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Studies on the Acid Unfolded and Molten Globule States of Catalytically Active Stem Bromelain: A Comparison with Catalytically Inactive Form

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We report the accumulation of an acid unfolded (UA) state and a molten globule (MG) state in the acid induced unfolding pathway of unmodified preparation of stem bromelain (SB) [EC 3.4.22.32], a cystein protease from *Ananas cosmosus*. The conformation of SB was examined over the pH 0.8–3 regions by circular dichroism, tryptophanyl fluorescence, 1-anilino-8-naphthalenesulfonate (ANS) binding, and tryptophanyl fluorescence quenching study. The pH 0.8–3.0 regions were selected to study the acid induced unfolding of SB because no autolysis of the enzyme was observed in these pH regions. The results show that SB at pH 2.0 is maximally unfolded and characterizes by significant loss of secondary structure (~80%) and almost complete loss of tertiary contacts. However, on further decreasing the pH to 0.8 a MG state was observed, with secondary structure content similar to that of native protein but no tertiary structure. We also made a comparative study of these acid induced states of SB with acid induced states of modified stem bromelain (mSB), reported by our group earlier [*Eur. J. Biochem.* (2002) 269, 47–52]. We have shown that modification of SB for inactivation significantly affects the N-UA transition but neither affects the UA-MG transition nor the stability of the MG state.

Key words: acid induced unfolding, circular dichroism, molten globule, fluorescence quenching, stem bromlain.

Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; U_A, acid unfolded state; CD, circular dichroism; C_m, mid point of transition; K_c, effective collisional quenching constant; f_a, fraction of accessible fluorophore; GnHCl, guanidine hydrochloride; MRE, mean residue ellipticity; MG, molten globule; mSB, modified stem bromlain (catalitically inactive form); NATA, *N*-acetyltryptophanamide; TCA, trichloroacetic acid; SB, unmodified stem bromelain (catalytically active form).

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 in protein science. 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*). One such intermediate state know as "molten globule" (MG) has attracted much attention in resent years. The molten globule is a state of the protein possessing native like "format" with no global tertiary structure. The common structural characteristics of MG state are as follow: (i) the presence of pronounced amount of secondary structure, (ii) the absence of most of the specific tertiary structure produced by the tight packing of side chains, (iii) the presence of loosely packed hydrophobic core that increases the hydrophobic surface accessible to solvent (*6–8*). Resent studies have shown that structure of the MG state is heterogeneous. One portion of the structure is more organized and native like with other portions of the structure being less organized and the detailed structural characteristics are remarkably depend on protein species (*4*).

The stem bromelain, from *Ananas cosmosus*, like other cysteine proteinases, belongs to the α + β protein class and the highly similar amino acid sequences of papain (*9*), actinidin (*10*), proteinase Ω (*11*), chymopapain (*12*), and stem bromelain (*13*) 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 (*14–16*). SB like other proteolytic enzyme, undergoes autocatalysis (*17*, *18*). 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 sulphahydril group of active site cysteine is blocked by caboxymethylation (*19*). This inactive enzyme cannot be native even if observed physical parameters are very similar to the active enzyme.

While a detailed study on the folding aspects of modified stem bromelain (mSB, catalytically inactive state) has been made by our group (*20*, *21*), no information about the general folding aspects of unmodified stem bromelain (catalytically active) is currently available. From our earlier studies on the folding of modified SB (mSB), a partially folded intermediate state was detected at pH 2.0, which refolds to molten globule like state around pH 0.8 (*20*).

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Further we have demonstrated that partially folded state of mSB obtained at pH 2.0 acquires different conformational states in the presence of salt and alcohols (*21*, *22*).

We present here a detailed investigation of the presence of a acid unfolded state (U_A) and a molten globule state of SB in acidic conditions. The U_A and MG states of SB have been compared with those of modified one, observed earlier. The significance of these results in the unfolding of stem bromelain is discussed in view of the structure of intermediate states of mSB and SB.

MATERIALS AND METHODS

Materials—Stem bromelain [EC 3.4.22.32] lot no. B 4882, guanidine hydrochloride and 1-Anilino 8-naphthalene sulfonic acid (ANS) were obtained from Sigma Chemical Co., St. Louis, and USA. All other chemicals used were of analytical grade.

Spectrophotometric Measurements—Protein concentration was determined spectrophotometrically using specific extinction coefficient $\varepsilon^{1}{}_{1\,\mathrm{cm}}$ of 20.1 by measuring the absorbance of protein solution at 280 nm on a Hitachi U-1500 spectrophotometer (*23*). The molecular weight of bromelain was taken as 23,800. A stock solution of ANS was prepared and concentration was determined using an extinction coefficient of ε_M = 5,000 M^{-1} cm^{-1} at 350 nm (*24*).

Determination of Extent of Autolysis—The extent of autolysis was measured by TCA precipitation method (*25*).

Acid/Guanidine Hydrochloride Denaturation—Acid induced unfolding of stem bromelain was carried out in 10 mM solutions of glycine/HCl buffer (pH 0.8–3.0). pH measurements were carried out on an Elico digital pH meter (model LI 610) with a least count of 0.01 pH unit. SB (10–20 μM) was incubated with the buffer of desired pH at 25°C and allowed to equilibrate for 4 h before spectrophotometric measurements.

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 1mm and 1cm respectively. The results are expressed as mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ defined as

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

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.* (*26*):

$$\%\ \mathrm{helix} = [(\mathrm{MRE}_{222\ \mathrm{nm}} - 2{,}340)/3{,}0300] \times 100.$$

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°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 or 280 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).

Fluorescence 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 296 nm in order to excite tryptophan residues only and the emission spectrum was recorded in the range 300–400 nm. The decrease in fluorescence intensity at λ_{max} was analyzed according to the modified Stern-Volmer equation (*27*).

$$F_0/(F_0 - F) = 1/f_a + 1/(f_a \cdot K_c \cdot [\mathrm{Q}])$$

Where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher respectively, K_c is the effective collisional quenching constant, f_a is the fraction of accessible fluorophore and [Q] is concentration of the quencher. A linear regression, $F_0/F_0 - F$ *vs.* 1/[Q] whose slope = $1/(f_a \cdot K_c)$ and intercept = $1/f_a$ was used for data analysis.

RESULTS AND DISCUSSION

The time courses of the autolysis process of SB in the pH range 0.8–7 were monitored by TCA precipitation method (*25*). Figure 1 shows the time dependent changes of OD at 275 nm of supernatant of TCA precipitated SB at pH 7, 3, and 0.8. No autolysis was observed upto 4 h in the pH region 0.8–3. Therefore, pH induced unfolding of SB can be determined in the pH region 0.8–3.0 without facing any complication due to autocatalysis.

Acidic pH induced unfolding transition of SB was monitored in the far UV CD region at 222 nm and 208 nm, probes for secondary structure, ANS fluorescence at 470 nm for the exposure of hydrophobic region of the protein and by multiple probes for tertiary structure *viz.* near-UV CD, intrinsic fluorescence intensity and wavelength of maximum emission.

Figure 2A shows acidic pH induced unfolding profile of SB as monitored by ellipticity measurements at 208 and 222 nm. The measurements of ellipticy at these wavelengths were used to monitor changes in the conformation of the polypeptide backbone. The ellipticities at 222 nm and 208 nm decreased markedly below pH 3 to a minimum value at around pH 2.0. A further decrease in the pH below 2.0 resulted in a second transition, corresponding to the formation of secondary structure, which became maximum at pH 0.8. Below pH 0.8, the protein was found to aggregate. In the first transition, 82.5% loss of secondary structure took place, calculated on the basis of the difference in the ellipticity at 222 nm between the native and denatured state in 6.0 M GnHCl taken to be 100%. However in the second transition, 81.8% secondary structure reformed. When the effect of pH was assessed by

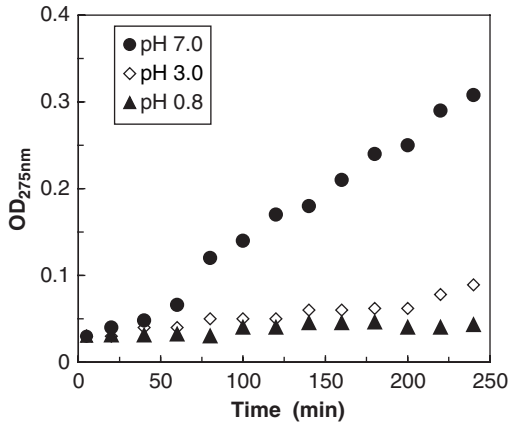

Fig. 1. **Extent of autolysis monitored by the time dependent changes of OD at 275 nm of supernatant of TCA precipitated SB at pH 7, 3, and 0.8.**

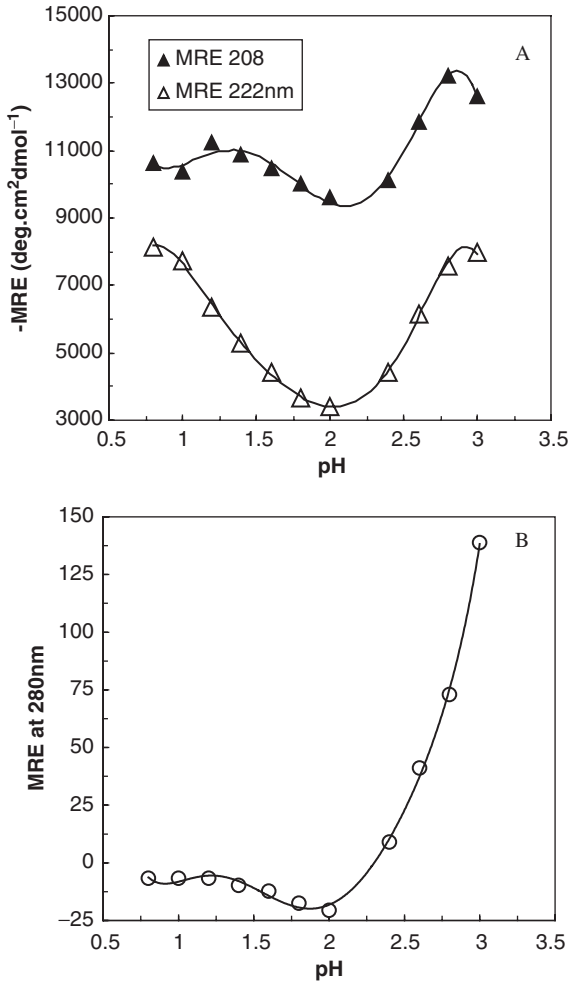

Fig. 2. **pH dependence of mean residue ellipticity (MRE) of unmodified stem bromelain at 222 nm, 208 nm (A) and 280 nm (B).**

MRE measurements at 285 nm, a probe for tertiary structure determination, a gradual decrease in the MRE values was observed on decreasing the pH below 3, reaching a minimum at pH 2.0 and remained unchanged down to pH 0.8 (Fig. 2B). A comparison with 6 M GnHCl denatured state of mSB, which is considered to exist in a randam coil conformation (*28*), SB is appeared to devoid of specific tertiary contacts in the pH range 2.0–0.8.

In order to study the exposure of hydrophobic clusters of the protein during acidic pH induced unfolding of SB, effect of pH was followed by ANS fluorescence at 480 nm after exciting the ANS-protein complex at 380 nm (Fig. 2B). The hydrophobic dye, ANS binds to hydrophobic patches in proteins and fluorescence emission is known to increase on binding (*29*). As can be seen from the figure, a gradual increase in the fluorescence intensity was observed on decreasing the pH below 2.4, reaching a maximum at around pH 0.8, where as no change in the ANS fluorescence was observed in the pH range 3–2.4. The transition obtained by ANS fluorescence measurements is not consistent with the transition followed by ellipticity measurements either in far-UV CD or near-UV CD regions (Fig. 2, A and B). Acid unfolded state at pH 2.0 retains small amount of secondary structure compared to completely unfolded protein in 6 M GnHCl but devoid of specific tertiary contacts. Thus small binding of ANS to the protein at pH 2.0 might be either due to small amount of hydrophobic clusters (present in residual secondary structure) or presence of small population of MG state. These results suggest the presence of large number of solvent accessible non-polar clusters in the protein molecule at pH 0.8 and retention of small amount of non-polar clusters also at pH 2.0.

In order to study the acid induced unfolding transition of SB in great detail, acid induced unfolding is also followed by intrinsic fluorescence properties of the protein. Stem bromelain contains five tryptophanyl residues (*13*) and extensive sequence homology with papain indicate that three Trp-residues are buried in the hydrophobic core where as two of them are located near the surface of the molecule. As the fluorescence properties (fluorescence intensity and maximum wavelength of emission) are highly sensitive to the polarity of its microenvironments, the pH induced changes in the conformation of SB are followed by measurements of relative florescence intensity (RFI) at 345 nm and λ_{max} of emission after exciting the protein at 295 nm (Fig. 3, A and B). Lowering the pH to 2.0 caused a red shift of 4 nm and remained unchanged down to pH 1.4, but when the pH was decreased below 1.4 a second transition corresponding to blue shift of 1 nm occurred which in turn tailed off at pH 1.0 (Fig. 3A). On the other hand, a gradual quenching of tryptophanyl fluorescence was observed in the pH region 3.0–0.8 (Fig. 3B). The observed red shift of λ_{max} and quenching of intensity in the pH region 3.0–2.0 could be ascribed to the exposure of tryphanyl residues to the polar environment due to unfolding of the protein. Whereas, slight blue shift (from 352 to 351) observed around pH 1.0–0.8 might be due to creation of slight hydrophobic environment around Trp residues due to reformation of secondary structure as discussed in the far UV-CD section.

Taken together these results it may be concluded that SB at pH 2.0 exists as acid unfolded state which lost around 80% of native secondary structure and almost complete loss of tertiary structure. However, SB at pH 0.8 exhibits characteristics of molten globue state (*6–8*) *i.e.* native like secondary structure contents, enhanced ability to

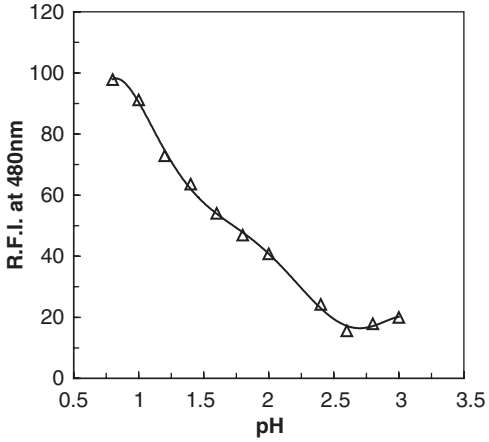

Fig. 3. **Effect of pH on the ANS fluorescence intensity at 480 nm of stem bromelain after exciting the ANS-protein complex at 380 nm.**

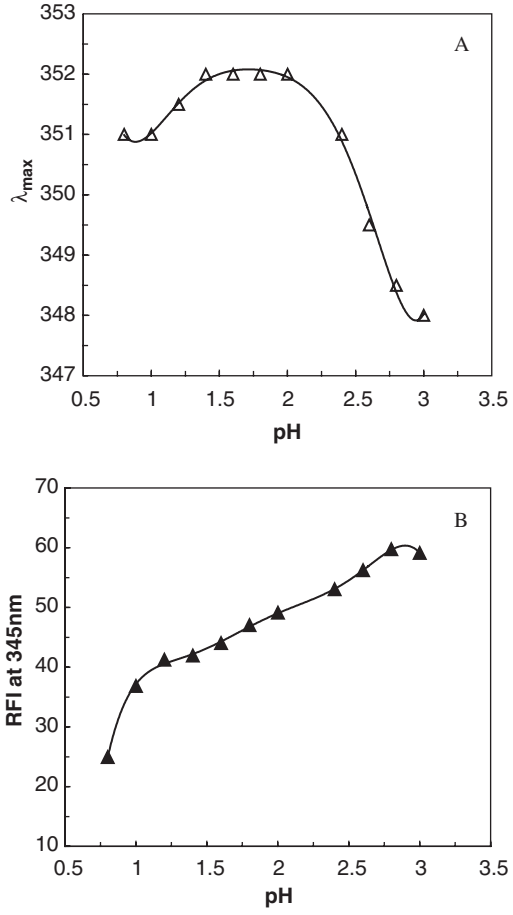

Fig. 4. **Effect of pH on tryptophanyl fluorescence of stem bromelain.** The protein was excited at 296 nm and the (A) maximum wavelength of emission (λ_{max}) and (B) relative fluorescence intensity at 345 nm was measured.

bind hydrophobic dye ANS and absence of specific tertiary contacts. The unfolding was found to be a irreversible process for all the transitions studied by different probes. Therefore, mechanism of acid induced unfolding of SB

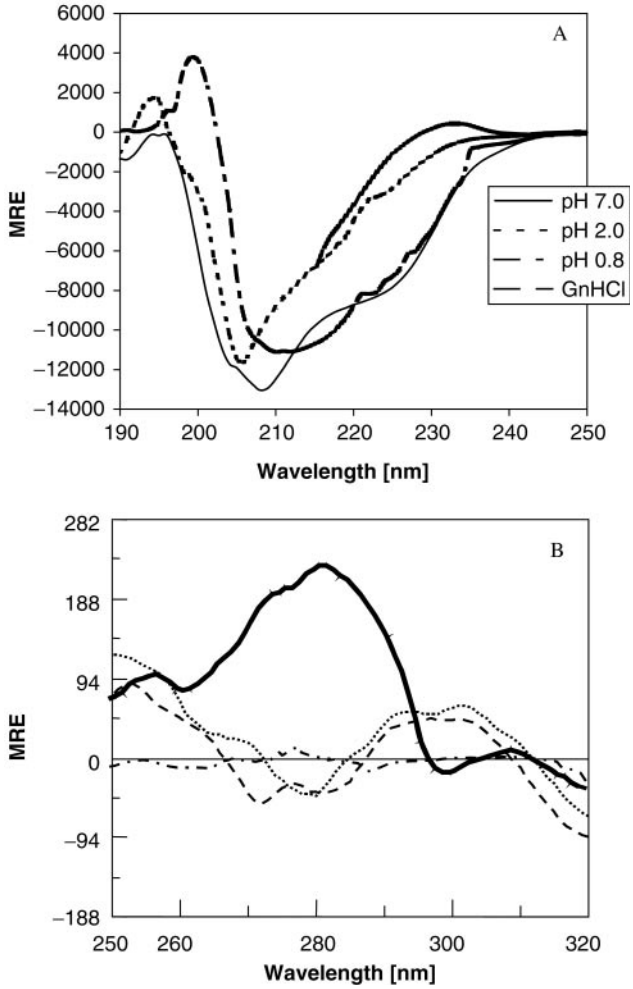

Fig. 5. **Far-UV CD (A) and near UV CD (B) spectra of native state at pH 7.0 (—) acid unfolded state at pH 2.0 (·····), molten globule state at pH 0.8 (–·–) and 6 M GnHCl denatured state (– – –) of stem bromlain.** Spectra were obtained at protein concentration of 0.31 mg/ml and 1.07 mg/ml in the far and near UV CD region respectively.

may be represented as

$$N \rightarrow \underset{pH\,2.0}{U_A} \rightarrow \underset{pH\,0.8}{MG}$$

where N, U_A and MG are the native, acid unfolded and molten globule states respectively. To ascertain whether the protein state observed at pH 2.0 and 0.8 represented the U_A and MG states, we compared the far UV CD spectra, near UV CD spectra, ANS and intrinsic fluorescence spectra of these states with those obtained at pH 7.0 (native state) and in the GnHCl denatured states.

Far-UV CD—Figure 5A shows the far UV CD of SB in the native, U_A, MG states, and completely denatured state in presence of 6 M GnHCl. Native SB revealed two negative peaks around 222 nm and at 208 nm with the signal pronounced in magnitude at the 208 nm, demonstrating typical of $\alpha + \beta$ proteins (*30*). The spectra for U_A acquired all the feature of GnHCl denatured state, although it was slightly more structured compared to GnHCl unfolded state. The spectrum of MG like state

was characterized by CD band at 222 nm and 208 nm indicating that it retained all the elements of secondary structure found in native protein, although there was a decrease in the ellipticity values at 208 nm suggesting loss of secondary structure without affecting basic format.

Near-UV CD—Figure 5B shows CD spectra of different states of SB in the near UV region, which provide information about the tertiary structure of the protein. The spectrum of native SB exhibits a negative band maximum at 298 nm and a broad positive band at 280 nm, which is in accordance with the previous findings (*30*, *31*). At pH 2.0 the negative band at 298 nm converts into a broad positive band and positive band at 280 nm into negative band. The spectrum at pH 2 of SB is essentially the same as that at pH 2 in the presence of 6 M GnHCl indicating that acid denatured stem bromelain has lost almost all its tertiary structural elements. As can be seen from the figure spectrum of MG state at pH 0.8 lacks all the features of native states and hence does not contains any specific tertiary contacts like MG states presents in many other monomeric proteins (*6–8*).

ANS Binding—ANS, a fluorescent hydrophobic dye is popularly used to monitor the exposure and/or disruption of hydrophobic patches of proteins during its unfolding/folding process (*29*, *32*). ANS has higher affinity for the intermediate state of protein than the protein in the native state or completely unfolded state. Because intermediate conformations of the protein has exposed hydrophobic pockets for ANS binding, which is less accessible for ANS binding in the native protein and is disrupted in the completely unfolded state (*6*). Figure 6 shows the fluorescence spectra of protein-ANS complex in the 400–600 nm range of N, U_A, MG and unfolded (pH 2.0 + 6 M GnHCl) states of stem bromelain. As can be seen binding of ANS to MG state produce a large increase in fluorescence intensity compared to native and unfolded states indicating sizeable amount of exposed hydrophobic clusters of MG state of SB.

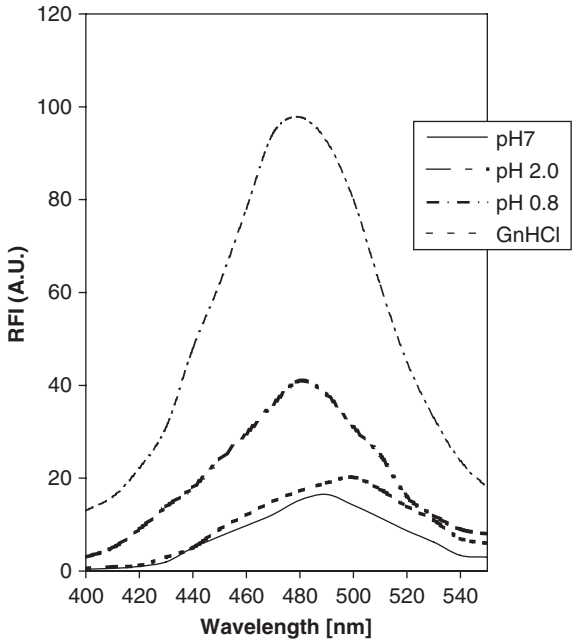

Fig. 6. **Fluorescence emission spectra of ANS bound to N, U_A, MG and unfolded in 6 M GnHCl states of stem bromelain.**

Tryptophanyl Fluorescence Quenching Studies—Measurement of the extent of quenching of tryptophanyl fluorescence by an external quencher is a very sensitive probe in determining the exposure of the tryptophanyl residues to the solvent (*27*). Uncharged molecules of acrylamide were used to quench the fluorescence of tryptophanyl residues. Analysis of quenching data by modified Stern-Volmer equation (see "MATERIALS AND METHODS") enable us to determine effective quenching constant K_c, and number of accessible tryptophan residues f_a. Stem bromelain contains five tryptophan residues at different locations throughout the polypeptide chain (*13*). Figure 7 shows the modified Stern-Volmer plots for acrylamide quenching studies for various states of stem bromelain. A comparison of parameters (K_c and f_a) provide information about the changes in the accessibility of tryptophanyl residues of SB during various treatments (Table 1). Results for the quenching of tryptophan analogue *N*-acetyltryptophanamide (NATA) are also included as a standard for complete accessibility to quencher. Quenching of *N*-acetyltryptophanamide (NATA), as a model compound that is fully accessible to the solvent, is characterized by effective quenching constant K_c = 22.35 and f_a = 1.05 ± 0.11. The effective quenching constant (K_c) for native SB (pH 7) was 11.2 M^{-1} and the fraction of accessible fluorophore

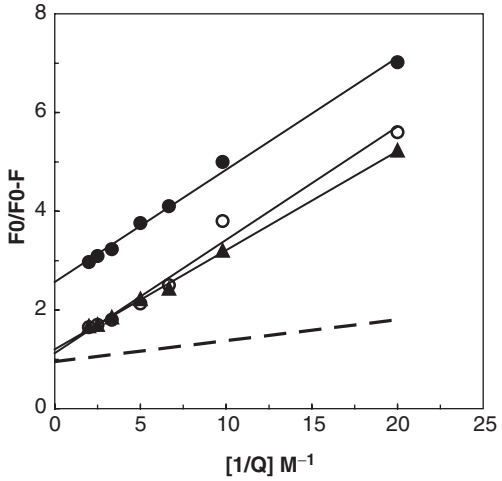

Fig. 7. **Modified Stern-Volmer plots for acrylamide quenching of tryptophan fluorescence of native (solid circles), acid unfolded (open circles) and molten globule (solid triangles) states.** For comparison, the acryl amide quenching of *N*-acetyle-L-tryptophanamide (NATA) is shown (dashed line).

Table 1. **Acrylamide quenching of tryptophanyl fluorescence.**

Stem bromelain states	K_c (M^{-1})	f_a	R^2	Accessible fluorophore*
Native (pH 7.0)	11.27	0.39 ± 0.03	0.994	1.95
U_A (pH 2.0)	3.89	0.89 ± 0.09	0.981	4.45
MG (pH 0.8)	6.5	0.83 ± 0.06	0.9969	4.15
NATA	22.35	1.05 ± 0.11	0.9823	–

R^2: Correlation coefficient obtained by linear regression. *: Calculated on the basis that f_a = 1.0 is indicating accessibility of all the 5 tryptophanyl.

Table 2. **Spectroscopic properties of different states of SB and mSB.**

Variables	Unmodified SB			Modified SB		
	pH 7.0	pH 2.0	pH 0.8	pH 7.0	pH 2.0	pH 0.8
$MRE_{222\,nm}$[a]	–8,404	–3,409	–8,151	–7,984	–4,764	–7,156
% Alpha helices[b]	20	3.5	19.3	18.6	8	15.9
$MRE_{280\,nm}$[a]	237	–20	–6	198	50	51.2
ANS-protein complex Fluorescence	1	2.4	5.7	1	5	15
Λ_{max}	348	352	351	347	344	346
$RFI_{345\,nm}$	1	0.75	0.38	1	0.41	0.48
C_m (M)[c]	–	–	2.3	3.5	–	2.3

[a]MRE, deg·cm^2·dmol^{-1}; [b]calculated by method of Chen *et al.*; [c]obtained from GnHCl induced normalized transition curves shown in Fig. 8.

fa 0.39 ± 0.03 indicating that 2 tryptophan residues are accessible to solvent, which agrees with crystal structure of papain that showed extensive sequence homology with SB (*14*). Both papain and SB belong to cystein proteases family of the protein. The fraction of exposed tryptophan residues of SB increased in U_A state to the value (f_a = 0.89 ± 0.09) corresponding to the exposure of 4 tryptophan residues to the solvent. The f_a of NATA is significantly higher than those for A_U and 6 M GnHCl unfolded states, indicating that these denatured conformations are not fully unfolded. As shown in Table 1, small difference in the f_a values between MG state and U_A state indicate that they have almost similar tryptophanyl microenvironment. This result together with intrinsic fluorescence and aromatic CD results suggested that MG state of SB is very similar to U_A state. The MG state is different from the U_A state only in possessing pronounced amount of secondary structures.

Effect of Modification (Inactivation) on the Acid Induced Unfolding Pathway of Stem Bromelain—To avoid any complication due to autolysis, active amino acid side chain of proteolytic enzymes is generally modified before performing unfolding refolding studies. This inactive enzyme cannot be native in the absence of activity even in the physiological condition. In view of this, here we are comparing the various spectroscopic properties of native (pH 7.0), U_A, and MG states of modified and unmodified stem bromelain (Table 2) from the results presented above and our previous reports on mSB (*20*).

The effect of modification on the secondary and tertiary structures of SB was monitored by far UV and near UV CD respectively. The far UV CD spectra were similar in both the cases as also reported elsewhere (*20*, *30*, *31*). The near UV CD spectrum of SB showed abroad positive maximum around 280 nm and a negative minimum at 298 nm. The absence of negative minimum at 298 nm from the spectrum of mSB together with small but significant changes in the fluorescence properties (Table 2) may be due to change in the environment of Trp residues because of alkylation of active site Cys-26. These results suggest that modification of SB to inactivate also affect the native conformation of the protein.

As summarized in Table 2, the effect of modification is more prominent at pH 2.0 where SB exists in U_A state. At pH 2.0, the mSB retains about 42% of the secondary structure while SB exhibits almost complete loss of secondary structure. Near UV CD, intrinsic fluorescence, ANS binding, and Tryptophanyl fluorescence quenching studies

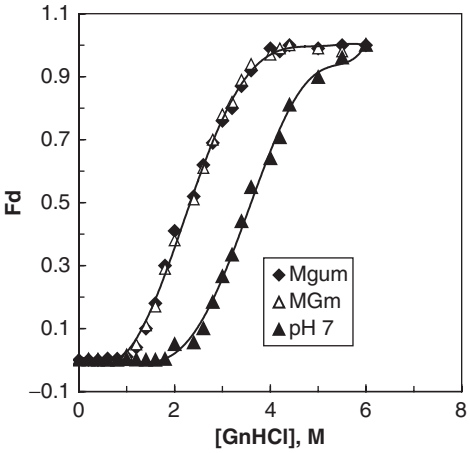

Fig. 8. **Normalized transition curves for GnHCl induced unfolding of molten globule states of mSB, SB and mSB at pH 7.0 as followed by MRE measurements at 222 nm.**

indicated that mSB exsits as partially folded intermediate and SB as unfolded states at pH 2.0. This indicated that alkylation impart stability to SB toward pH denaturation, although it does not affect the charge state of the protein.

It is interesting to note that in spite of significant differences between the acid unfolded states of mSB and SB, molten globules states have almost same spectroscopic properties (Table 2). The structural stability of the molten globule states of mSB and SB were determined by GnHCl induced unfolding followed by MRE measurements at 222 nm. Figure 8 showed the normalized transition curves for GnHCl induced unfolding of mSB at pH 7.0 and molten MG states of mSB and SB at pH 0.8. GnHCl induced unfolding of both native and MG states are cooperative and the transitions curves are sigmoidal. The C_m values have been used as a measure of stability as delta G_U could not be determined because of the irreversibility of the unfolding process. The C_m values of mSB at pH 7.0, MG states of mSB and SB are found to be 2.4, 2.3 and 3.5 M respectively. The Cm values for MG states of both preparations are almost equal but lower than mSB at pH 7.0. Since both pseudo-native and MG states contains almost equal amount of secondary structure, lower stability of MG states compared to pseudo-native state might be due to loosely ordered secondary structure of MG states. Although, MG

states generally unfold non-cooperatively (*33*), cooperative unfolding of MG states has also been reported in some proteins (*34–36*). Usually, the chemical denaturation of MG state is cooperative, but thermal unfolding is either non-cooperative or relatively cooperative. A possible explanation of this behavior as suggested by Arai and Kuwajima (*4*) is that the thermal unfolding more effectively disrupts the structures and interactions responsible for the origination of native state from molten globule state (stage II of hierarchical folding) while the denaturant induced unfolding disrupt all the structure and interactions organized at stage I (formation of MG from unfolded state) and stage II.

In the study presented here, we have shown that stem bromelain acquired a MG state at pH 0.8 from native state through an U_A state occurring at pH 2.0. The structure of acid unfolded states may be different depending on the states of modification, but no significant differences were noted either in structure or stability of the MG states. Thus, these observations lead us to believe that MG state is an essential intermediate in the unfolding pathway the stem bromelain. Our study on the acid induced unfolding of SB reveal that it exhibits unfolding behavior characteristic of type I proteins as classified by Fink *et al.* (*37*).

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