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Insights into the interactions between enzyme and co-solvents: Stability and activity of stem bromelain

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In present study, an attempt is made to elucidate the effects of various naturally occurring osmolytes and denaturants on BM at pH 7.0. The effects of the varying concentrations of glycerol, sorbitol, sucrose, trehalose, urea and guanidinium chloride (GdnHCl) on structure, stability and activity of BM are explored by fluorescence spectroscopy, circular dichroism (CD), UV–vis spectroscopy and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Our experimental observations reveal that glycerol and sorbitol are acting as stabilizers at all concentrations while sucrose and trehalose are found to be destabilizers at lower concentrations, however, acted as stabilizers at higher concentrations. On the other hand, urea and GdnHCl are denaturants except at lower concentrations. There is a direct relationship between activity and conformational stability as the activity data are found to be in accordance with conformational stability parameters (ΔG_u, T_m, ΔC_p) and BM profile on SDS-PAGE.

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1. Introduction

Considerable time in nearly all fields of biochemical sciences is devoted in developing empirical formulations and protocols for retaining the stability and activity of proteins. Being protein stabilization a raised and puzzled issue in scientific research, it is not a small and simple problem in living systems [1–4]. Proteins are very sensitive and highly complex molecules which are stabilized as a result of a balance between the intramolecular interactions of protein functional groups and their interactions with solvent environment [5–8]. This balance can be disturbed by the addition of the osmolytes due to their special properties by which they can affect electrostatic interactions, steric interactions, conformational entropy, hydrogen bonding and hydrophobic interactions contributing towards protein stability [9–12]. Thus, these physiologically important small compounds can be used to better understand the thermodynamics of protein folding/unfolding studies, which reveals the interaction of the different osmolytes functional groups with the protein side chains and peptide backbone so that a long term stability can be aroused which is a big problem encountered in the preparation and administration of the protein therapeutics.

Stem bromelain (BM) is a major basic cysteine proteinase, a glycoprotein isolated from pineapple (*Ananas cosmosus*) which contains a asparagine linked single hetero oligosaccharide unit per molecule comprising six neutral sugars located at the middle of this enzyme with residues Asn-Glu-Ser (117–119), demonstrating closely related proteinases in vitro and in vivo [13–16]. Glycosylation of BM protein is of biological importance for its stability and activity which was elucidated by Khan et al. [17]. BM, which is administrated orally, absorbed through gastrointestinal tract and found to be in highest concentration after 1 h of administration, is antidematous, antiflammatory, antithrombotic, fibrinolytic, antitumoral, immunomodulator, antimetastic, antiinvasive, etc. [18,19]. BM, like other cysteine proteinases, belongs to the $\alpha+\beta$ protein class with 23% helix, 18% antiparallel sheet, 5% parallel sheet, 28% turns and the remaining 35% as other structure [20,21]. BM is 212 amino acid residues enzyme with molecular weight 23,800 Da containing three disulphides and a single free cysteine residue (Cys) and has amino acid sequences highly similar to chymopapain, papain, actinidin and proteinase [13,16,19]. BM is autocatalytic in nature [18,19]. Aromatic residues such as phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr) are six, five and fourteen in number, respectively [13].

The thermal denaturation of BM is completely irreversible and apparently follows two state mechanism of folded to denatured conformation type [20]. Moreover, according to Gupta et al. [22] unfolding/folding behavior of BM is independent of the disulphide bonds. From the last few studies on the folding of BM, a partially

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folded structure is detected at pH 2.0 and molten globule state is around pH 0.8 [23,24]. A number of investigations have examined the polyhydroxy osmolytes as stabilizers for various biomolecules [25–28]. Habib et al. [29] reported trehalose and sucrose acted as a destabilizer for BM. According to Khan et al. [17,30], alcohols are destabilizers and deactivators of native state of BM while urea and GdnHCl induced structures of BM are functionally active at lower concentration. Despite widespread efforts (>40 years) on the influence of these osmolytes on protein stability, the exact mechanism of the modulation of the water structure of proteins by polyhydroxy osmolytes is still not well understood.

BM is quite important enzyme from chemical and pharmaceutical point of view. BM kills the lymphatic cells, probacteria, parasite and bacillus tuberculosis, help in diminishing inflammation, normalize functioning of gall bladder, alleviate pain, promote digestion and clean soft lens. It is also used to synthesize aspartame, antitumor compounds and bioactive peptides [19,31]. These wide applications in various fields of chemical and pharmaceutical industries, have drawn our interest in studying the complete thermodynamic profile associated with the conformations of BM in presence of osmolytes and denaturants. The information derived from thermodynamic profile about the interactions between protein and osmolytes/denaturants can be utilized to induce a better form of BM with more therapeutic properties than native form. Moreover, understanding the structural and functional properties of BM provides insight into the mechanism underlying its clinical utility and also fulfills the purpose of storage, transport and shelve-life during industrial applications.

To the best of our knowledge, this work represents a complete structural and thermal stability study of BM and the results shed new light on the mechanism of stabilization of protein in folded state ensemble. In this context, we analyze the structure, stability and activity of the BM as a function of co-solvents with the objective of determining structure–function relationship of BM by using biophysical methods.

2. Materials and methods

2.1. Materials

Bromelain (E. C. 3.4.22.32) lot No. B4882 from Ananas cosmosus, glycerol, sorbitol, sucrose, trehalose, urea and GdnHCl were purchased from Sigma–Aldrich, USA. Anhydrous sodium phosphate monobasic and sodium phosphate dibasic dihydrate of highest purity and analytical grade purchased from Sisco Research Lab (SRL), India. For the study of proteolytic activity, casein (refined) and coomassie brilliant blue (CBB R-250) were also purchased from the SRL, India. Acrylamide, glycine, ammonium per sulphate and sodium dodecyl sulphate from Sigma and bis acrylamide and N,N,N′,N′-tetramethylethylenediamine from Himedia (India) were used for sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). All other chemicals used were of analytical grade with highest purity.

2.2. Sample preparations

The enzyme samples were prepared at 0.5 mg/mL concentration of BM in 0.2 M sodium phosphate buffer at pH 7 for all measurements. A different concentration of BM was used for gel electrophoresis i.e. ∼13 μg/10 μl. Mettler Toledo balance with a precision of ±0.0001 g was used for all gravimetric measurements. All mixture samples were prepared using distilled deionized water with resistivity of 18.3 Ωcm. After completely dissolving the enzyme in the solution, the mixture was filtered with 0.45 μm disposal filter (Millipore, Millex-GS) through syringe before performing experiments. All samples were incubated for 15 min at 25 °C in order to obtain complete equilibrium. The stability and activity of BM were studied in presence and absence of 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M of osmolytes except upto 2 M for trehalose for all experiments because of its solubility problem. In case of denaturants, all experiments were performed till 6 M and 8 M for GdnHCl and urea, respectively.

2.3. Thermal equilibrium unfoldings of BM in presence of osmolytes or denaturants

Osmolytes and denaturants induced equilibrium unfolding studies of the BM are conducted over a temperature range 15–90 °C, using Trp fluorescence as a probe. These thermal denaturation studies are carried out in Cary Eclipse fluorescence spectrofluorimeter (Varian optical spectroscopy instruments, Mulgrave, Victoria, Australia) equipped with an intense Xenon flash lamp as the light source equipped with a Peltier-type temperature controller with a precision of ±0.05 °C at a heating rate of 1 °C min^{-1} providing 15 min for equilibration. The change in absorbance with increasing temperature is followed at an excitation wavelength of 295 nm in order to avoid the radiation energy transfer from other fluorescent residues to the tryptophan (Trp) residues for the overall fluorescence emission. All unfolding thermal transitions of BM at pH 7.0 are averaged values of three concordant readings and are analyzed by assuming this small globular protein closely approaching to the two state folding mechanism such as shown in Eq. (1).

$$\mathrm{N} \rightleftharpoons \mathrm{U} \tag{1}$$

$$f_u = \frac{(y_f - y)}{y_f - y_u} \tag{2}$$

An essential precondition for extracting the complete thermodynamic profile of protein unfolding is to determine the dependence of intrinsic Trp fluorescence intensity in native and denatured ensembles on temperature. Sigmoidal fluorescence intensity curves are obtained for BM in presence of the various osmolytes and denaturants. Experimentally observed properties i.e. y_f and y_u are assumed to be linearly temperature dependent intensities of native and denatured state, respectively, which are depicted by the extrapolations of the pre and post-transition baselines of thermal denaturation curve using linear fitting method. A significant amount of folded and unfolded protein concentration is present at T (K) with total protein composition ($f_f + f_u$) unity where f_f and f_u are the fraction of folded and unfolded ensembles under experimental conditions by using y_f, y_u and $y(T)$ as shown in Eq. (2). The equilibrium constant (K) can be obtained by using following equation:

$$K = \frac{f_u}{f_f} = \frac{f_u}{(1 - f_u)} = \frac{(y_f - y)}{(y - y_u)} \tag{3}$$

$$\Delta G = -RT \ln K \tag{4}$$

By using equilibrium constant (K), free energy change of unfolding (ΔG_u) at a temperature T (K) can be obtained according to the relation shown in Eq. (4).

The value of transition temperature (T_m) and the enthalpy change of unfolding (ΔH_m) at a condition where ΔG_u is zero, can be obtained by analysis of the plot of ΔG_u versus T (°C). The slope of this plot at T_m yields the entropy change of unfolding (ΔS_m). The ΔH_m was calculated using the following equation.

$$\Delta H_m = T_m \Delta S_m \tag{5}$$

Other thermodynamic parameters such as enthalpy change of unfolding (ΔH_u) and entropy change of unfolding (ΔS_u) at any temperature, are associated with the value heat capacity change (ΔC_p)

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