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Resistance of bromelain to SDS binding

Reema Bhattacharya, Debasish Bhattacharyya *

Division of Structural Biology and Bioinformatics, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Jadavpur, Kolkata — 700032, India

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ABSTRACT

Interaction of the plant cysteine protease bromelain with SDS has been studied using CD spectroscopy, intrinsic fluorescence emission, extrinsic fluorescence probe pyrene, isothermal calorimetric (ITC) investigations and inhibition of hydrolyzing activity. Results exhibit number of synchronous transitions when plotted against the total SDS concentration. SDS at submicellar level caused conformation change of bromelain leading to a stable entity. ITC and pyrene experiments suggest that the structural modifications below 5 mM, the cmc_{app} of SDS solutions containing bromelain, are the result of alterations of solvent hydrophobicity or non-specific weak binding and/or adsorption of SDS monomers. Melting temperature (T_m) and the free energy change for thermal unfolding (ΔG_{unf}) of the SDS induced conformers was decreased by 5 °C and 0.5 kcal/mol respectively, compared to native bromelain. Below 5 mM, SDS caused large decrease in V_{max} without affecting K_m for the substrate Z-Arg-Arg-NHMec. Analysis of kinetic data imply that SDS acts as a partial non-competitive inhibitor since even at 100 mM, the residual activity of bromelain was retained by 3%. Inhibition studies show an IC_{50} of 0.55 mM and a high K_i of 0.145 mM. These demonstrate that bromelain is resistant to SDS binding and denaturation, a property known for β-sheet rich kinetically stable proteins.

1. Introduction

The study of surfactant–protein interaction creates much interest for many physicochemical as well as conformational phenomena. From a technological perspective, studies of protein–surfactant interactions are intriguing because they modulate the functional properties of proteins. In some situations, these interactions may be detrimental to the product quality; while in others may be beneficial [1]. Such interaction has been widely studied for many years because of its applications in industry, chemical, biological, pharmaceutical and cosmetic laboratories. Surfactants can bind to proteins both as monomers and as micelles depending on the nature of the interaction and the surfactant concentration [2–4]. Non-ionic or 'soft' surfactants are usually used for solubilization of membrane proteins. In principle all proteins retain their structures and activities in the presence of non-ionic detergents, although their stability vary [5]. In contrast ionic surfactants bind in large quantities and denature most soluble enzymes and membrane proteins. Among these surfactants, SDS is most commonly used. The forces involved between protein and ionic detergents are both electrostatic and hydrophobic in nature [6,7]. Ionic surfactants can bind to proteins and alter the conformation substantially that often leads to denaturation. Different and partly contradictory structural models have been proposed for protein–surfactant complexes, in particular for SDS–globular protein complexes, but their structure has not yet been determined. It is therefore important to improve our understanding of the origin and nature of protein–surfactant interactions.

Surfactants have been divided into those, which bind and induce protein unfolding and those, which slightly interact without protein denaturation. In the literature, however, several surfactants are reported to enhance activity and stability of some enzymes. The increase in enzyme activity can occur as the result of surfactant–substrate or surfactant–product interactions. Enzyme activation in the presence of surfactants can be a consequence of increased solubility of the substrate or an unfavorable surfactant–substrate interaction, which in turn favors the formation of enzyme–substrate complex. Surfactants showing a strong interaction with the product may accelerate product release from the enzyme as well as prevent the reversible conversion of the product back to the substrate and hence increase the turnover of the enzyme [8–10]. The detergent can also bind to the enzyme and cause a conformational change to a more active form and generally surfactant head groups have a determining role. This can not only affect the kinetic parameters but also cause a change of the reaction mechanism [11].

The use of proteolytic enzymes in detergent formulations is now very common; with over half of all detergents presently available containing enzymes. Proteases of broad specificity hydrolyze precipitated and aggregated proteins into smaller fragments that are otherwise insoluble or sparingly soluble in detergents [12]. To achieve this, supplemented enzymes should be functionally stable in the presence of the detergent. The functionality of an industrial enzyme is governed mainly by its 'kinetic stability' where a large barrier of

Abbreviations: MRE, mean residue ellipticity; cmc, critical micelle concentration

* Corresponding author. Fax: +91 33 2473 5197/0284.
E-mail address: debasish@iicb.res.in (D. Bhattacharyya).

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energy of activation prevents the native enzyme from unfolding leading to very slow rate of denaturation.

'Bromelain', an extract from the fruit and stem of pineapple (*Ananas comosus*), is a rich source of cysteine proteases. The major protease present in the extract is also called bromelain. It offers a wide range of therapeutic efficacies and is increasingly being accepted as a phytotherapeutical drug [13]. Similar to the model plant cysteine protease papain, 'bromelain' is remarkably heat stable, retaining proteolytic activity between 40 and 60 °C where most enzymes are destroyed, or denatured. The optimal temperature for the proteolysis of stem bromelain ranges from 35–50 °C, in one study [14], and up to 60 °C, in later studies [15]. Bromelain, unlike most enzymes, has a very wide effective range of activity in both acidic and alkaline conditions that allows it to remain active in a variety of biological environments. It is stable between pH 5.0 and 10.0 and is of broad specificity [16]. This enzyme is a good alternative for microbial protease like subtilisins from *Bacillus licheniformis* and *Bacillus amyloliquifaceins* that are enzymes of choice for detergents [17].

SDS is generally considered a denaturing surfactant, although there are enzymes that resist its binding and unfolding ability. The present study is encouraged by the observation that bromelain could be classified as a kinetically stable protein. Characters of this class of proteins include resistance to SDS binding and proteinase K digestion, rich in β-sheet structure and compact rigid globular conformation [18]. These features are present in bromelain (Bhattacharya, R. and Bhattacharyya, D., unpublished data). To gain an insight into the property of resistance to SDS binding and to evaluate bromelain as an aid to detergents, we sought information on the mechanism of their interaction. Here it is demonstrated that at low concentrations of SDS, the detergent does not bind to bromelain. Inactivation of the enzyme appears to be due to structural deformity induced by SDS monomers.

2. Materials and methods

2.1. Materials

Stem bromelain (EC 3.4.22.4, crude extract), 7-aminomethyl coumarin (NHMec), Z-Arg-Arg-NHMec (where Z is a N-terminal blocking group), dithiothreitol (DTT) and EDTA were from Sigma, USA. Na-tetrathionate, dihydrate (Spectrum, USA) and SDS (DNAase, RNAase and protease free molecular biology grade; SRL, Mumbai, India) were purchased as indicated.

2.2. Enzyme purification

Crude stem bromelain (25 mg/ml) was dissolved in 10 mM Na-phosphate, pH 7.5 containing 5 mM of Na-tetrathionate for reversible inactivation of the protease. This preparation contains very low amount of other enzymes that are incapable of interfering with the spectral data of bromelain. To remove contaminating organic molecules and peptides, if any, it was centrifuged and the supernatant was passed through a pre-calibrated Sephadex G-50 column (1.5×90 cm) equilibrated with the same buffer at 25 °C. Flow rate was 12 ml/h. Elution of proteins was followed from $A_{280\ nm}$ and proteolytic activity. The major fraction that corresponded to 25 kDa was pooled and was concentrated 10-fold by lyophilization to serve as stock. Bromelain concentration of this purified preparation was determined using $\varepsilon^{1\%}_{280\ nm}=20.1$ [19]. The Mw of bromelain was taken as 22.828 kDa [20]. Unless otherwise mentioned, in all experiments tetrathionate inactivated bromelain was used. It was reactivated after incubation with 8 mM of DTT and 4 mM of EDTA in the same buffer (referred to as activation buffer) for 2 min at 25 °C. All UV–visible optical measurements were done using a WPA Lightwave S2000 diode array spectrophotometer (Biochrom, U.K).

2.3. Enzyme assay

The amidolytic activity of bromelain was measured using Z-Arg-Arg-NHMec as substrate [21]. A working solution of the substrate (360 μM) was prepared by dilution of the stock (7.2 mM in DMSO) in water as required. Assays were performed in activation buffer with peptide substrate delivered from the 360 μM stock at 25 °C. The reaction was initiated by addition of enzyme from a 1.5 μM (0.03 mg/ml) stock. The volume of the final reaction mixture was 1 ml. The fluorescence of the released NHMec was followed continuously for 3 min (ex: 360 nm; em: 460 nm). Reaction rates were calculated from the linear portion of the progress curve. For quantification of the fluorescence data, a standard solution of NHMec in DMSO (3.0 mg/ml or 0.017 M) was prepared gravimetrically. A calibration curve correlating fluorescence emission versus concentration of NHMec was constructed that followed a linear dependency ($R^2=0.9998$). This relation was used to convert initial rates of amidolysis from fluorescence intensity change/min to μM of substrate converted/min.

2.4. Inhibition of amidolysis

Bromelain in buffer was incubated with 0–80 mM of SDS for 2 h at 25 °C. For the determination of kinetic parameters of hydrolysis of Z-Arg-Arg-NHMec by the preincubated enzyme at each SDS concentration, measurements of the initial rates of reaction were made as described above. By varying the enzyme volume between 10 and 100 μl in lieu of the activation buffer in the assay mixture, detection of as low as 3% of enzyme activity was possible. After activation of the preincubated enzyme, the reaction was initiated by addition of the substrate (5–50 μM) to a final volume of 1 ml. The K_m and V_{max} values were determined from Lineweaver–Burk plots ($1/v$ versus $1/[S]$) of the initial reaction rates.

2.5. Data analysis

Initial rates of enzyme hydrolysis in the presence and absence of SDS were fit by non-linear least squares analysis to Michaelis–Menten equation (Eq. (1)),

$$v = \frac{V_{max}\cdot[S]}{K_m + [S]} \tag{1}$$

where v, V_{max}, K_m and $[S]$ represent the initial reaction velocity, maximum velocity, Michaelis–Menten constant and substrate concentration respectively.

The effect of SDS as an inhibitor on the initial reaction velocities of amidolysis by bromelain at a fixed concentration of the substrate was characterized using dose response curves, best represented by Eq. (2) [22].

$$y = \frac{y_{max} - y_{min}}{1 + \frac{[I]}{IC_{50}}} + y_{min} \tag{2}$$

where y is the fractional activity (v_i/v_0) of the enzyme in the presence of inhibitor at concentration $[I]$. Here v_i and v_0 represent the initial velocity in the presence of inhibitor at concentration $[I]$ and initial velocity in the absence of the inhibitor respectively. y_{max} is the maximum value of y that is observed at zero inhibitor concentration, y_{min} is the minimum value of y that can be obtained at high inhibitor concentrations and IC_{50} is the inhibitor concentration required to achieve 50% inhibition. The dose response curves at different substrate concentrations were plotted using Eq. (2), on a linear y-axis scale and logarithmic x-axis scale i.e. v_i/v_0 versus log $[I]$. The IC_{50} values at the corresponding substrate concentrations were determined graphically from these plots. The type of inhibition was determined from a