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Concentration-dependent antagonistic persuasion of SDS and naphthalene derivatives on the fibrillation of stem bromelain

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ABSTRACT

Sodium dodecyl sulfate, a biological membrane mimetic, can be used to study the conversion of globular proteins into amyloid fibrils *in vitro*. Using multiple approaches, the effect of SDS was examined on stem bromelain (SB), a widely recognized therapeutic protein. SB is known to exist as a partially folded intermediate at pH 2.0, situation also encountered in the gastrointestinal tract (its site of absorption). In the presence of sub-micellar SDS concentration (500–1000 μM), this intermediate was found to exhibit great propensity to form large-sized β-sheeted aggregates with fibrillar morphology, the hall marks of amyloid structure. We also observed inhibition of fibrillation by two naphthalene-based compounds, ANS and bis-ANS. While bis-ANS significantly inhibited fibril formation at 50 μM, ANS did so at relatively higher concentration (400 μM). Alcohols, but not salts, were found to weaken the inhibitory action of these compounds suggesting the possible involvement of hydrophobic interactions in their binding to protein. Besides, isothermal titration calorimetry and molecular docking studies suggested that inhibition of fibrillation by these naphthalene derivatives is mediated not just through hydrophobic forces, but also by disruption of π–π interactions between the aromatic residues together with the inter-polypeptide chain repulsion among negatively charged ANS/bis-ANS bound SB.

Introduction

The self-assembly of proteins caused by their aberrant misfolding and subsequent aggregation into amyloid fibrils is associated with several clinical disorders including Alzheimer's, Parkinson's, Type II diabetes and many others [1,2]. In addition to their *in vivo* formation, the amyloid fibrils can also be generated from many different unrelated peptides and proteins under carefully chosen condition. This observation suggests that fibril formation is an inherent property of polypeptide chain, though individual propensity may vary with the amino acid sequence as well as environmental conditions [3]. In past, there has been intense discussion on how a normally soluble protein spontaneously converts into pathogenic amyloid structure. A number of structurally unrelated compounds have been probed for their ability to weaken the intermolecular interactions that stabilize the fibrils and to inhibit the self-assembly of protein [4]. However despite immense investigation the comprehensive details of the mechanism underlying protein aggregation is yet not clear and currently there is no real cure available for treating the devastating diseases.

Stem bromelain (SB)[1] [EC 3.4.22.32], a member of C1A family of proteolytic enzymes is obtained from *Ananas comosus* [5]. SB is widely accepted as a potential phytotherapeutic drug due to its broad medicinal applications such as inhibition of platelet aggregation, angina pectoris, bronchitis, sinusitis, surgical traumas, thrombophlebitis, pyelonephritis and enhanced absorption of drugs particularly antibiotics, analgesic, anti-inflammatory, antitumoral

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[1] *Abbreviations used:* ANS, 1-anilino-8-napthalene sulfonate; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate; CD, circular dichroism; CMC, critical micellar concentration; ITC, isothermal titration calorimetry; PFI, partially folded intermediate; RLS, Rayleigh light scattering; SDS, sodium dodecyl sulfate; SB, stem bromelain; TEM, transmission electron microscopy; ThT, thioflavin T.

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and antituberculosis activity etc. [6–8]. It has been proposed that these effects of SB may originate from its unusual ability to traverse membranes [9,10]. Previous report from our lab has revealed that SB exists as partially folded intermediate (PFI) at pH 2.0, a state characterized by ~42% of the native secondary structure, disrupted tertiary contacts and exposed hydrophobic clusters [11]. A similar situation is also encountered by this protein inside the acidic environment of stomach (pH ~2.0) following oral administration. The partially folded intermediates and other related states are known to possess greater propensity for aggregation owing to the fact that they have less ordered conformation and exposed hydrophobic clusters [12]. Since SB has to traverse through the gut membrane before its absorption into the blood and the fact that peptides/proteins become fibrillar and toxic upon interaction with membrane components such as lipids [13,14], the study of SB interaction with membrane become all the more important.

Sodium dodecyl sulfate (SDS), an anionic surfactant, has long been used to mimic the *in vivo* interaction of proteins with biological membranes and their subsequent conversion into amyloid fibrils [15–18]. SDS solution greatly reduces turbidity and light scattering, normally encountered in lipid–peptide interaction studies, thereby making spectroscopic studies easier [19]. Using a combination of spectroscopic techniques as well as microscopy, we demonstrate that in the presence of sub-micellar concentration of SDS, the PFI of SB exhibits high propensity to form aggregates with prominent β-sheet structure and fibrillar morphology.

Further, we monitored the effect of two naphthalene derivatives i.e. ANS (1-anilino-8-napthalene sulfonate) and bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate) on the SDS-induced fibrillation of SB. Studies on usage of ANS and bis-ANS (Fig. 1) in identifying the folding intermediates and detection of protein aggregates are abundant in literature [20]. However, only few studies have focused on their effect on inhibition of amyloid formation [21–23]. With brief explanation, such inhibitory potential of these compounds, particularly bis-ANS, is attributed to their hydrophobic interaction with protein thereby preventing aggregation. Our data, however, demonstrates that the protective effect of bis-ANS and ANS against the self-assembly of SB is exerted not only through hydrophobic interactions but there is possible involvement of other noncovalent forces particularly π–π interactions.

Materials and methods

Materials

SB from *A. comosus* (B4882), SDS (sodium dodecyl sulfate), ANS (1-anilino-8-naphthalene sulfonate), bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate) and ThT (thioflavin T) were purchased from Sigma–Aldrich Chemical Co., St. Louis, MO, USA. All other reagents used were of analytical grade.

Sample preparation

SB was dissolved in 20 mM sodium phosphate buffer pH 7.4 containing 5 mM sodium tetrathionate for inactivation of the proteolytic activity. The protein solution was dialyzed extensively and subjected to size-exclusion chromatography as described elsewhere [24]. Protein concentration was determined using $\varepsilon^{1\%}_{280\,nm} = 20.1$ on Perkin Elmer Lambda 25 UV–Visible spectrophotometer [25]. The molecular weight of protein was taken as 23,800 Da [11]. The stock solution of SDS was prepared in 20 mM Gly–HCl buffer pH 2.0. Concentration of ANS and bis-ANS was determined using $\varepsilon_{350\,nm} = 5000\,M^{-1}\,cm^{-1}$ and $\varepsilon_{385\,nm} = 16,790\,M^{-1}cm^{-1}$ respectively [26,27]. The stock solutions and buffers were filtered through 0.45 μm syringe filter.

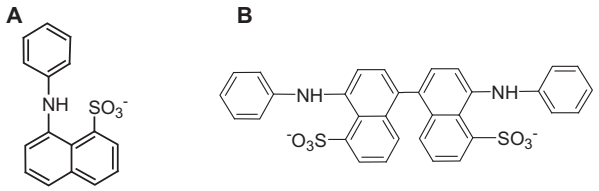

Fig. 1. Chemical structures of naphthalene derivatives. (A) 1-anilinonaphthalene-8-sulfonate (ANS) and (B) 4,4′-dianilino-1,1′-binaphthyl-5,5′-sulfonate (bis-ANS).

To obtain partially folded intermediate (PFI) state, 5 μM SB was incubated in Gly–HCl buffer pH 2.0 for 1 h at 25 °C. Aggregation was induced by incubating the protein samples (pH 2.0) with desired range of SDS concentration (0–8000 μM) for 4 h. For study of aggregation inhibition, the PFI of SB was incubated with desired concentration of ANS and bis-ANS for 1 h prior to the addition of SDS. Unless otherwise mentioned, the protein concentration in all the experiments was 5 μM.

The samples for pKa determination of naphthalene derivatives were prepared by incubating desired concentration of ANS/bis-ANS in 20 mM of following buffers: KCl–HCl (pH 0.6–1.5), Gly–HCl (pH 2.0–3.0), sodium acetate (pH 3.5–5.5), sodium phosphate (pH 6.0–7.5), tris–HCl (pH 8.0–8.5), Gly–NaOH (pH 9.0–10.0) and also in 0.1 M HCl or NaOH for at least 4 h in dark. Samples for assaying the effect of salts and alcohols on PFI were prepared by incubating the PFI with 500 μM $NaCl/Na_2CO_3/Na_2SO_4$ or 30% methanol/ethanol/isopropanol for at least 4 h.

pH determination

pH measurements were carried out on Mettler Toledo pH meter (Seven Easy S20–K) using Expert "Pro3 in 1" type electrode. The least count of the pH meter was 0.01 pH unit.

Conductivity measurements

The conductivity measurements were performed on an ELICO (type CM 82T) bridge equipped with platinized electrodes (cell constant = 1.02 cm^{-1}). The experiment was carried out in thermostated water bath. The buffer solution (20 mM Gly–HCl pH 2.0) was equilibrated at 25 °C for 30 min before the addition of the suitably prepared concentrated stock solution of SDS. After each addition, the solution was mixed carefully ensuring that no foam is formed and conductivity was recorded. The critical micellar concentration, CMC was determined from the break point in the specific conductance (κ) versus [SDS] profile assuming the conductivity to be linearly related to the surfactant concentration. The experimental error in the temperature was 0.5 °C.

Rayleigh light scattering

Rayleigh light scattering (RLS) experiment was performed on Hitachi F-4500 fluorescence spectrophotometer at 25 °C in 1 cm path length cuvette. Protein samples under desired conditions were excited at 350 nm and spectra were recorded from 300 to 400 nm. Both excitation and emission slits were fixed at 5 nm. The equilibrium data obtained from light scattering measurements was fitted using Sigma plot 12.0 to single exponential equation:

$$F = F_o e^{-\Lambda[I]} \quad (1)$$

where F_o and F are the fluorescence intensity at 350 nm in the absence and presence of inhibitor, Λ is the inhibition constant and $[I]$ is the concentration of inhibitor (ANS/bis-ANS).