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pH-Dependent Urea-Induced Unfolding of Stem Bromelain: Unusual Stability against Urea at Neutral pH

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Abstract—Equilibrium unfolding of stem bromelain (SB) with urea as a denaturant has been monitored as a function of pH using circular dichroism and fluorescence emission spectroscopy. Urea-induced denaturation studies at pH 4.5 showed that SB unfolds through a two-state mechanism and yields ΔG (free energy difference between the fully folded and unfolded forms) of ~5.0 kcal/mol and C_m (midpoint of the unfolding transition) of ~6.5 M at 25°C. Very high concentration of urea (9.5 M) provides unusual stability to the protein with no more structural loss and transition to a completely unfolded state.

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Denaturation studies have long been used to elucidate the folding–unfolding transitions in proteins. They have contributed considerably to the understanding of the role of various stabilizing and destabilizing forces that are responsible for their unique folded structure in solution [1-3]. This helps us to elucidate the molecular mechanism of the spontaneous folding of proteins from a random polypeptide chains to a well-ordered native conformation. Results of *in vitro* refolding experiments as well as theoretical considerations suggest that folding of large proteins is a sequential hierarchical process, in which protein folding proceeds according to the established hierarchy of the native structure, i.e. involving secondary and tertiary structures [4, 5]. The partially folded intermediates can be made to accumulate in equilibrium by mild concentrations of chemical denaturants, low pH, covalent trapping, or by protein engineering [6].

Stem bromelain (SB) belongs to the ($\alpha + \beta$)-protein class as other cysteine proteinases do, and the highly similar amino acid sequences of papain [7], actinidin [8], proteinase Ω [9, 10], chymopapain [11], and SB [12] indicate a common fold. This has been confirmed for the first three proteinases by detailed X-ray diffraction studies [13-15]. These proteins are rich in hydrophobic and uncharged amino acid residues and contain only 16-18% charged amino acid residues. X-Ray diffraction studies on these proteins reveal that the entire polypeptide chain folds into two domains, L and R. The domains interact with each other through hydrogen bonds, hydrophobic interactions, and salt bridges, in which residues from both domains participate [12-16].

Very little information about the general folding aspects of plant cysteine proteases is available. Extensive studies on the folding of papain [17, 18] as well as other cysteine proteases (procerain and ervatamin C) have been carried out by the group of Jagannadham [19-26]. They have demonstrated that cysteine proteases unfold through an intermediate state. On the other hand, the thermal denaturation of bromelain, as studied by means of circular dichroism (CD) and differential scanning calorimetry, is completely irreversible and apparently follows a simple two-state mechanism of the F–D type (where F is folded and D is denatured states) [2]. The SB molecule, when exposed to increasing alkalinity, exhibits conformational response through at least three different stages leading to the ionization of Tyr hydroxyl groups [27]. Folding studies on more cysteine proteases from different sources would certainly help in generalizing the folding behavior of cysteine proteases as well as complementing the proposed mechanism. With this view, unfolding studies of SB have been initiated. In our earlier studies on the folding of SB, a partially folded intermediate was detected at pH 2.0, which refolds to molten globule state around

Abbreviations: CD, circular dichroism; D, denatured state; F, folded state; I, intermediate state; MRE, mean residue ellipticity; SB, stem bromelain.

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pH 0.8 [28, 29]. Further, we have demonstrated that the partially folded intermediate at pH 2.0 acquires molten globule-like conformations in the presence of salt, alcohols, and low molecular weight polyethylene glycol [30-32].

In this paper, the pH-dependent stability of SB has been characterized by urea-induced unfolding studies. We show that SB is fully resistant against urea around neutral pH (5.5 to 10) and unfolds only below pH 5.0. This information will be helpful in understanding the basis of structural stability of SB against urea denaturation.

MATERIALS AND METHODS

Materials. Stem bromelain (EC 3.4.22.32) lot No. B4882 and ultra pure urea were obtained from Sigma Chemical Co. (USA). All the other reagents were of analytical grade. The commercial stem bromelain was purified to homogeneity by gel filtration chromatography.

Spectrophotometric measurements. SB concentration was determined spectrophotometrically using specific extinction coefficient $\varepsilon^{1\%}_{1cm}$ of 20.1 by measuring the absorbance of protein at 280 nm on a Hitachi U-1500 (Japan) spectrophotometer [2]. The molecular weight of bromelain was taken as 23.8 kDa [33].

pH measurements. pH was measured using an Elico digital pH meter (model L1610) with the least count of 0.01 pH unit. The pH meter was routinely calibrated at room temperature with 0.05 M potassium hydrogen phthalate buffer (pH 4.0) in the acidic range and with 0.01 M sodium tetraborate buffer (pH 9.2) in the alkaline range.

Inhibition of autolysis. To avoid complications due to autocatalysis, protein samples were irreversibly inactivated by the procedure of Sharpira and Arnon [34] with a modification as reported earlier by our group [18]. The protein was reduced with 0.32 M 2-mercaptoethanol for 1 h at room temperature followed by addition of solid iodoacetamide to final concentration 43 mM. After stirring for 30 min at 4°C, the solution was subjected to extensive dialysis in respective buffers. This inactive preparation of SB was used through the present study.

Denaturation studies. Stock protein solutions were prepared by exhaustive dialysis of modified SB against 60 mM sodium phosphate buffer (pH 6.0, 7.0, and 8.0), 10 mM sodium acetate buffer (pH 5.0, 4.5, 4.0, and 3.5), and 10 mM glycine-NaOH buffers (pH 9.0 and 10.0). To 0.5 ml of stock protein solution, different volumes of the desired buffer were added first, followed by the addition of stock urea solutions (10 M) prepared in their respective buffer to get the desired concentration of denaturant. The final solution mixture (3 ml) was incubated for 10 to 12 h at room temperature before optical measurements.

Circular dichroism (CD) measurements. CD was measured with a Jasco J-720 spectropolarimeter (Japan) equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulfonic acid. All the CD measurements were made at 25°C with a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of ±0.1°C. Spectra were collected with scan speed of 20 nm/min and response time of 1 sec. Each spectrum was the average of four scans. Far UV CD spectra were taken at protein concentrations of 1.8-2.0 μM with a 1-mm pathlength cell. The results were expressed as MRE (mean residue ellipticity) in $deg \cdot cm^2 \cdot dmol^{-1}$, which is defined as $MRE = \theta_{obs}/(10n \cdot l \cdot C_p)$, where θ_{obs} is the CD in millidegrees, n is the number of amino acid residues (212), l is the pathlength of the cell, and C_p is mole fraction. Helical content was calculated from the MRE values at 222 nm using the following equation as described by Chen et al. [35]: $\% \ \alpha\text{-helix} = (MRE_{222nm} - 2340/30300) \times 100$.

Fluorescence measurement. Fluorescence measurements were performed on a Shimadzu RF-540 (Japan) spectrofluorimeter equipped with a DR-3 data recorder. 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 280 or 295 nm, and emission spectra was recorded in the range of 300-400 nm.

Data analysis. For the unfolding transition of type F↔D the ΔG_D was determined by the method of Pace et al. [36] using equation:

$$\Delta G = -RT\ln[(Y - Y_F)/(Y_D - Y)]. \quad (1)$$

For the unfolding transition of the type F–I–D, where I is an intermediate state, each step can be assumed to follow a two-state mechanism. The data monitored by tertiary structure probes and MRE measurements at 222 nm were used to determine the value of ΔG_1 associated with the first transition using the relation:

$$\Delta G_1 = -RT\ln[(Y_1 - Y_F)/(Y_I - Y_1)]. \quad (2)$$

The ΔG_2 of the second transition, which involved melting of secondary structure, was determined using the equation:

$$\Delta G_2 = -RT\ln[(Y_2 - Y_I)/(Y_D - Y_2)]. \quad (3)$$

Here R is the universal gas constant, T is the temperature in Kelvin, Y_1 and Y_2 are the observed optical property corresponding to the first and second transition, respectively, and Y_F and Y_D are the optical properties of the SB at pH 3.5 and denatured state. Least square analysis of Eqs. (2) and (3) as a function of denaturant concentration [D] was used to fit the data to the following equation for the determination of ΔG^{H_2O}, the free energy change in the absence of urea:

$$\Delta G = \Delta G_D^{H_2O} - m[D],$$

where m is the measure of the dependence of ΔG on denaturant concentration in cal·mol^{-1}·M^{-1}.

RESULTS AND DISCUSSION

The stability of a native protein is a function of external variables such as pH, temperature, ionic strength, and solvent composition as they disrupt the different kind of bonds that are responsible for the intrinsic stability of the protein. Therefore, a quantitative analysis of the role of such variables in the formation of the structure of a protein is a prerequisite in describing the forces that are responsible for the conformational stability. To understand the stability of stem bromelain, the pH-dependent stability has been characterized by urea-induced unfolding of SB over the pH range 3.5-10.0.

Far-UV CD. Figure 1 shows MRE at 222 nm of SB in the absence and presence of 9.5 M urea over the pH range 3.5-10.0. Changes in the ellipticity at this wavelength are a useful probe for visualizing varying α-helical contents. We do not observe any apparent difference in the ellipticity between the pH region 6-10 both in presence and absence of 9.5 M urea. At pH 5.0, increase in the MRE values at 222 nm from −8140 to −5618 deg·cm^2·dmol^{-1} was noted in the presence of 9.5 M urea, indicating 30% loss of the α-helical content of the protein. Below pH 5.0, i.e. at pH 4.5, 4.0, and 3.5, almost complete loss of secondary structure of SB was detected in the presence of 9.5 M urea. The mean residue ellipticity at 222 nm of the SB in the pH range 3.5-4.5 is around −1000 deg·cm^2·dmol^{-1}, which is similar to the value (−1200 deg·cm^2·dmol^{-1}) expected for random coil conformation of a polypeptide chain [35]. The secondary structure of the protein remains unaffected even at 9.5 M urea, indicating an extreme resistance to urea-induced perturbations in the wide range of pH 6-10. On the other hand, the stability of the secondary structure of SB towards urea denaturation decreases below pH 5.0.

Tryptophan fluorescence. SB contains five tryptophan residues distributed in the entire polypeptide chain [12]. To minimize radiation energy transfer from the tyrosine residues, the protein was excited at 296 nm instead of 280 nm. The pH profile of the fluorescence intensity and the wavelength at the respective emission maximum (λ_{max}) in the absence and presence of 9.5 M urea are shown in Figs. 2a and 2b. In the absence of urea, no significant changes either in the intensity or wavelength maxima are observed in the pH range between 5 and 10, whereas from pH 3.5 to 4.5 we observe a slight decrease in both λ_{max} (2 nm) and fluorescence intensity (10%). In the presence of 9.5 M urea over the pH range 6-10, ~20% increase in the fluorescence intensity without any detectable change in λ_{max} was observed as compared to the SB in the absence of urea. At pH 5.0 in presence of 9.5 M urea, neither intensity enhancement nor marked shift in maximum emission (1 nm only) was observed. In the pH region 3.5-4.5, the fluorescence intensity is ~2.5-fold lower, and the emission maximum is shifted from 348 to around 355 nm in the presence of 9.5 M urea compared with the SB in the absence of urea. The observed increase in the λ_{max} and pronounced quenching of fluorescence intensity indicate the full exposure of tryptophan residues to the solvent.

Urea did not cause any structural perturbation over the pH range 5-10. This shows that the protein is susceptible to urea unfolding at lower pH. Therefore, to investigate the structural stability of SB, equilibrium denaturation curves were obtained at pH 4.5, 4.0, and 3.5 using both MRE at 222 nm and fluorescence intensity at 348 nm as probes for urea-induced unfolding of SB.

Unfolding transitions monitored by far UV-CD and tryptophan fluorescence at low pH. The urea-induced unfolding profiles of SB at pH 4.5, 4.0, and 3.5 monitored by MRE measurements at 222 nm and relative fluorescence intensity measurements at 348 nm are shown in Figs. 3a and 3b, respectively. The unfolding profiles at pH 4.5 and 4.0 were found to be cooperative, monophasic, and following two-state unfolding models where the protein exists either in folded or in unfolded state [37], while at pH 3.5 the transitions are cooperative but biphasic. To evaluate the state character of the unfolding transitions of SB, fraction unfolded data (F_d) obtained from measurements of both MRE at 222 nm and relative fluorescence intensity at 348 nm were overlapped. At pH 4.5, the unfolding transitions were found to be almost overlapping and cooperative, and all changes occur between 4.5

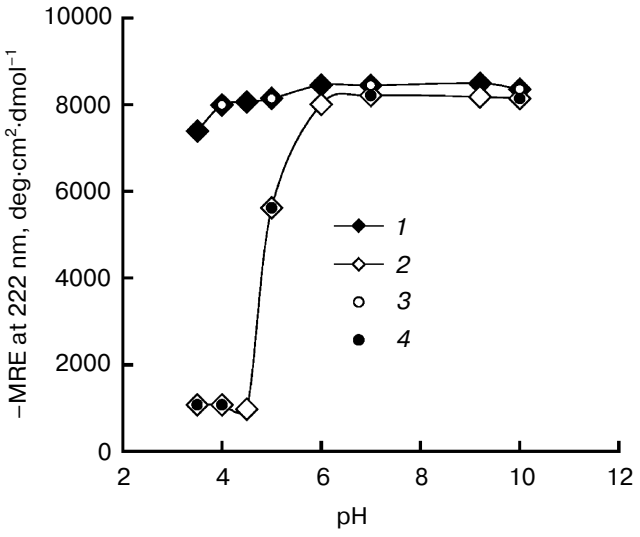

Fig. 1. Effect of pH on MRE at 222 nm of SB in the absence (*1*) and presence (*2*) of 9.5 M urea. The reversibility points in the absence (*3*) and presence (*4*) of 9.5 M urea are designated.

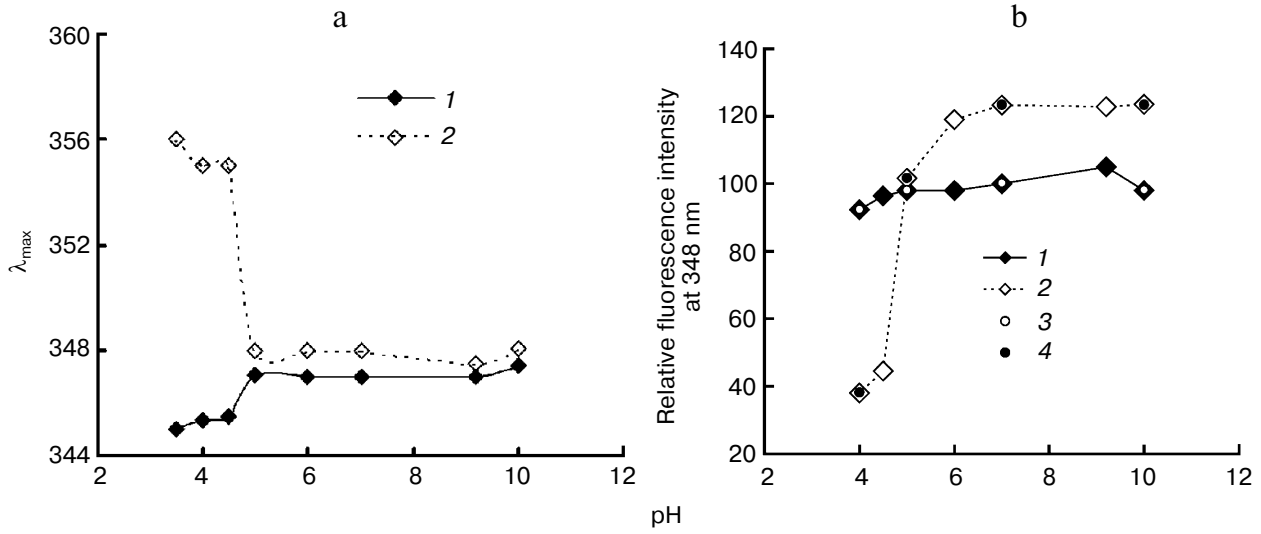

Fig. 2. Effect of pH on wavelength maximum (a) and relative fluorescence intensity at 348 nm (b) of SB in the absence (*1*) and presence (*2*) of 9.5 M urea. The protein was exited at 295 nm. The reversibility points in the absence (*3*) and presence (*4*) of 9.5 M urea are designated.

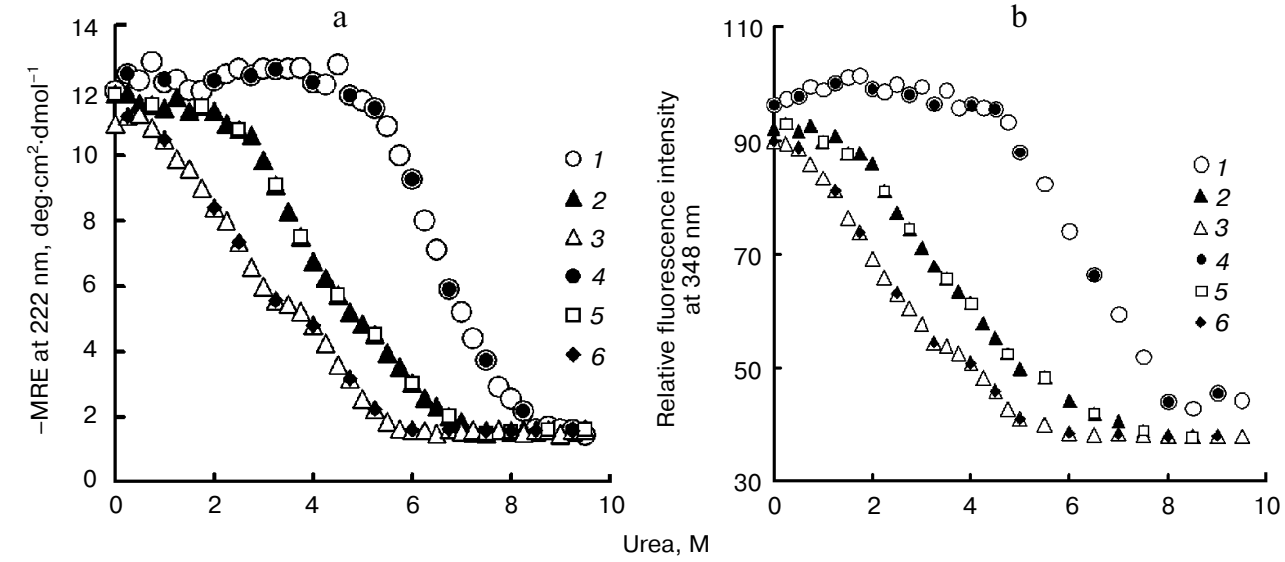

Fig. 3. Urea-induced unfolding profiles of SB as monitored by (a) MRE measurements at 222 nm at pH 4.5 (*1*), 4.0 (*2*), and 3.5 (*3*) and (b) tryptophan fluorescence measurement at 348 nm (λ_{ex} is 295 nm) at pH 4.5 (*1*), 4.0 (*2*), and 3.5 (*3*). The reversibility points at pH 4.5 (*4*), 4.0 (*5*), and 3.5 (*6*) are designated.

and 8.5 M urea (Fig. 4a). Linear regression analysis of the data yielded the value of ΔG^0_D and m (Fig. 4b). The table shows the thermodynamic parameters of SB for both secondary and tertiary probes. Dividing ΔG^0_D values by the slope (m) gives an approximate value of the midpoint of the transition. This gives similar values of midpoint of transition whether transition is followed by MRE at 222 nm (6.5 M urea) or fluorescence measurements (6.4 M urea). These results indicate that urea-induced unfolding of SB at pH 4.5 is a two-state process. This might be due to high cooperativity of the urea-induced unfolding process, as the protein is structurally stable with no loss of either secondary or tertiary structure even after prolonged exposure in the urea concentration ranging from 0 to 5.0 M.

At pH 4.0 urea-induced unfolding of SB was found to be cooperative, but the transition curves monitored by fluorescence and CD data were non-coincidental (Fig. 5a). Such non-coincidental transitions indicate the probable existence of an intermediate in the unfolding SB at pH 4.0 [37]. The change in free energy depending on the concentration of urea was calculated by assuming the

Thermodynamic parameters of stem bromelain at 25°C

pH	Method	C_m, M	ΔG_I, kcal/mol	$\Delta G_D^{H_2O}$, kcal/mol	m, cal·mol^{-1}·M^{-1}
4.5	$MRE_{222\ nm}$	6.5		5.4	821
	$RFI_{348\ nm}$	6.4		5.0	783
4.0	$MRE_{222\ nm}$	4.1		2.9	702
	$RFI_{348\ nm}$	3.3		−1.9	573
3.5	$MRE_{222\ nm}$	1.9		3.2	1011
	I–D	4.5	1.9		1383
	$RFI_{348\ nm}$	1.8		3.1	998
	I–D	4.3	1.7		1337

Note: Each ΔG value is the mean of three independent experiments with standard deviation ranging from ±0.31 to ±0.87 kcal/mol. RFI, relative fluorescence intensity.

unfolding process as a two state mechanism (Fig. 5b), and the conformational stability of the protein by both fluorescence and CD measurements was found to be 1.9 and 2.9 kcal/mol, respectively. As suggested by earlier investigators [38], the process is no longer two-state and the change in free energy is highly underestimated. The urea-induced denaturation of SB at pH 3.5 as monitored by the measurements of intrinsic fluorescence at 340 nm (on exciting the protein at 280 nm) and MRE at 222 nm are shown in Figs. 3a and 3b, respectively. As can be seen from the figures, urea-induced denaturation of SB at pH 3.5, which started from 0.75 M urea and completed around 5.5 M urea, followed a two-step three-state transition with accumulation of an intermediate state (I) around 3.25-3.75 M urea concentration. The I-state was characterized by abundant secondary structure, i.e. 10% α-helix as compared to ~28% α-helix found in the SB at pH 3.5 protein as calculated from MRE values at 222 nm. These results are in agreement with earlier observations made for a number of multidomain proteins [39-41]. To determine the stability of SB at pH 3.5 in the absence of urea and I-state in the presence of 3.5 M urea, thermodynamic parameters for the F–I and I–D processes were calculated according to the three-state model of unfolding. The values of ΔG_I and $\Delta G_D^{H_2O}$ and their respective m values are shown in the table, and dependence of free energy on urea concentration is presented in Fig. 6. Since $\Delta G_D^{H_2O}$ is a thermodynamic property that does not depend

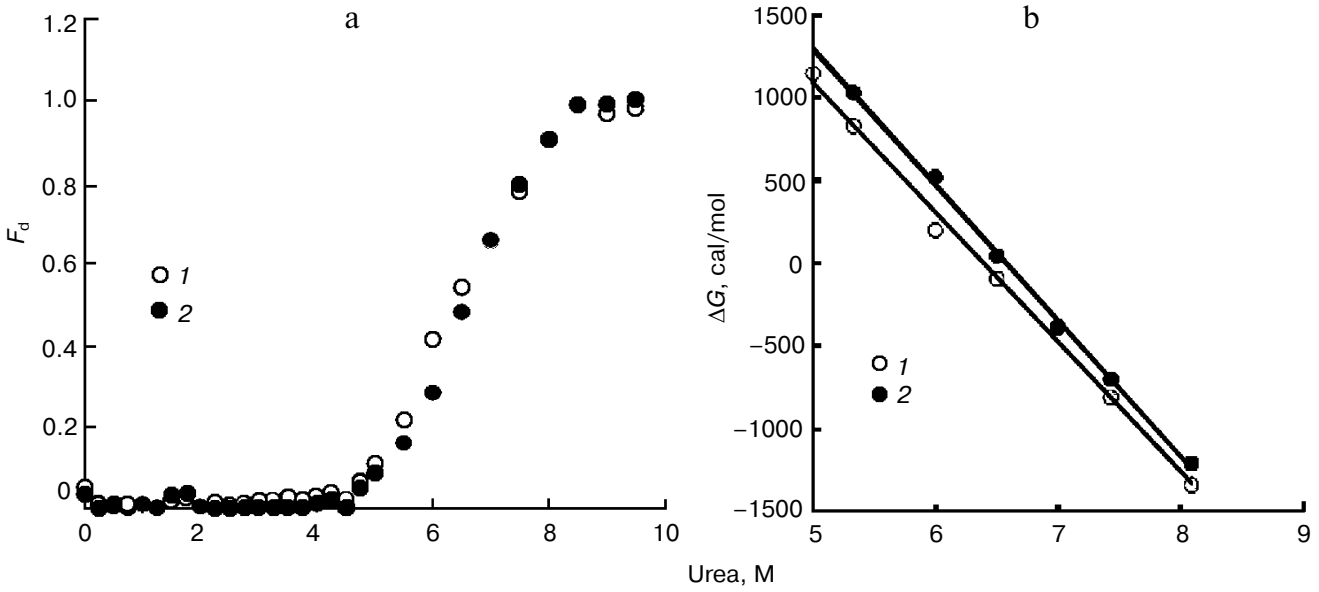

Fig. 4. a) Normalized urea-induced unfolding profiles of SB at pH 4.5 monitored by fluorescence intensity at 348 nm (*1*) and MRE at 222 nm (*2*). b) Dependence of free energy change of SB at pH 4.5 as a function of urea concentration for the transition shown in Fig. 4a.

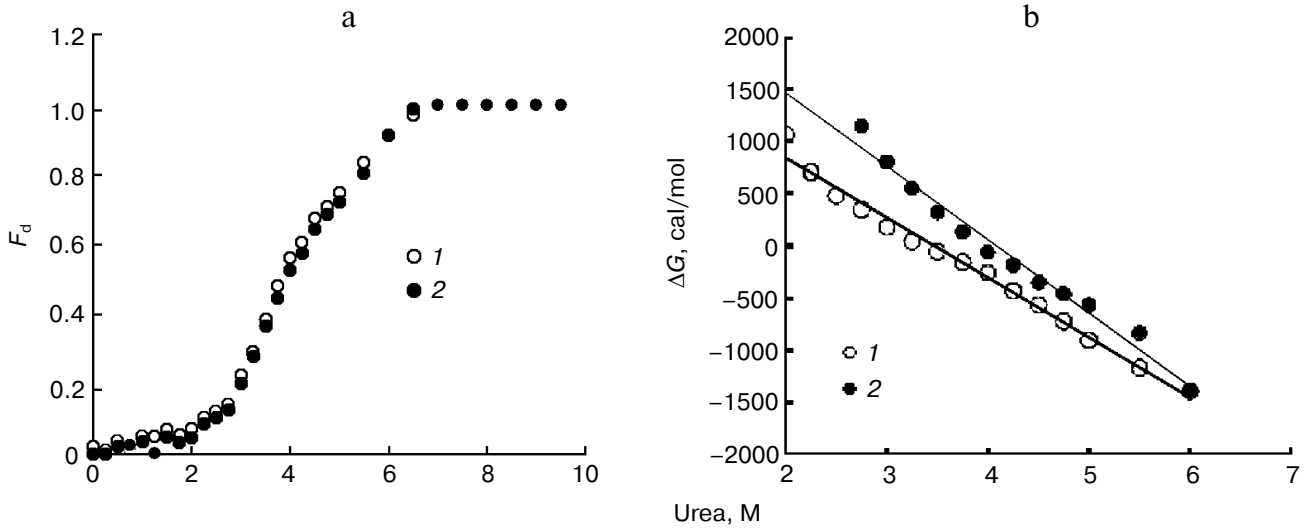

Fig. 5. a) Normalized urea-induced unfolding profiles of SB at pH 4.0 monitored by fluorescence intensity at 348 nm (*1*) and MRE at 222 nm (*2*). b) Dependence of free energy change of stem bromelain at pH 4.0 as a function of urea concentration for the transition shown in Fig. 5a.

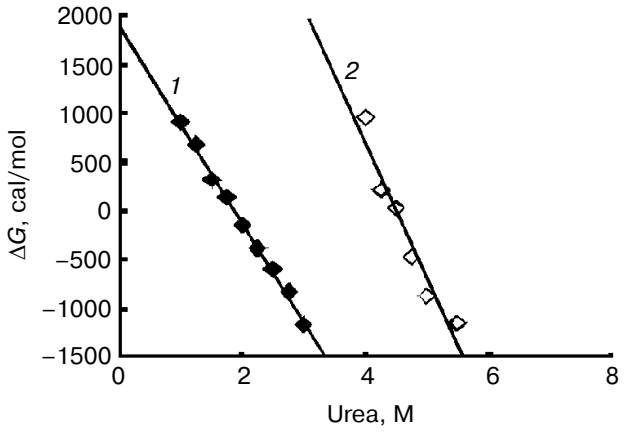

Fig. 6. Dependence of free energy change of stem bromelain at pH 3.5 as a function of urea for the F–I (*1*) and I–D (*2*) transitions.

on path, it was obtained by summation of individual steps, i.e. ΔG_{F-I} and ΔG_{I-D}. Thus the free energy change associated with F–I through I-state was 3.2 and 2.8 kcal/mol by CD and fluorescence measurements, respectively.

Thus, the urea-induced equilibrium unfolding of SB over the pH range 3.5-10.0 was investigated by amide CD and intrinsic fluorescence measurements. Over the pH range 5.5-10.0, the protein was found to be structurally stable with no loss either in secondary or tertiary structure. On lowering the pH to 5.0, the protein showed unusual stability in concentrated urea solution (9.5 M) with no more structural loss and transition to a completely unfolded state. At pH 4.5, the unfolding of SB appeared to follow a two-state mechanism of protein unfolding. Stability of the protein at this pH was found to be ~5 kcal/mol, and the midpoint of the transition was at ~6.5 M urea. At pH 4.0, although unfolding profiles were monophasic, the overlaps of the normalized transitions were non-coincidental, suggesting that the unfolding process of SB at pH 4.0 is a not a two-state process. On further lowering of pH to 3.5, a clear intermediate is visible around 3.25-3.75 M urea in the unfolding path of the protein, suggesting loss of interactions between SB domains that lead to their independent unfolding.

This information could be helpful in understanding the structure–function relationship of SB and cysteine proteases in general. Understanding the structure–function relationship of a protein under different solvent conditions can provide insight into the molecular basis of the stability, which can be further used to design protocols and/or an enzyme with special properties for biotechnological application.

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