Dominant effect of ethanol in thermal destabilization of bovine serum albumin in the presence of sucrose

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Abstract. The thermal melting of bovine serum albumin (BSA) in the presence of excipients like ethanol and sucrose was studied by circular dichroism spectroscopy at physiological pH in phosphate buffered saline. Calculated apparent T_m values were used to assess the thermal stability using two state fitted experimental curves. 0.5 M sucrose stabilized the BSA indicated by the increase in T_m of $\sim 8^\circ\text{C}$ when compared to the T_m of the same solution measured in the absence of sucrose. Conversely, in the presence of varying concentrations of ethanol (2–20%), the protein was destabilized by a decrease of $\sim 3\text{--}10^\circ\text{C}$ of T_m . In the binary mixture of sucrose and ethanol, the T_m values showed that ethanol dominantly destabilized BSA in the presence of sucrose, possibly by weakening the hydrophobic interactions in the protein.

Keywords: Ethanol, sucrose, bovine serum albumin, circular dichroism spectroscopy

1. Introduction

Alcohols such as ethanol critically affect many biological processes which includes neurotransmission and immune responses [6,9]. Studying the effect of ethanol on the conformational stability of proteins like albumin is essential since albumin is the most abundant blood plasma protein that plays key role in the transport of hormones, anesthetics, endogenous ligands and fatty acids in blood [5]. Bovine serum albumin (BSA), which is homologous to human serum albumin (HSA), is a single polypeptide chain with 583 amino acid residues and three domains named I–III. Each domain is subdivided into two more domains (A and B) that are predominantly helical and cross-linked by several disulfide bridges [4]. Direct binding of ethanol to the hydrophobic binding sites on fatty acid free bovine serum albumin (BSA) was reported [1]. The study showed that the presence of ethanol inhibited the binding of 1-anilinonaphthalene-8-sulfonic acid (1, 8-ANS) and *cis*-parinaric acid to BSA. Also, ethanol's effect on the thermal stability of HSA was studied by Differential scanning calorimetry (DSC) very recently [13]. The study showed that the presence of ethanol decreased the thermal stability of HSA. The effect of ethanol in decreasing the thermal stability of proteins is attributed to the perturbation of the intramolecular hydrophobic interactions [8].

While ethanol thermally destabilizes these proteins, sugars such as sucrose are generally known to stabilize proteins. Sucrose stabilization is explained by the preferential exclusion phenomenon [12].

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Proteins are preferentially hydrated in the presence of sucrose which enhances the thermal stability of the same [2]. Understanding protein stabilization and destabilization mechanisms in a multi solvent system is garnering attention specifically in industries due to formulation efforts [16]. The effects of stabilizing and destabilizing cosolvents on proteins have been studied separately but their combined effects on altering protein's physicochemical properties are limited. Here we have attempted to study the combined effect of ethanol and sucrose on the thermal stability of BSA by circular dichroism spectroscopy.

2. Materials and methods

2.1. Materials

Bovine serum albumin (globulin free of 96% purity) was purchased from Sigma Aldrich. The protein was dialyzed overnight against 20 mM sodium phosphate buffer, pH 7.0. The concentration of BSA (0.2 mg/ml) was measured by UV Spectrophotometer using $E1\% 1\text{ cm} = 6.8$ at 280 nm [10]. The sucrose concentration was 0.5 M while the ethanol concentrations varied between 2% and 20% v/v. BSA samples were incubated with ethanol and sucrose for 30 minutes before the measurements. The pH of the albumin solutions was not affected by ethanol and sucrose.

2.2. Circular dichroism spectroscopy

CD measurements were taken with a Jasco 715 Spectropolarimeter equipped with a computer-controlled water bath, and samples were placed in a cylindrical jacketed cuvette of 1 mm optical path length. The thermal melting was monitored at 222 nm from 25°C to 95°C at 1°C/min rate. The thermal unfolding of BSA is only partially reversible when cooled back. Therefore the T_m values that were calculated in this study by fitting with two state equations [15] are apparent T_m values. Also, the stability that is mentioned throughout this article is related to the α -helical structure of BSA resisting thermal denaturation.

3. Results and discussion

3.1. Effect of 0.5 M sucrose on the thermal stability of BSA

The CD thermal melting curves for both BSA and BSA in the presence of 0.5 M sucrose (BSA_{suc}) are shown in Fig. 1. To facilitate the comparison, the mdeg values were normalized for BSA concentration. The starting ellipticity value is consistent with the literature [11]. Both samples were heated from 25°C to 95°C at 1°C/min rate, and cooled back to check for the protein's reversibility. The cooling curves were only partially reversible (data not shown). The apparent T_m values that were calculated using two state fitting equations are shown in Table 1. BSA does not lose its secondary structure even when heated to 95°C probably due to the constraint of disulfide bonds. The presence of sucrose stabilizes BSA by about 8°C. One similar study where BSA was heated in the presence of 40% sucrose by DSC showed an increase of 7°C [2]. Sucrose does not induce conformational changes at lower temperatures, as evident by the overlay of the melting curves of both samples up to 55°C, which is the onset temperature of

Fig. 1. Thermal melting curves of BSA monitored at 222 nm by CD spectroscopy. The change of mean residue ellipticity at 222 nm with temperature upon continuous heating ($1^{\circ}\text{C}/\text{min}$) is shown both in the absence (solid) and presence (dot) of 0.5 M sucrose. The apparent T_m values calculated from the two state fitting of experimental curves are shown in Table 1. Presence of sucrose increased both the onset and melting temperatures of BSA.

Table 1

Apparent T_m values calculated for BSA and BSA_{suc} in varying concentrations of ethanol

Sample	Apparent T_m ($^{\circ}\text{C}$)
BSA	70.2 ± 0.1
In ethanol	
2%	67.9 ± 0.07
5%	66.7 ± 0.06
10%	67.0 ± 0.07
20%	59.9 ± 0.05
BSA _{suc}	78.1 ± 0.3
In ethanol	
2%	70.5 ± 0.04
5%	69.4 ± 0.12
10%	69.4 ± 0.10
20%	60.0 ± 0.05

unfolding for BSA. The onset temperature of BSA_{suc} is higher by $5\text{--}7^{\circ}\text{C}$. Sucrose is preferentially depleted from proteins surface which in turn strengthens the water structure around the protein molecules and hence intensifies the hydrophobic interactions in the protein [3]. This explains the enhanced thermal stability of BSA_{suc}.

3.2. Effect of ethanol on the thermal stability of BSA

Figure 2 shows the effect of increasing the concentration of ethanol on the thermal stability of BSA. While the apparent T_m value of BSA is 70.2°C , the presence of ethanol destabilizes BSA thereby de-

Fig. 2. Thermal melting curves of BSA monitored at 222 nm by CD spectroscopy in the presence of 0% (filled circle), 2% (open circle), 5% (cross), 10% (filled triangle) and 20% (open triangle) of ethanol. The apparent T_m values (Table 1) calculated from the fitting of the experimental curves show that as the concentration of ethanol increases, the thermal stability of BSA decreases.

creasing the T_m values (Table 1). An ethanol concentration less than 1% v/v does not have an effect on the thermal stability of BSA. Thus the data shown here are from 2% v/v concentration, which decreased the T_m by 3°C. The thermal stability is not affected between 5% and 10% v/v ethanol concentration. T_m values remain constant between those concentrations of ethanol, but 20% v/v ethanol decreases the T_m by almost 10°C. Direct binding of ethanol to BSA was studied by Fluorescent and ^{13}C NMR [1]. In this study, it was found that ethanol prevents the binding of *cis*-parinaric acid to three out of five fatty acid binding sites in BSA. It was concluded that ethanol is selective in its interaction with the hydrophobic sites of BSA because the methyl group of ethanol was discovered to bind to the hydrophobic pocket of BSA. Our study found that binding of ethanol decreases the thermal stability of BSA which can be explained as follows. Ethanol competes with the bound endogenous fatty acids in BSA and most likely replaces them. This substitution disrupts the intramolecular hydrophobic interactions. Disruption of hydrophobic interactions naturally leads to a decrease in the thermal stability. We have not observed any induction of non-native helical structures of BSA in the presence of ethanol. Because it is highly hydrophobic with a lower dielectric constant, it might not have a strong influence in inducing non native secondary structure except for protein denaturation [14].

3.3. Effect of ethanol on BSA_{suc}

In order to understand the combined effects of sucrose and ethanol, we studied the influence of varying concentrations of ethanol on the thermal stability of BSA_{suc} (Fig. 3). The dominant effect of ethanol in destabilizing BSA_{suc} is clearly shown by the apparent T_m values (Table 1). Despite of being stabilized by 0.5 M sucrose, the presence of even 2% v/v ethanol decreases the T_m by almost 8°C. As observed in Fig. 2, the T_m values remain constant between 5% and 10% v/v ethanol and decreased further by 20°C in 20% v/v ethanol. Stabilizing solvents maintain or increase the hydration of protein while destabilizing solvents directly bind to the protein and dehydrate them. Ethanol directly binds to the proteins disrupting the hydrophobic interactions while sucrose is preferentially depleted from proteins surface enhancing

Fig. 3. Thermal melting curves of BSA_{suc} monitored at 222 nm by CD spectroscopy in the presence of 0% (filled circle), 2% (open circle), 5% (cross), 10% (filled triangle) and 20% (open triangle) of ethanol. Ethanol dominantly decreased the thermal stability of BSA which was otherwise stabilized by sucrose.

the hydration and hence the stability. However from our study, when considering the combined effect, it looks like effect of ethanol dominates the effect of sucrose on the stability of BSA. Solvent interactions provide complex contributions to protein folding through hydration, hydrogen bonding, hydrophobic interaction, van der Waals forces and electrostatic interactions. Out of which, hydrophobic interaction is thought to be the dominant force in governing the stability of proteins [7]. Our study made the same observation. In the presence of sucrose, hydrophobic interactions are enhanced due to hydration effects. On the other hand, the addition of ethanol disrupts the hydrophobic interactions, thermally destabilizing the protein more than in the absence of sucrose. Ethanol is much stronger in destabilizing BSA even in the presence of a stabilizer.

4. Conclusions

Serum albumins are excellent targets not only for biochemical investigations but also for pharmaceutical industries to characterize the effect of different solvents on their stability as they are used as therapeutic agents. Here we have used BSA to study the combined effect of ethanol and sucrose. Our CD thermal melting experiments highlight the dominant influence of ethanol in destabilizing BSA_{suc} by disrupting the hydrophobic interactions. These kinds of studies are important to understanding the role played by the combined use of binary cosolvents in protein stabilization.

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