37**, 361–374
20. Ahmad, B., Ansari, M.A., Sen, P., and Khan, R.H. (2006) Low versus high molecular weight poly ethylene glycol induced states of stem bromelain at low pH: stabilization of molten globule and unfolded states. *Biopolymers* **81**, 350–359
21. Vanhoof, G. and Cooreman, W.B. (1997) *Pharmaceutical Enzymes* (Lauwers, A. and Scharpe, S. eds) Marcel Dekker Inc., New York
22. Khurana, R. and Udgaonkar, J.B. (1994) Equilibrium unfolding studies of barstar: evidence for an alternative conformation which resembles a molten globule. *Biochemistry* **33**, 106–115
23. Demeester, J., Dekeyser, P.M., Samyn, N., Sierens, W., and lauwers, A. (1997) Bromelain in *Pharmaceutical Enzymes* (Lauwers, A. and Scharpe, S. eds) pp. 365–368s, Marcel Dekker Inc., New York
24. Jiang, L., He, L., and Fountoulakis, M. (2004) Comparison of protein precipitation method for sample preparation prior to proteomic analysis. *J. Chromatogr. A.* **1023**, 317–320
25. Chen, Y.H., Yang, J.T., and Martinez, H. (1972) Determination of the secondary structure of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* **11**, 4120–4131
26. Eftink, M.R. and Ghiron, C.A. (1982) Fluorescence quenching studies with proteins. *Anal. Biochem.* **114**, 199–227
27. Determann, H., Jaworek, D., and Kotitscks, A.W. (1969) Products from the autolytic decomposition of pepsin. *Hoppe. Seylers. Z. Physiol. Chem.* **350**, 379–388
28. Rajagopalan, T.G., Moore, S., and Stein, W.H. (1966) Pepsin from pepsinogen. Preparation and properties. *J. Biol. Chem.* **242**, 4940–4950
29. Sharpira, E. and Arnon, R. (1969) Cleavage of one specific disulfide bond in papain. *J. Biol. Chem.* **244**, 4989–4994
30. Tanford, C. (1970) Protein denaturation, Theoretical models for the mechanism of denaturation. *Adv. Protein. Chem.* **24**, 1–95
31. Semisotonov, G.V., Rodionova, N.A., Razgulyaev, O.I., Uversky, V.N., Gripas, A.F., and Gilmanshin, R.I. (1991) Study of the molten globule intermediate state by a hydrophobic fluorescent probe. *Biopolymers* **31**, 119–128
32. Arroyo-Reyna, A., Hernandez-Arana, A., and Arreguin-Espinosa, R. (1994) Circular dichroism of stem bromelain a third spectral class within the family of cysteine proteinases. *Biochem. J* **300**, 107–110
33. Wolosiuk, R.A. and Stein, M. (1990) Modulation of spinach chloroplast NADP glyceraldehyde-3-phosphate dehydrogenase by chaotropic anions. *Arch. Biochem. Biophy* **279**, 70–77

34. Fan, Y.-X., Ju, M., Zhou, J.-M., and Tsou, C.-L. (1996) Activation of chicken liver dihydrofolate reductase by urea and guanidine hydrochloride is accompanied by conformational change at the active site. *Biochem. J.* **315**, 97–102
35. Narayanasami, R., Nishimura, J.S., McMillan, K., Roman, L.J., Shea, T.M., Robida, A.M., Horowitz, P.M., and Masters, B.S.S. (1997) The influence of chaotropic reagents on neuronal nitric oxide synthase and its flavoprotein module. Urea and guanidine hydrochloride stimulate NADPH-cytochrome *c* reductase activity of both proteins. *Nitric Oxide* **1**, 39–49
36. Das, M. and Dasgupta, D. (1998) Enhancement of transcriptional activity of T7 RNA polymerase by guanidine hydrochloride. *FEBS Lett* **427**, 337–340
37. Inui, T., Ohkubo, T., Urade, Y., and Hayaishi, O. (1999) Enhancement of lipocalin-type prostaglandin D synthase enzyme activity by guanidine hydrochloride. *Biochem. Biophys. Res. Commun.* **266**, 641–646
38. Jaenicke, R. and Böhm, G. (2001) Thermostability of proteins from *Thermotoga maritima*. *Methods Enzymol.* **334**, 438–469
39. Dubey, V.K. and Jagannadham, M.V. (2003) Difference in unfolding of procerain induced by pH, guanidine hydrochloride, urea and temperature. *Biochemistry* **42**, 12287–12297
40. Shoichet, B.K., Baase, W.A., Kuroki, R., and Matthews, B.W. (1995) A relationship between protein stability and protein function. *Proc. Natl. Acad. Sci. USA.* **92**, 452–456