Archives of Biochemistry and Biophysics 499 (2010) 26–31

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Specific molten globule conformation of stem bromelain at alkaline pH

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ARTICLE INFO

Article history:
Received 5 April 2010
and in revised form 5 May 2010
Available online 9 May 2010

Keywords:
Protein folding
Alkaline denaturation
Bromelain
Molten globule
Temperature endotherm
Thermodynamics

ABSTRACT

Stem bromelain (SBM), a therapeutic protein, is rapidly absorbed across the gut epithelium. Because SBM encounters an alkaline pH at its principal site of absorption, we investigated the alkaline-induced denaturation of SBM. From pH 7 to 10, the protein's secondary structure remained the same, although a slight loss of tertiary structure was observed. Above pH 10, there was a significant and irreversible loss of secondary and tertiary structure. At pH 10, SBM showed enhanced tryptophan fluorescence, however, the number of accessible tryptophans remained the same. The thermodynamics of temperature transition at pH 7 and 10 were strikingly different, with the former showing a two-phase transition endotherm, and the latter a broad non-two-state transition. At pH 10, SBM showed a significant increase in 8-anilino-1-naphthalene-sulfonate binding relative to the native state, suggestive of a specific molten globule (SMG) state. These studies suggest a distinct conformational rearrangement in SBM, at the protein's isoelectric point.

Introduction

The amino acid sequence of a protein stores the information required for it to spontaneously adopt its native three-dimensional form [1]. However, the conformation of a protein can be influenced by many factors, including solute concentration, mechanical forces, chemical denaturants and pH. Extremes of pH can sometimes induce nonnative forms, partially folded intermediates or aggregation of the native protein [2–5]. Although protein folding has been studied extensively at low pH, documentation on protein folding in alkaline pH is sparse. Most proteins do not encounter such conditions, so the physiological relevance of such studies is debatable. Extremely high pH [11,12,5] invariably results in unfolded proteins as a result of Coulombic forces of repulsion that counteract the stabilizing forces within a protein [6]. In some cases, proteins are cleaved at high pH [7]. Also, the deprotonation of tyrosines (pK = 10.1) complicates the study of folding behavior.

Although extremely high pH conditions usually are not physiologically relevant, moderate alkaline conditions (pH 7–10), often are encountered by proteins in physiological settings and can induce stable folding intermediates such as the molten globule (MG) state. Indeed, several proteins have been shown to exist in an intermediate/MG state at moderately alkaline pH [7–10]. The MG state is a compact collapsed form of a protein with pronounced secondary structure but lacking rigid tertiary structure [11,12,5]. However, recent evidence supports the idea that the MG might also possess well-defined tertiary contacts, i.e., a "specific molten globule" (SMG) state [13–15]. Proteins in the SMG state could thus contain a high level of secondary structure and a rudimentary or fluctuating, native-like tertiary topology. Structural similarities between the MG and the native form of a protein could provide insight into the nature of protein folding problem [12,16].

Stem bromelain (SBM) [3.4.22.32], a widely accepted phytotherapeutic drug, is a member of the bromelain family of proteolytic enzymes obtained from *Ananas comosus* [17]. SBM reversibly inhibits platelet aggregation, angina pectoris, bronchitis, sinusitis, thrombophlebitis, and pyelonephritis, and enhances absorption of drugs, particularly antibiotics [18–20]. The diverse effects of SBM appear to depend on its unusual ability to traverse membranes [21,22]. Because of its therapeutic utility and unique properties, it is crucial to understand its structural and folding properties under the conditions to which it is exposed when administered orally: SBM encounters low pH in the stomach, followed by an alkaline pH in the intestine, the main site of its absorption. Previous studies in our laboratory demonstrated that SBM adopts a partially folded intermediate state at pH 2.0 and a MG-like state at pH 0.8 [23,24]. In this study, we investigate the conformational rearrangement of SBM at alkaline pH. The results indicate that SBM adopts an SMG-like state near its isoelectric point.

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doi:10.1016/j.abb.2010.05.005

Materials and methods

Reagents

Stem bromelain [EC 3.4.22.32], guanidine hydrochloride (GdnHCl), 1-anilino-8-naphthalene sulfonic acid (ANS)[1] were obtained from Sigma Chemical Co., St. Louis, USA. All other chemicals used were of analytical grade.

Spectrophotometric measurements

Protein concentrations were determined by measuring absorbance at 280 nm using a Hitachi U-2900 spectrophotometer and calculated using a specific extinction coefficient ($\varepsilon^{1\%}_{1\text{-cm}}$) of 20.1 [25]. The molecular weight of bromelain was taken as 23,800 Da. The concentration of the ANS stock solution was determined at 350 nm using an extinction coefficient of $\varepsilon_M = 5000\ M^{-1}\ cm^{-1}$ [26].

Determination of extent of autolysis/enzyme assays/aggregation

The extent of autolysis was measured by trichloroacetic acid precipitation [27]. Enzyme assays were performed as described previously [28]. To measure the level of protein aggregation, samples were incubated for 18 h at room temperature at different pH and the resulting turbidity was monitored at 350 nm.

Sample preparation

SBM solutions were prepared, dialyzed extensively and subjected to size exclusion chromatography. SBM solutions (2–20 μM) were incubated with buffers of the desired pH or solvent–water mixtures at 25 °C and allowed to equilibrate for 4 h before spectrophotometric measurements. SBM as monitored by size exclusion chromatography is monomeric in solution [29].

CD measurements

CD measurements were performed using a Jasco model J-810/J-720 spectropolarimeter. The instrument was calibrated with D-10-camphorsulfonic acid. All CD measurements were performed at 25 °C with a thermostatically controlled cell holder. Far-ultraviolet (UV) CD spectra were measured at a protein concentration of 2 μM and near-UV CD spectra were measured at protein concentrations of 20–30 μM in 30 mM Tris NaOH buffer. Because high pH shows high dynode voltage (dynV) in the lower far-UV range, spectra were recorded in the 200–250 nm range. The path lengths were 1 mm and 1 cm, respectively. Results were expressed as mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$, defined as $MRE = \theta_{obs}/(10 \times n \times C_p \times l)$, where θ_{obs} is the CD in millidegrees, n is the number of amino acid residues, l is the path length of the cell in cm and C_p is the molar fraction. Helical content was calculated from the MRE values at 222 nm using the following equation [30]: $\%_{helix} = [MRE_{222nm} - (2340/30{,}300)] \times 100$. Reversibility of these alkaline-induced states was monitored by gradual dialysis back to physiological pH and corroborating the MRE values.

Fluorescence measurements

Fluorescence measurements were performed on a Cary Eclipse spectrofluorometer (Varian). 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 solutions at 295 nm and emission spectra were recorded in the 300–400-nm range. For ANS binding fluorescence experiments, the excitation wavelength was set at 380 nm and the emission spectra were taken in the 400–600-nm range. The molar ratio of protein to ANS was 1:50. ANS fluorescence studies at different pH and in the presence of GdnHCl were performed using a fixed protein concentration (2.0 μM). Appropriate blanks were subtracted.

Fluorescence quenching experiments

Aliquots of 5 M acrylamide stock solution were added to 2-μM protein solutions to achieve quencher concentrations ranging from 0.1 to 0.5 M. Excitation was set at 296 nm to excite tryptophan residues only. Emission spectra were recorded in the 300–400-nm range. Decreases in fluorescence intensity at λ_{max} were analyzed according to the modified Stern–Volmer equation [31], $F_0/(F_0 - F) = 1/f_a + 1/(f_a \cdot K_c \cdot [Q])$, where F_0 and F are the fluorescence intensities at an appropriate wavelength measured 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 is equal to $1/(f_a \cdot K_c)$ and whose intercept is $1/f_a$, was used for data analysis.

Differential scanning calorimetry (DSC)

Calorimetric scans were performed on a Nano DSC differential scanning calorimeter (TA Instruments). Stem bromelain solutions (0.80–1.0 mg/ml in 10 mM glycine–NaOH buffer) were degassed under vacuum for 20 min. Calorimetric scans were conducted at 1 °C/min under a total pressure of 3.0 kg/cm^2. Baseline buffer tracings were obtained under the same conditions and subtracted from the sample curves. The DSC analyzer software package (TA Instruments) was used for automatic baseline subtraction and determination of the total enthalpy and T_m changes to the midpoint of transition. Also, deconvolution of spectra for non-two-state transitions were verified using other DSC analysis software obtained online. The ΔH_{VH} and the ΔH of denaturation were determined according to the Van't Hoff and Kirchhoff equation, respectively.

Fractional denaturation studies

The fractional denaturation (f_D) of SBM at different pH values was calculated as follows:

$$f_D = (Y_N - Y)/(Y_N - Y_D),$$

where Y_N, Y_D and Y are the mean residue ellipticity at 222/280 nm ($MRE_{222/280}$) of the native, denatured and transition states of the protein, respectively.

Results

Circular dichroism (CD) spectra of SBM at alkaline pH

The changes in the secondary structure of SBM as a function of pH were monitored by far-UV CD (Fig. 1A). Although SBM retains most of its secondary structural features up to pH 10, a transition from the native to the unfolded state occurs at higher pH. As expected, near-UV CD spectral analysis showed that the native state of the protein was different from that of the denatured state (i.e., in the presence of guanidine hydrochloride [GdnHCl]). Interestingly, it also was different from that of the alkaline-induced state at pH 10 (Fig. 1B). At pH 7–9, tertiary contacts were characteristic of na-

[1] *Abbreviations used:* ANS, 1-anilino-8-naphthalene sulfonic acid; CD, circular dichroism; GdnHCl, guanidine hydrochloride; MG, molten globule; MRE, mean residue ellipticity; NATA, *N*-acetyl-l-tryptophanamide; SBM, stem bromelain; SMG, specific molten globule.