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Amyloidogenic behavior of different intermediate state of stem bromelain: A biophysical insight

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ABSTRACT

Stem bromelain, a cysteine proteases from *Ananas comosus* is a widely accepted therapeutic drug with broad medicinal application. It exists as intermediate states at pH 2.0 and 10.0, where it encountered in gastrointestinal tract during adsorption (acidic pH) and in gut epithelium (alkaline pH), respectively. In this study, we monitored the thermal aggregation/amyloid formation of SB at different pH intermediate states. Thermal treatment of stem bromelain at pH 10.0 favors the fibrillation in which the extent of aggregation increases with increase in protein concentration. However, no fibril formation in stem bromelain at pH 2.0 was found at all the concentration used at pH 10.0. The fibril formation was confirmed by various techniques such as turbidity measurements, Rayleigh light scattering, dye binding assays and far UV circular dichroism. The Dynamic light scattering confirmed the formation of aggregates by measuring the hydrodynamic radii pattern. Moreover, microscopic techniques were performed to analyze the morphology of fibrils. The aggregation behavior may be due to variation in number of charged amino acid residues. The less negative charge developed at pH 10.0 may be responsible for aggregation. This work helps to overcome the aggregation related problems of stem bromelain during formulations in pharmaceutical industry.

1. Introduction

Protein aggregation is associated with various neurodegenerative disorders including prion disease and several form of amyloidosis as Alzheimer's disease [1–3]. One of the major hurdle in the field of biotechnology is protein aggregation, during the process of protein purification, processing, adsorption and storage [4]. Additionally, aggregation of proteins remains an important factor behind thermo-inactivation [5], which is undesirable especially for industrially important enzymes. Aggregation occurs when unassembled or misfolded proteins improperly expose hydrophobic surfaces that are normally buried in the protein interior's region or takes place at the interface of subunits on exposure of extreme pH and temperature [6]. Under *in vitro* conditions aggregation occurs by employing harsh conditions such as acidic or alkaline pH, by the use of cosolvents, high temperature, high pressure, metal ions, lipid assemblies and surfactants [7–9]. Among these, temperature and pH are most recurrent factor that affects the progression of aggregation process [10–14]. This is because of unfolding and hydrophobic exposure at high temperature [15,16]. Conformational changes also occur in proteins due to change in pH followed by heat treatment. They are commonly recognized to play a pivotal role in the aggregation process, since they may promote the onset of new intermolecular interactions. Besides these, any conformational or structural alterations in the free –SH group also lead to aggregate formation, as they are more exposed to new intermolecular interaction [17]. At elevated temperature, hydrophobic interactions as well as frequency of molecular collision increase that lead to enhancement of protein aggregation process [18]. Large aggregates formed as a consequence of new intermolecular interactions, arises due to increase in flexibility of nature and compact structure of proteins at high temperature [19].

Stem bromelain [EC 3.4.22.32], which is a crude extract from pineapple *Ananas comosus*. It is widely accepted as a potential phytotherapeutic drug due to its broad medicinal application such as reversible inhibition of platelet aggregation, bronchitis, sinusitis, angina pectoris. enhances adsorption of drugs specially antibiotics [20,21]. In addition to antithrombotic and fibrinolytic effects it has also anti-inflammatory and analgesic actions. Anti-inflammatory

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activity of SB is mediated by various factors like increased serum fibrinolytic activity, reduced plasma fibrinogen levels and reduced vascular permeability due to decreased bradykinin levels [22–24]. Like other cysteine proteases SB belongs to the α+β protein class with 23% alpha helix, 5% parallel beta sheet, 18% anti-parallel β-sheet, 28% turns and rest other secondary structures [25,26]. Stem bromelain, unlike most enzymes, has a very wide effective range of activity at both acidic and alkaline conditions that allows it to remain active in a variety of biological environments [27]. During their adsorption, when administered orally, SB encounters low pH in the stomach followed by an alkaline pH in the intestine. Previous studies have demonstrated that SB adopts partially folded intermediate in acidic [28], as well as specific molten globule state at basic pH [29]. At acidic pH the occurrence of PFI state of SB was inferred by various approaches like conformational changes in the vicinity of surface of the exposed hydrophobic tryptophan that results in internalization in the hydrophobic environment [28]. Further, retention of some secondary structural features as well as presence of large number of solvent accessible non-polar clusters in the protein molecules indicates occurrence of PFI as reported in earlier reports [28]. At basic pH (10.0) stem bromelain undergoes conformational rearrangement in which the hydrophobic core becomes more compact and accessible [29]. Further, retention of most of the secondary structural features with disruption of tertiary contacts also indicates that SB acquires molten globule state at basic pH [29]. Although considerable progress have been made in studying the behavior of SB at different state, as well as an effect of various surfactants for aggregation induction, the effect of temperature on SB at both (acidic and basic) conditions is still not fully deciphered. Due to different structure and composition of these intermediates, they show different aggregation behaviors at different pH during heat treatment.

Here, we attempted to find out effect of temperature on aggregation behavior of SB at both pH 2.0 (net positive charge) and 10.0 (net negative charge) by employing various techniques. The protein secondary structural changes were monitored through far-UV circular dichroism spectroscopy. The nature of aggregated particles were analyzed by turbidity measurements, ThT binding, fluorescence microscopy and transmission electron microscopy. Particle size was measured by using dynamic light scattering.

2. Material and methods

2.1. Reagents

Stem bromelain (from *Ananas comosus* B 4882), 1-anilino-8-naphthalene sulfonate (ANS) and Thioflavin T (ThT) were procured from Sigma–Aldrich Chemical Co., St. Louis, MO, USA. All other reagents used were of analytical grade. Double deionized water, free from all fluorescent contaminant, was used throughout the study. Glycine-HCl buffer of pH 2.0 and Glycine-NaOH buffer of pH 10.0 were used. Prior to use buffers and stock solutions were filtered using PVDF 0.45 μm syringe filters (Millipore Milex-HV).

2.2. Sample preparation

Stem bromelain was dissolved in sodium phosphate buffer (20 mM) at pH 7.4. To avoid complications due to autocatalysis, sodium tetrathionate (5 mM) was added to buffer prior to protein addition, for inactivation of its proteolytic activity. Protein solution was dialyzed extensively against the 20 mM sodium phosphate buffer and subjected to size-exclusion chromatography [30]. Protein concentration was determined by Perkin-Elmer Lambda 25 double beam UV–vis spectrophotometer and extinction coefficient ($E^{1\%}_{280nm}$ = 20.1) was used. Molecular weight of protein was taken as 23,800 Da [21]. Protein samples at 25 °C at both pH served as control for all measurements.

2.3. pH measurements

pH measurements were carried out on a Mettler Toledo pH meter (seven easy S 20-K) using an Expert "Pro3 in 1" type electrode. pH was adjusted by HCl and NaOH for pH 2.0 and pH 10.0 respectively.

2.4. Protein charge determination

The charge on stem bromelain at various pH was calculated using the program protein calculator v3.4 [31].

2.5. Turbidity measurements

Turbidity measurements of SB (5 μM and10 μM) at pH 2.0 and 10.0 were taken from 0 to 24 h at 65 °C. The measurement of aliquots was carried out at 350 nm on a PerkinElmer Lambda 25 double beam UV–vis spectrophotometer, in a cuvette of 1 cm path length.

2.6. Rayleigh light scattering measurements

Rayleigh scattering experiments were performed on a Shimadzu (RF-5301PC) fluorescence spectrophotometer. Samples were incubated with specified pH (pH 2.0 and 10.0) at 65 °C for 0–24 h. To obtain reliable data the scattered intensity was averaged for 3 samples for each concentration. Samples were excited at 350 nm and spectra were recorded from 300 to 400 nm. Both the excitation and emission slit widths were set at 1.5 nm.

2.7. Thioflavin T (ThT) binding assay

Thioflavin T (ThT) was dissolved in double distilled water and filtered through 0.45 μM syringe filter. Concentration of ThT was determined by using a molar extinction coefficient (E_M = 36,000 M^{-1} cm^{-1}) at 412 nm [32]. Protein samples were incubated for 0–12 h at both pH conditions i.e., at pH 2.0 and 10.0. ThT was added to aggregate samples of protein in a molar ratio of 2:1 followed by 30 min of incubation. ThT fluorescence spectra were recorded on Shimadzu (RF-5301PC) fluorescence spectrofluorometer. The intensity of the excitation was 440 nm and emission intensity was in the range of 450–600 nm. Both excitation and emission slit widths were fixed at 5 nm. Spectra were subtracted from appropriate blanks.

2.8. ANS fluorescence measurements

ANS is the extrinsic fluorescent dye with susceptibility towards exposed hydrophobic patches and therefore a means for symbolizing the molten globule states and certainly the surface hydrophobicity in proteins could be resolved smoothly [33,34]. In order to scrutinize the exposed hydrophobic patches upon heat induced aggregation of Stem bromelain, ANS binding assays were executed. ANS was properly dissolved in distilled water and filtered with 0.45 μM syringe filter followed by measurement of concentration using molar extinction coefficient, E_M = 5000 M^{-1} cm^{-1} at 350 nm. The ANS fluorescence spectra of aliquots incubated for varying time (0–12 h) at different pH (pH 2.0 and 10.0) were observed on Shimadzu (RF-5301PC) fluorescence spectrophotometer equipped with water circulator (Julabo Eyela). Prior to measurements, aliquots were incubated with 20 fold molar excess of ANS for 30 min in dark. For ANS fluorescence, emission spectra