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Research paper

Role of benzyl alcohol in the prevention of heat-induced aggregation and inactivation of hen egg white lysozyme

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ABSTRACT

The aim of the study was to investigate the stability of a model protein, lysozyme, in the presence of the commonly used preservative benzyl alcohol. Techniques including lytic assay, size exclusion chromatography, circular dichroism, differential scanning calorimetry, native polyacrylamide gel electrophoresis and dynamic light scattering were used to study the overall stability of lysozyme in the presence of benzyl alcohol. The stability of lysozyme against thermal stress was higher in the presence of benzyl alcohol. In the presence of 0.5%, 0.9% and 2% v/v benzyl alcohol, the enzyme showed 33%, 42% and 75% residual activity, respectively, when exposed to 75 °C for 2 h, as compared to the 22% activity of control sample. A gradual increase in the size of aggregates was observed for the control sample relative to the samples containing benzyl alcohol, as a result of loss of monomer concentration. The effect was found to be concentration-dependent with 2% benzyl alcohol showing maximum prevention of heat-induced unfolding and aggregation. This effect is remarkable since the thermal transition temperature of the enzyme decreases in the presence of benzyl alcohol. Benzyl alcohol favours the thermal denaturation of lysozyme but stabilizes the lysozyme against the heat-induced aggregation.

1. Introduction

Protein aggregation is a frequently encountered hurdle in all stages of protein production [1] including its storage [2], and is also a cause of many neurodegenerative disease conditions like Alzheimer's and Parkinson's [3]. A large number of peptide/protein drug candidates are being discovered and developed due to advancements made in biotechnology, most of which are administered parenterally. Since the development of protein products involves large investments in terms of research effort as well as money, there is a strong need to develop cost-effective multi-dose protein formulations, which allow removal of several doses from the same vial and thus, minimize product wastage and cost of therapy. Multi-dose parenteral formulations require antimicrobial preservative(s) to prevent microbial growth [4,5]. Antimicrobial preservatives including phenol, *m*-cresol and benzyl alcohol are most widely used in protein multi-dose formulations, and about 25% of the protein products currently available in the market contain such preservatives [6].

It has been realized for some time that addition of small molecules or excipients, mainly polyols such as sugars, increases the storage stability of protein drugs [7]. The use of aromatic alcohols has been restricted to their antimicrobial activity. That is probably because these molecules have been reported to cause aggregation of proteins, and are hence not ideal candidates as protein stabilizers. Protein aggregation not only has an adverse effect on the functionality of a protein but can also trigger severe immune response in some patients [8–10]. Therefore, interaction of the preservative with the protein/peptide needs to be critically assessed in order to ensure stability of the protein in the final formulation. Numerous reports have appeared in the literature on protein-preservative interactions, which demonstrate the incompatibility of preservatives with proteins [11–14].

Benzyl alcohol is considered to be one of the least toxic preservatives and can be used up to 2% v/v for parenteral applications [5]. It also has potential antioxidative properties [15]. However, benzyl alcohol and *m*-cresol have been shown to accelerate aggregation by binding to proteins and favouring the formation of aggregation-prone molecular population. This was observed as lower melting temperature of proteins like recombinant human interleukin-1 receptor antagonist (rhIL-1ra), recombinant human growth hormone (rhGH), and monoclonal antibody in the presence of benzyl alcohol [16–18]. It has also been shown that the effect of benzyl

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alcohol-induced aggregation was pH-dependent; an acceleration in the rate of aggregation of recombinant human granulocyte colony stimulating factor (rh GCSF) was seen at pH 7.0, whereas a high positive charge on the same protein at pH 3.5 resulted in strong protein–protein electrostatic repulsion, thus inhibiting benzyl alcohol-induced aggregation [19]. We therefore thought to use a positively charged protein.

Lysozyme is used in a variety of food products as well as in pharmaceutical products due to its antimicrobial properties [20,21]. It has also been used extensively as a model protein for several studies [22,23]. Except for the study reported above with rhGCSF at a non-physiological pH, no report is available on the interaction of benzyl alcohol with a basic protein (i.e. those carrying a positive charge at the physiological pH) till date. The present work aims to study the stabilization effect of benzyl alcohol on a positively charged protein, viz. lysozyme (isoelectric point of 11) [24], with respect to its biological activity, aggregation properties and structural changes, when exposed to 'elevated temperature conditions'. This work can further be extended to other positively charged proteins like ribonuclease A (pI 9.6), cytochrome *C* (pI 10.0–10.5), egg avidin (pI 10.5) and basic pancreatic trypsin inhibitor (pI 10.5) as well as therapeutically relevant proteins carrying a high positive charge such as the HIV TAT protein and HSV-I VP22 protein, both of which present antigenic sites important for designing vaccines against pathogens.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (3× crystallized) and *Micrococcus lysodeikticus* cells were purchased from Sigma Chemical Co. (St. Louis, USA) and used as received without further purification. Benzyl alcohol (extra pure, 99% v/v) was purchased from Merck Specialities Private Limited (Mumbai, India). All solvents were of HPLC grade and other chemicals were of analytical grade or higher.

2.2. Preparation of formulations

Protein solution (5 mg ml^{-1}) was prepared by dissolving lysozyme in 10 mM sodium phosphate buffer (pH 6.2). The concentration of lysozyme was determined by UV-absorption measurements (Beckman single beam UV/Vis spectrometer, model DU 640) at 280 nm, using the value of the specific absorptivity as 2.6 (1 mg/ml, 1 cm path length) [25]. Benzyl alcohol was added at different final concentrations to the protein solution as shown in Table 1.

2.3. Enzyme activity assay

Substrate (2.5 ml) *M. lysodeikticus* (0.015% w/v in 10 mM sodium phosphate buffer, pH 6.2) was taken in a spectrophotometer cell maintained at 25 °C. One hundred microliter of lysozyme sample was added, mixed and the decrease in absorbance at 450 nm for 5 min, at 30 s interval was recorded [26]. Enzyme activity was calculated by obtaining the change in A_{450nm}/min from the slope of the linear portion of the plot of absorbance against time at the specified pH and temperature. Unit of biologically active lysozyme was determined by using the following formula:

$$\text{Units of lysozyme/ml sample} = \frac{(\Delta A_{450nm}/\text{min Test} - \Delta A_{450nm}/\text{min Blank})(\text{df})}{(0.001)(0.1)}$$

where df is the dilution factor; 0.001 is the change in absorbance as per unit definition; 0.1 is the volume (in ml) of the sample used.

In the absence of lysozyme, benzyl alcohol did not show any antimicrobial activity towards *M. lysodeikticus* cells (substrate of lysozyme). In all the experiments where the stability and other characteristics of the enzyme have been evaluated at higher temperature, 100% activity refers to the enzyme activity at 25 °C, as described above.

2.4. Evaluation of tertiary structure by circular dichroism

Circular dichroism measurements were carried out on a Jasco spectropolarimeter, model J-815, equipped with a Peltier type temperature control system. Quartz cuvettes with path lengths of 5 and 10 mm were used. Scans were obtained with band width of 0.5 nm and response time of 4 s with 100 mdeg sensitivity. Samples were diluted tenfold before carrying out the wavelength scan. Triplicate scans were recorded for each sample and corrected for background dichroism. The effect of benzyl alcohol on the thermal unfolding of lysozyme was measured by recording the change in ellipticity at a wavelength of 288 nm (the maxima of near-UV-CD spectrum) within a temperature range of 25–85 °C, at a heating rate of 1 °C min^{-1}. The melting temperature of lysozyme from thermal unfolding transition curve was determined from the point of inflection of the curve by sigmoidal curve fitting [27] using OriginPro 8 SrR2 software version v8.0891, B891 (Originlab).

2.5. Thermal denaturation by differential scanning calorimetry (DSC)

The calorimetric measurements were performed in a Mettler-Toledo calorimeter, model DSC-821, using 100 µl medium pressure crucibles; the instrument was calibrated with indium prior to use. The samples were measured in aluminium pans containing 50 µl of lysozyme solution. As a reference, the same amount of the buffer solution was used. The sample was held at 32 °C for 2 min, and then heated to 100 °C at different heating rates (ranging 0.2–20 °C min^{-1}). After the end of the first heating cycle, the protein sample was quickly cooled (the cooling rate set to the maximum value of −100 °C min^{-1}) to 32 °C, and rescanned again at 32 °C for 5 min. Base lines, obtained by filling both cells with the corresponding buffer and excipient concentrations, were subtracted from the sample experimental trace. Thermograms were evaluated using Star® software version 5.1 (Mettler-Toledo). Measurements

Table 1
Formulation compositions of different samples

S. No.	Formulation code	Ingredients			
		Lysozyme (% w/v)	Benzyl alcohol (% v/v)	Vehicle	pH at 25 °C
1	Control	0.5	0	Phosphate buffer (10 mM)	6.2 ± 0.02
2	BA1	0.5	0.5	Phosphate buffer (10 mM)	6.2 ± 0.02
3	BA2	0.5	0.9	Phosphate buffer (10 mM)	6.2 ± 0.02
4	BA3	0.5	2	Phosphate buffer (10 mM)	6.2 ± 0.02

were carried out on three separate samples (replicates) and were reported as the average.

2.6. Dynamic light scattering (DLS) measurements

DLS measurements were performed on Zeta sizer (Nano ZS, Malvern Instruments), taking the average of five measurements. Protein samples were filtered through pre-sterilized and disposable 0.1 μm polyether sulphone membrane syringe filters before measurement to remove impurities and dust particles.

2.7. Quantification of soluble protein using SEC-HPLC

The HPLC system (Shimadzu Corporation) comprised of a system controller (CBM-20A), a pump (LC-20AT), a degasser (DGU-20A5), an autosampler (SIL-20AC), a column oven (CTO-10A VP) and a diode array detector (SPD-M20A) with Class-VP software (version 6.1). A sample volume of 20 μl was introduced into an analytical column GF-250, ZORBAX (4.6 × 250 mm, 4 μm) attached with a GF-250 guard column (4.6 × 12.5 mm, 4 μm) (Agilent Technologies, USA). Chromatograms were obtained with 0.1 M phosphate buffer containing 0.15 M NaCl (pH 7.0) as the mobile phase, at a flow rate of 0.3 ml/min. Peak areas were used to quantify the amount of soluble proteins, by setting the detector wavelength at 280 nm. All samples were diluted up to 50 μg ml^{-1} and filtered through 0.45 μm nylon filters before injection.

2.8. Turbidity measurement

The effect of benzyl alcohol on the aggregation of lysozyme was analyzed by monitoring the turbidity of the resultant solution at 600 nm using Beckman single beam UV/Vis spectrophotometer, model DU 640.

2.9. Native polyacrylamide gel electrophoresis

Electrophoresis of lysozyme sample was carried out using a mini gel electrophoresis unit (GE Healthcare, Hong Kong), employing a reverse electrode assembly [28]. The electrophoretic run was carried out at pH 4.3 in a 15% polyacrylamide gel (30% acrylamide, 0.8% bis acrylamide) with a discontinuous buffer system (200 V and 25 mA). Acetate–KOH buffer (1.5 M, pH 4.3) was used for the resolving gel while 0.25 M acetate–KOH buffer (pH 6.8) was used for the stacking gel. After the completion of the run, gels were removed and stained with Coomassie blue dye. Freshly prepared lysozyme was loaded as the standard to compare the intensity of bands in all the samples. Densitometric analysis was performed with the help of the software Image Quant TL (GE Healthcare, Hong Kong).

3. Results and discussion

3.1. Effect of benzyl alcohol on the biological activity of lysozyme

The biological activity assay of the enzyme incubated at elevated temperature provides information about the effect of added excipient(s) on the stability of the protein. The excipient was found not to affect the activity of lysozyme to a significant extent at 25 ± 0.25 °C up to 3 days (Fig. 1A). Samples were also incubated for longer periods; aliquots were withdrawn at different time intervals and the residual enzyme activity was measured. The control sample showed more than 80% residual activity even after incubation for 21 days. In the presence of 0.5%, 0.9% and 2% v/v benzyl alcohol, the enzyme showed 86%, 98% and 99% residual activity, respectively, after 21 days. The effect of benzyl alcohol on the activity of lysozyme was further studied at 40 ± 0.25 °C and the enzyme showed 60%, 67%, 74% and 89% residual activity

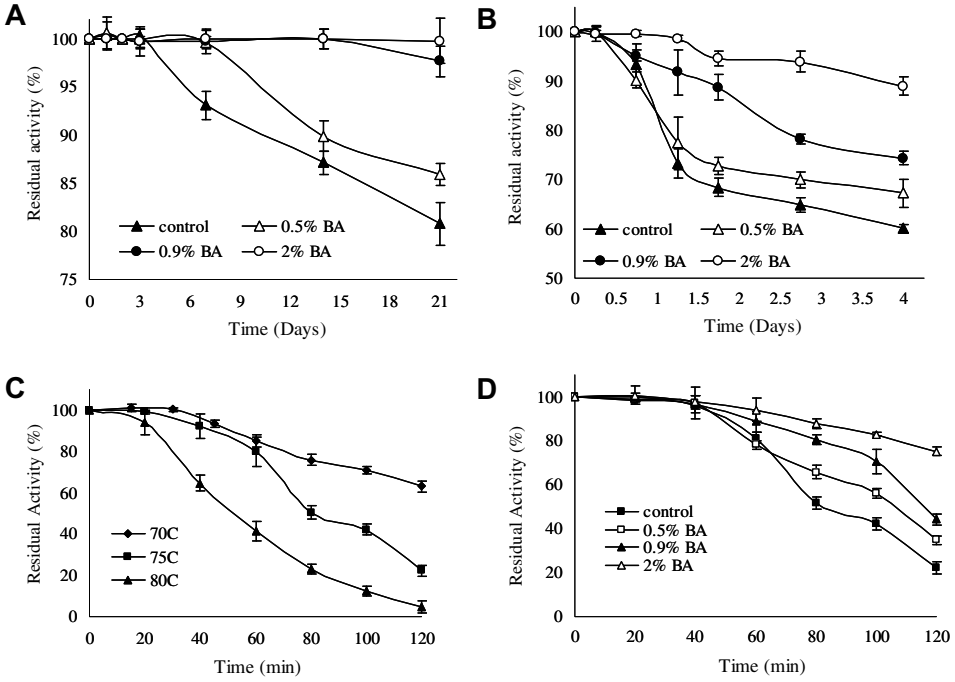


Fig. 1. Thermal inactivation curve of lysozyme (5 mg ml^{-1}) in the presence of benzyl alcohol as a function of temperature. The precision (coefficient of variation) of enzyme activity assay was found to be 0.85% for $n = 6$. Error bars represent the data for mean ± SD for triplicates incubated samples. (A) Lysozyme in the presence and absence of different concentration of benzyl alcohol at 25 °C; (B) lysozyme in presence and absence of different concentration of benzyl alcohol at 40 °C; (C) loss of residual activity of lysozyme in absence of benzyl alcohol at three elevated temperatures; (D) lysozyme in presence and absence of different concentration of benzyl alcohol at 75 °C. Aliquots were withdrawn at different time intervals, cooled to room temperature and checked for residual enzyme activity. The activity recovered from incubated samples was expressed as percentage of activity with respect to activity at initial time point.

for control, BA1, BA2 and BA3, respectively (Fig. 1B). The above results indicate increased stability of lysozyme in the presence of benzyl alcohol.

The elevated temperature is used as a means to mimic accelerated storage condition to enhance protein degradation. Stability assays were carried out at three elevated temperatures. The control enzyme, without any excipient, was found to be stable at 70 °C while it lost activity very fast at 80 °C (Fig. 1C). This is in agreement with earlier reported results [29]. Thus, 75 °C was chosen as the temperature for further detailed studies, since the differential effect of benzyl alcohol as a stabilizer of the control enzyme was found to be significant at this temperature.

All samples were incubated at 75 °C and aliquots were withdrawn after regular time intervals up to 2 h, cooled to room temperature and checked for residual enzyme activity. The stability of the lysozyme in the presence of different concentrations of benzyl alcohol is shown in Fig. 1D. All samples showed similar activity up to 20 min, showing a lag period of inactivation, which agrees well with previous reports of lag period of inactivation during heat incubation of lysozyme [30]. But after this, the activity of lysozyme alone decreased drastically and the control sample showed substantially low activity of about 22% after 2 h. In the presence of 0.5%, 0.9% and 2% v/v benzyl alcohol, the enzyme showed 33%, 42% and 75% residual activity, respectively, after 2 h. Further increase in the concentration of benzyl alcohol did not result in any improvement in the stabilization effect. Thus, benzyl alcohol shows a protective action against heat denaturation of lysozyme, which depends on the concentration of the excipient up to a certain level. It may be noted that benzyl alcohol has also been used as a stabilizer for lysozyme in controlled release systems of smart

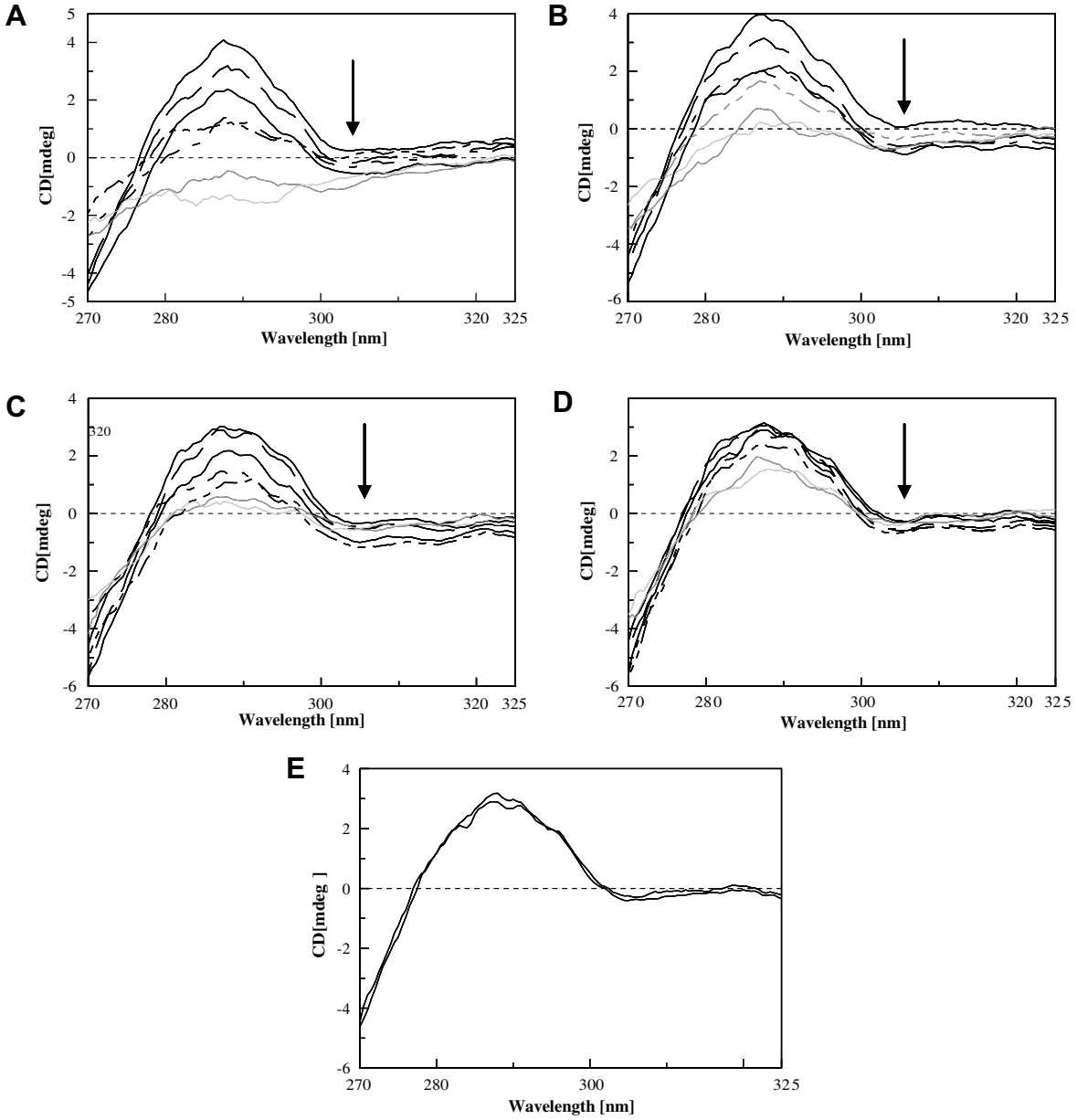


Fig. 2. Near-UV CD spectra of lysozyme (0.5 mg ml^{-1}) after incubation at 75 °C: effect of heating on conformational changes of lysozyme as a function of time (up to 2 h). (A) Lysozyme alone; (B) lysozyme with 0.5% benzyl alcohol; (C) lysozyme with 0.9% benzyl alcohol; (D) lysozyme with 2% benzyl alcohol. Downward arrow indicates the subsequent samples at different time points. Solid dark black coloured line, 0 min; broken line, 20 min; dot-dot line, 40 min; dash-dot-dash line, 60 min; dash-dot-dot-dash line, 80 min; solid dark grey coloured line, 100 min; solid light grey coloured line, 120 min for all samples. (E) Overlay of CD spectra of lysozyme with 2% benzyl alcohol should be represented via dot-dot line.

polymers [22]. Partial evidence for the higher conformational stability of the enzyme in such a preparation has been provided.

3.2. Evaluation of conformational changes

Circular dichroism has been widely used to analyze the changes in secondary and tertiary structures of proteins caused by changes in solvent polarity, pH, temperature and added excipients [31]. We could not use far-UV CD spectroscopy to monitor secondary structure changes because of a high degree of interference by benzyl alcohol in this region. Thus, only changes in the tertiary structure were monitored using near-UV CD spectroscopy.

The near-UV CD spectrum of the control sample was characterized by a positive maxima at 288 nm, which agrees well with the reported spectrum of lysozyme in this region [32]. The heating of the protein at 75 °C resulted in a gradual loss of CD signal intensity with time as a consequence of unfolding, which appeared as a decreased ellipticity (Fig. 2). A greater decrease in the ellipticity was observed in the absence of benzyl alcohol (control sample); after incubation for 100 min at 75 °C, the peak at 288 nm disappeared completely. The presence of benzyl alcohol decreased the rate of unfolding, suggesting a greater resistance to unfolding in response to thermal stress (Figs. 2 and 3). This effect of benzyl alcohol was concentration-dependent, with 2% concentration showing the maximum effect, and correlated well with the retention of enzyme activity with increasing concentration of benzyl alcohol. The probable cause for such behaviour is proposed below in conjunction with data obtained from DSC measurements.

3.3. Effect of benzyl alcohol on prevention of aggregation of lysozyme

The concentration of residual monomer in the presence of different concentrations of benzyl alcohol at 40 and 75 °C at different time points, as determined by size exclusion chromatography, is depicted in Fig. 4A and B. No appreciable loss of monomer concentration was observed in any of the samples at 40 °C. Heat inactivation studies revealed that lysozyme loses a major fraction of its monomeric form after a lag period of about 20 min at 75 °C, which is probably the time period associated with nucleation before aggregation commences [1]. The observation of lag period is consistent with previous reports showing a single rate-limiting step in the inactivation of lysozyme [33]. This rate-limiting step is attributed to the conversion of the native to the aggregation-prone intermediate state and is referred to as the nucleation step [1,34,35]. Occurrence of this step is an indication that aggregation is nucleation-dependent. The control sample showed a residual monomer concentration of 33% at the end of 2 h study. This correlated well with the 22% residual activity observed at the end of the same time period for the control sample (Fig. 5). In contrast, benzyl alcohol was able to preserve a higher fraction of the enzyme in the monomeric and functionally active form at 75 °C; the monomer concentration was found to be 38%, 58% and 95% for BA1, BA2 and BA3, respectively. The samples were filtered through 0.45 μm filters prior to injection so as to remove dust particles and oligomers greater than 0.45 μm. Though the monomer peak area decreased significantly over time (Fig. 4C and D), no soluble aggregates were detected in any of the samples up to 2 h of heat incubation, suggesting the formation of higher oligomers, with decreased solubility. Our inability to isolate lower oligomers also supports the fact that the transition from partially to completely unfolded one is very fast at 75 °C. The observations are thus in agreement with the activity assay data of lysozyme, suggesting the highest stability of lysozyme at 75 °C, in the presence of 2% benzyl alcohol.

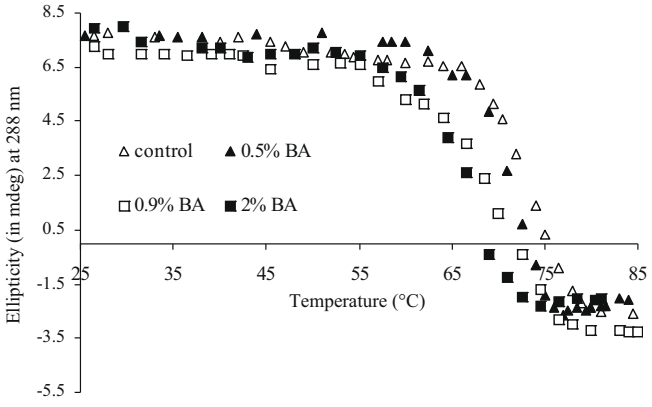


Fig. 3. Representative data for thermal unfolding curves of lysozyme (1 mg ml^{-1}) in the presence of different concentration of benzyl alcohol obtained by heating of samples from 25 to 85 °C at 288 nm at scan rate of 1 °C min^{-1} determined by circular dichroism spectroscopy. Sigmoidal curve represents fits to a two-state unfolding model. T_m values determined from point of inflection are listed in Table 3.

For further confirmation of the above results, the samples were run on native PAGE and the densitometric analysis was performed to compare the intensity of the enzyme bands obtained for each formulation (Fig. 6A and B). The run was carried out under acidic conditions due to positive charge on lysozyme. As expected, the least intense band for the monomer was seen in the case of the control sample. The intensity increased with increasing concentration of benzyl alcohol, with values of 42%, 54% and 78% for BA1, BA2 and BA3, respectively, compared to 23% for the control. These values correlated well with the biological activity (Fig. 1D). However, no high molecular weight aggregates were seen in any of the lanes, which agrees well with the data reported earlier.

Furthermore, samples were analyzed for the presence of aggregates using DLS which detects early states of aggregation [36]. The mean sizes of particles of both the control and the sample containing 2% benzyl alcohol were compared to study the change in particle size due to aggregation. Mean particle size denotes the mean size of particles distributed by percentage intensity. The DLS measurements showed a significant difference in the distribution of particles which was expressed as mean size of particles (Fig. 7A). The size of lysozyme monomer at initial time point was found to be around 3.55 nm which matches the reported value for diameter of monomeric lysozyme [37]. This diameter increased significantly due to the association of monomers after heat incubation of 2 h pointing to the formation of higher molecular weight species. In the case of control, the population density of monomeric particles (initially present) decreased with time since they combine to form larger particles. As expected, the control sample showed shifting of particles population towards higher size for each subsequent time point which reached 13.26 nm after 120 min of incubation at 75 °C (Fig. 8). However, in the presence of 2% benzyl alcohol, the mean size remained less than 4 nm even at the end of 2 h and no lysozyme aggregates were formed. The loss of biological activity of lysozyme was correlated with the mean particle size of lysozyme (Fig. 7B) using both parametric (Karl Pearson's correlation) and non-parametric (Spearman correlation) tests with the assumptions of presence and absence of normal distribution, respectively. All calculations were performed using Sigma Stat software (version 2.0, SPSS Inc., Chicago, IL). Karl Pearson's correlation coefficient and Spearman correlation coefficient were found to be −0.95 and −1.0, respectively, with p values less than 0.01 in both cases, showing a good inverse correlation between the two parameters. Hence, the activity of lysozyme in the control sample was lost due to the aggregation of the functionally active monomeric form. Polydispersity index values (listed in Table 2) showed no significant difference at subsequent time points for both control and BA3. DLS

technique provides autocorrelation functions of the scattered light by fit of the data points by cumulants analysis. Initially, autocorrelation function indicated small particles in solution, but with the heating of control samples for subsequent time points, the decay in the autocorrelation function was slower, which indicated formation of aggregates and thus was reflected in the increased mean size at each subsequent time point (Fig. 9). However, for BA3 samples, autocorrelation function decayed at an almost constant rate for all the time points, indicating absence of aggregates as evident by distribution of particles in Fig. 8.

Having established that in the presence of benzyl alcohol, a higher fraction of the population remains in the monomeric form as compared to the control, the samples were further incubated at 75 °C for a longer period and analyzed for the presence of insoluble aggregates by light scattering measurement at 600 nm. The principle behind the measurement is that protein solution has negligible absorbance at this wavelength, and any increase in the absorbance is indicative of turbidity developed due to protein aggregation. For the control sample, the formation of insoluble aggregates was observed after 3.5 h, as indicated by an increase in the light scattering signal. The absorbance values at 600 nm in the control, BA1, BA2 and BA3 are depicted in Fig. 10. The samples were observed visually, and an increase in turbidity with incubation time was found to be inversely correlated to the concentration of benzyl alcohol. The control sample became turbid at around 3.5 h which turned into insoluble aggregates on further incubation, while BA3 remained clear up to 7 h of heat incubation. Thus, unlike the studies reported with other proteins earlier where benzyl alcohol has been shown to induce aggregation, in the case of lysozyme, the additive exhibits a protective effect towards aggregation when the protein is subjected to thermal stress.

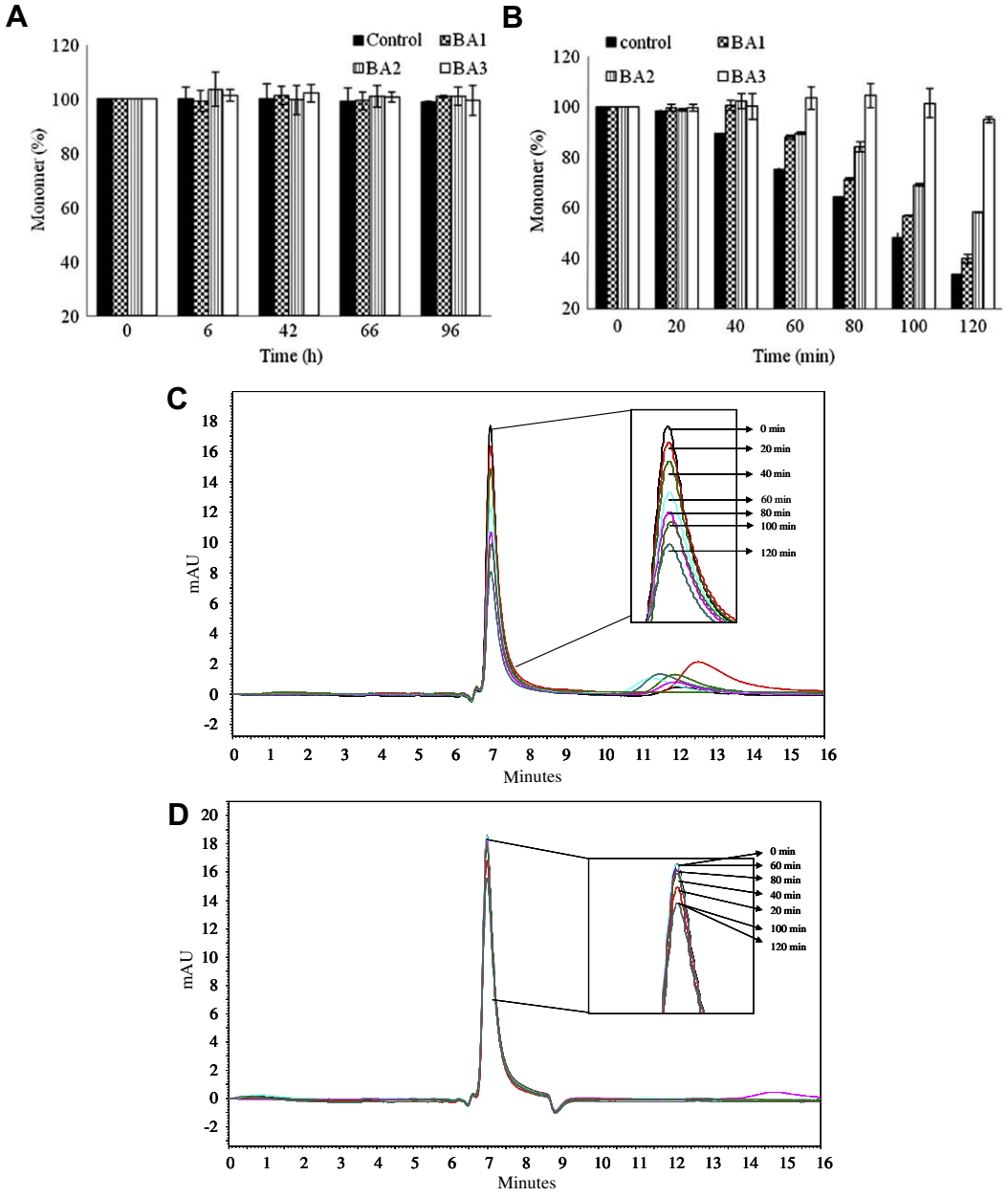


Fig. 4. Residual monomer concentration (mean ± SD for $n = 3$) of lysozyme samples incubated at 40 °C (A) and 75 °C (B) in presence and absence of different concentrations of benzyl alcohol, as determined by size exclusion chromatography (SEC)-HPLC. Chromatograms showed relative decrease in monomer peak area at subsequent time points for control (C) and BA3 (D) samples. In both cases, no aggregates were detected which indicate formation of higher aggregates.

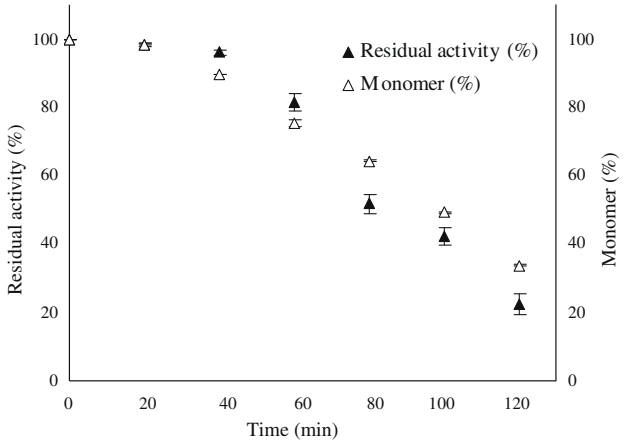


Fig. 5. Correlation of loss of enzymatic activity and monomer concentration of lysozyme at subsequent time points of heat incubation at 75 °C. The protein concentration in both cases was 5 mg ml^{-1}.

3.4. Thermal denaturation

Differential scanning calorimetry (DSC) is an ideal tool for determination of heat absorption at elevated temperatures, since the technique directly measures the forces stabilizing the native conformation of the protein and the effect of additives in solution on these forces. The endothermic transition observed is believed to be due to the melting of the hydrogen bonded structure of the protein, in which water successfully surrounds the peptide backbone and the side chain groups [38]. The characteristic parameters, T_m (melting temperature) and ΔH (change in enthalpy) of DSC thermograms of lysozyme, measured at a heating rate of 10 °C min^{-1}, are listed in Table 3. The results showed heating rate dependency, and the thermal unfolding temperature increased with increasing heating rate, indicating that the thermal denaturation of lysozyme is kinetically controlled [39]. T_m corresponds to the peak maximum and greater ΔH values have been correlated with higher conformational stability of lysozyme preparations stabilized with benzyl alcohol [22]. In the present case, however, benzyl alcohol was not found to significantly alter the value of ΔH of lysozyme denaturation (Table 3) though there was a marginal decrease in the thermal denaturation temperature. This is probably because at a higher heating rate, when the denaturation process is kinetically controlled, the rate of binding of benzyl alcohol to the partially unfolded form cannot match the rate of collapse of this structure to the completely denatured state. The thermal denaturation temperature was not affected by the initial protein concentration (Table 4), implying the possibility of a rate-limiting protein unfolding step [40]. The reversibility of thermal transition of lysozyme was checked by cooling and reheating the sample with the same temperature program, and the process of lysozyme denaturation was found to be fully reversible, indicating

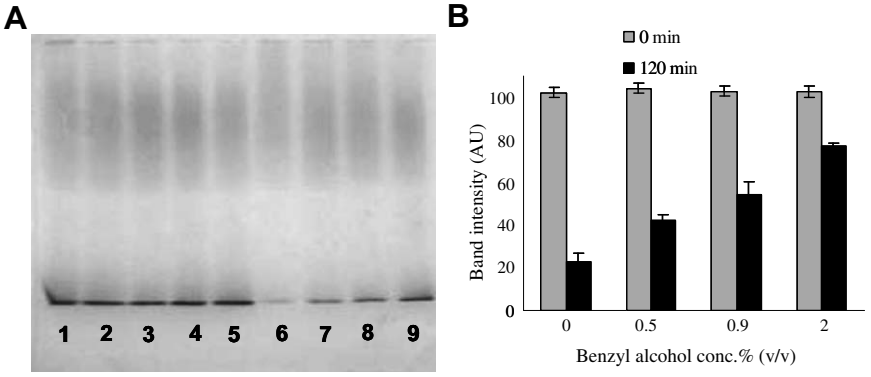


Fig. 6. Non-denaturing PAGE of lysozyme samples incubated at 75 °C. (A) The effect of benzyl alcohol on the intensity of band for monomeric lysozyme after heating at 75 °C after 0 and 120 min. Lane 1: control, lane 2: BA1, lane 3: BA2, lane 4: BA3, all at 0 min, lane 5: freshly prepared lysozyme, lane 6: control, lane 7: BA1, lane 8: BA2, lane 9: BA3, all after 120 min. (B) Densitometric analysis of the bands obtained after 0 and 120 min. Error bars represent the data for mean ± SD for triplicates incubated samples. The intensity of the band in lane 5 has been taken to be 100%.

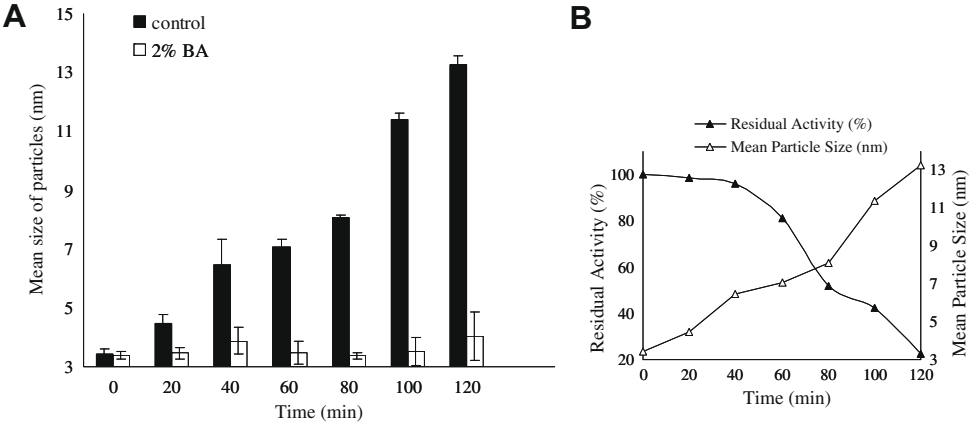


Fig. 7. Mean size of the lysozyme particles in the presence and absence of 2% BA after incubation at 75 °C for different time intervals (A). Data represented as mean ± SD for triplicate samples. (B) An inverse correlation between particle mean size and residual activity of lysozyme after heating at 75 °C indicating loss of biological activity of control sample due to aggregation.

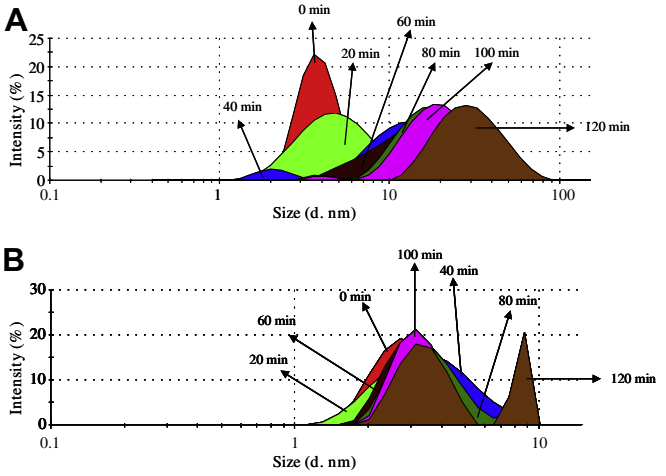


Fig. 8. Relative particle size distribution of control (A) and BA3 (B) samples measured after heat incubation at 75 °C at each time point. The protein concentration was 5 mg ml^{-1}. Control sample showed shifting of particle population towards higher size at each time point.

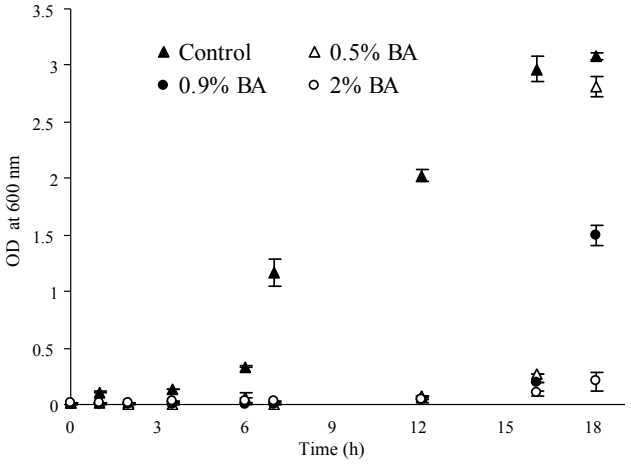


Fig. 10. Light scattering measurements of control, BA1, BA2 and BA3 at 600 nm. Data represented as mean ± SD for triplicate samples. The aggregation was monitored by light scattering at 600 nm as a function of incubation time.

Table 2
Polydispersity index values of lysozyme in presence and absence of 2% benzyl alcohol after heat incubation at 75 °C (mean ± SD, $n = 3$)

Time	Control	BA3
0	0.2 ± 0.03	0.3 ± 0.07
20	0.2 ± 0.00	0.3 ± 0.02
40	0.3 ± 0.04	0.3 ± 0.05
60	0.2 ± 0.01	0.3 ± 0.01
80	0.2 ± 0.00	0.3 ± 0.01
100	0.2 ± 0.01	0.3 ± 0.01
120	0.2 ± 0.05	0.3 ± 0.04

Table 3
Thermodynamic parameters (mean ± SD, $n = 3$) of lysozyme in presence of benzyl alcohol

Sample	Thermodynamic parameters determined by DSC				Determined by CD
	Onset (°C)	Midpoint (T_m) (°C)	Endpoint (°C)	ΔH (kJ mol^{-1})	T_m (°C)
Control	72.6 ± 0.0	78.0 ± 0.2	84.1 ± 0.7	431.5 ± 9.4	73.1 ± 0.4
BA1	71.1 ± 1.2	76.2 ± 0.3	82.2 ± 0.3	427.7 ± 10.1	70.9 ± 0.1
BA2	68.6 ± 1.2	75.1 ± 0.5	82.0 ± 0.4	435.4 ± 9.6	69.2 ± 0.4
BA3	65.0 ± 0.4	71.8 ± 0.9	80.0 ± 0.3	424.3 ± 7.2	66.6 ± 0.5

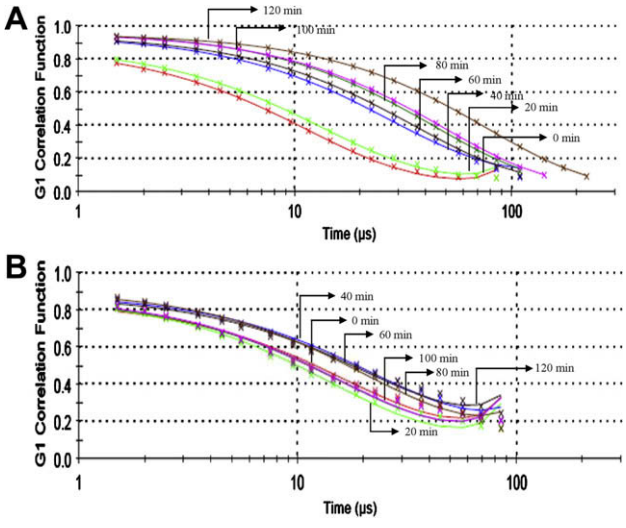


Fig. 9. Cumulant fit graph showing correlation functions of control (A) and BA3 (B) after heat incubation at 75 °C at each time point. The data points used in the analysis are from the actual data in the correlation function obtained by selecting a subset of points geometrically spaced and normalizing these by subtracting the baseline.

Table 4
Effect of protein concentration on melting temperature (T_m) of lysozyme (mean ± SD, $n = 3$)

Protein Conc. (mg ml^{-1})	T_m (°C)
As determined by CD[a]	
0.5	73.1 ± 0.3
1	73.1 ± 0.4
As determined by DSC[b]	
5	78.0 ± 0.2
7.5	78.3 ± 0.2
10	78.2 ± 0.6

[a] At a heating rate of 1 °C min^{-1}.
[b] At a heating rate of 10 °C min^{-1}.

a two-state behaviour kinetically driven upon thermal denaturation and suggesting that the equilibration between the two states proceeds slowly, compared to the high scan rate of the instrument [41]. The reversibility of thermal denaturation was retained in the presence of benzyl alcohol. This also ruled out the formation of aggregates during the scan period as reported for other proteins. Protein aggregates usually show reduced or no activity [1] though in a very few cases, protein aggregates have been shown to retain activity [42]. In a majority of the cases, association of exposed hydrophobic groups/patches on unfolding/folding intermediates initiates the aggregation process [1,43]. These aggregates are usually non-productive in nature and their presence in the pharmaceutical product renders the latter unfit for use.

The typical single endothermic peak was observed for all the samples. T_m of the control sample was found to be 78 °C (Fig. 11 and Table 3) at a heating rate of 10 °C min^{-1}. The faster heating rate results in faster absorption of heat. Lysozyme contains four disulphide bonds. It has already been reported that thermal denaturation at 75 °C results in the predominant formation of three isomers, as identified by disulphide scrambling [44]. These authors had been unable to detect any intermediate in the heat-mediated unfolding

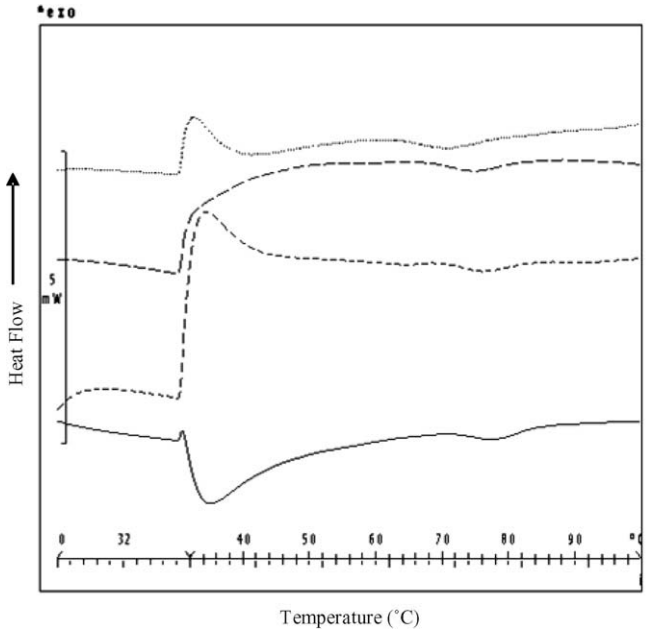
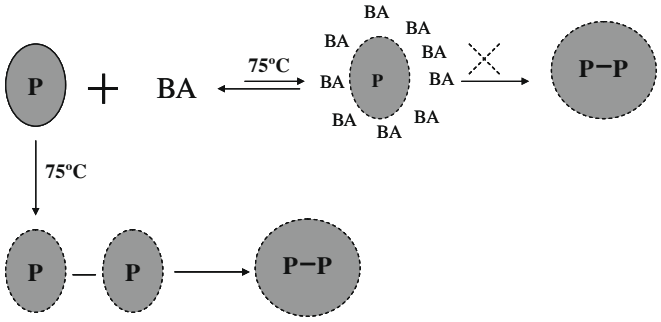


Fig. 11. Thermal stability of lysozyme (5 mg ml^{-1}) in the presence of benzyl alcohol as detected by DSC. The samples were heated at a scan rate of 10 °C min^{-1}. Solid line, control (lysozyme alone); dash-dot-dot line, 0.5% benzyl alcohol; dash line, 0.9% benzyl alcohol; dotted line, 2% benzyl alcohol. The thermodynamic parameters obtained by DSC are listed in Table 3.

pathway of lysozyme at 75 °C but have proposed the existence of a partially unfolded intermediate with two native and two non-native disulphide bonds. The non-linear nature of inactivation curve of lysozyme in the presence of benzyl alcohol at 75 °C (Fig. 1D) does seem to support the existence of such a state. We can thus speculate on the mechanism of protective action of the additive. Benzyl alcohol probably binds to the partially unfolded form and slows down the rate of inactivation of lysozyme. It thus prevents the unfolding of the enzyme by slowing down the rate of association of partially unfolded forms via (mainly) hydrophobic interactions. Due to the high partial positive charge on this partially unfolded species, at a higher rate of heating, the denaturation process is kinetically controlled and this hypothetical, partially unfolded form, probably collapses into the completely denatured form before benzyl alcohol can bind to it. However, more experimental evidence is needed to validate this hypothesis. Our results show that T_m (corresponding to maximum heat flux) decreases linearly in the presence of benzyl alcohol, with values of 76.16, 75.07 and 71.77 °C for samples BA1, BA2 and BA3, respectively. This is similar to the results seen with rhGH in the presence of benzyl alcohol [17], though the latter process is reversible. These results suggest that benzyl alcohol preferentially binds to the unfolded molecules of lysozyme, thereby suppressing the aggregation and destabilization of the native structure during thermal scanning [30,45]. However, the enthalpy of unfolding is not significantly different in the presence of benzyl alcohol, suggesting that the initial equilibrium between the folded and unfolded moieties of lysozyme is not affected by benzyl alcohol [46]. Enthalpy change is a cumulative resultant of a number of parameters and it can be taken to be a measure of protein stability. The similar values of ΔH in the presence and absence of benzyl alcohol imply that the excipient exerts its effect via kinetic rather than thermodynamic route. Taken together with the fact that benzyl alcohol protects lysozyme against changes in the tertiary structure of the protein (Fig. 2) and the earlier reported result that rhGCSF remains impervious to benzyl alcohol-induced aggregation at pH 3.5, it can be speculated that the high partial positive charge on lysozyme at pH 6.2 helps it to survive benzyl alcohol-induced aggregation.

Thermal unfolding was further studied with the help of near-UV CD spectroscopy by heating lysozyme and monitoring the changes at a fixed wavelength (288 nm), at a heating rate of 1 °C min^{-1}. T_m was calculated from the point of inflection of thermal unfolding curve obtained after heating the lysozyme, which fitted well to two-state reversible transition model (Fig. 3 and Table 3). The thermal unfolding of the control sample started around 66 °C and was found to have a T_m of 73.09 °C. The thermal unfolding temperatures of lysozyme were observed as 62.5, 55 and 52.5 °C for BA1, BA2 and BA3, respectively. The reason for the difference in T_ms obtained by the two techniques (CD & DSC) may lie in the different rates of heating involved in the two cases. However, the trend is similar to the decrease in thermal transition temperature observed with increase in concentration of benzyl alcohol by DSC.

The inverse relationship of thermal unfolding temperature of lysozyme with benzyl alcohol concentration, as determined by DSC and near-UV CD spectroscopy, reflects the destabilization effect of benzyl alcohol on the native structure of lysozyme. Taken together with the activity data, this supports the hypothesis that the thermal denaturation of lysozyme in the presence of benzyl alcohol is a kinetically controlled process, wherein the rate of conversion of partially unfolded to fully unfolded form of the enzyme is slowed down by benzyl alcohol as shown in the schematic representation below.



As per Tanford's transfer model, the mechanism of interaction of benzyl alcohol with lysozyme may involve preferential binding of benzyl alcohol to the partially unfolded state of lysozyme, thereby decreasing the free energy of unfolding of the enzyme [47]. According to Auton and Bolen, in such a situation, the solubility ratio of the partially unfolded protein (in water and 1 M excipient) is less than 1, the apparent transfer free energy is negative and the interaction between this protein intermediate and the excipient is energetically favourable [48]. Stabilization of the partially unfolded form by benzyl alcohol also prevents its collapse to the completely unfolded form, thus slowing down its rate of denaturation.

According to mass action law, preferential binding of benzyl alcohol to the unfolded state will lead to destabilization of protein [30], which is shown by a linear decrease in thermodynamic parameters of lysozyme in Table 2. Similar observations of decrease in T_m with increasing concentrations of excipients have been reported elsewhere. It has been shown, for example, that stabilizers like ammonium salts, polyamines, arginine ethyl ester and cyclodextrins prevent the heat-induced aggregation and inactivation of lysozyme but favour its thermal degradation [23,33,45,46]. A positive correlation has been found between T_m and aggregation propensity of lysozyme [33], which may explain the results obtained here.

4. Conclusion

The results described in the present study point to a novel role for the antimicrobial preservative benzyl alcohol, viz. that of a pro-

tein stabilizer. This is counter-intuitive to the expected behaviour, wherein preservatives are reported to be incompatible with protein stability. Since protein aggregation is a critical and inescapable problem in the development of protein drugs, benzyl alcohol could be explored as a new candidate for suppression of protein aggregation and retention of biological activity. We further conclude that benzyl alcohol is one of the very few stabilizing agents of lysozyme like ammonium salts, arginine ethyl ester and cyclodextrins that prevent the heat-induced aggregation and inactivation of lysozyme but favour its thermal degradation. In summary, this work shows that the presence of preservatives is not always detrimental to the stability of the protein; this relationship depends on various properties of the protein, including its charge, hydrophobicity, solubility, etc. and how these properties are modulated in the presence of excipients.

Together, these results support that this work has potential implications for development of multi-dose formulations of proteins.

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