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Research paper

Role of trehalose in moisture-induced aggregation of bovine serum albumin

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Abstract

Moisture-induced aggregation has been identified as a key problem in the long term storage stability of therapeutic proteins. In the present work, we have investigated the impact of the disaccharide trehalose on the aggregation behavior of a model protein, bovine serum albumin (BSA) under moist conditions. About 50% aggregation of BSA was observed at a moisture level of 8 μl/10 mg protein. Including trehalose in the protein sample caused a significant reduction in aggregation. We address the probable mechanisms for the protective effect of trehalose by considering the various hypotheses that have been proposed in the literature. The techniques that have been used include denaturing and non-denaturing gel electrophoresis and tryptophan intrinsic fluorescence. The nature of the aggregates was studied by carrying out electrophoresis of the aggregated protein in the presence of reducing and chaotropic reagents. The interaction studies of aggregated BSA with Thioflavin T and CongoRed indicate the possibility of amyloid type of character in the former. These studies may explain the protective role of trehalose under conditions where the storage stability of therapeutic proteins is compromised.

Keywords: Aggregation mechanism; Biopharmaceuticals; Moisture-induced aggregation; Protein stability; Trehalose

1. Introduction

Aggregation is an emerging issue in the field of biopharmaceutical formulations. It is the main hurdle in proteins becoming successful commercial drug candidates [1–3]. The numerous ways in which a protein can interact with its neighboring molecules (covalent, non-covalent bonds, etc.) may be the possible reason. Among the various modes by which protein drugs can aggregate, induction by moisture is by far the most troublesome [4–8]. It generally arises during storage of the final dosage form. The role of water in the stability of proteins is quite critical. Proteins are more stable in solid state. On exposure of solid proteins to increased levels of moisture, the flexibility/mobility of the proteins increases, resulting in chemical reactions between accessible, vulnerable groups. Water itself may also participate in several protein degradation pathways, viz. β-elimination, etc. Thus, with increased hydration during storage under humid atmosphere, the stability of the protein drug falls considerably. Upon reconstitution, these turn into hazardous aggregates which may have altered structure and increased immunogenicity and are thus unsafe for use. For any molecule to become a drug takes years of research and lots of money. If it is unsuccessful in commercialization due to problems associated with the stability, it will have serious consequences on the economics of drug discovery [1,9]. Therefore, the mechanistic study of protein aggregation and its prevention against elevated moisture conditions are necessary.

A number of strategies have been used to stabilize proteins. Changing their immediate environment using various excipients is the most commonly used approach [2,7,10–

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12]. Among the different excipients available, trehalose, a naturally occurring non-reducing disaccharide has many properties which make it superior for protection of biomolecules under various stress conditions [13–15]. It has potential applications in the areas of stabilization of proteins, vaccines, liposome, etc., preservation of human organs, platelets and membranes, as therapeutics for diseases caused by protein aggregation, dry skin in humans, dental caries, etc. [14–18].

In the present work, we have studied the moisture-induced aggregation behavior of bovine serum albumin (BSA) in the presence and absence of trehalose. Serum albumins have been used as model proteins for studying the stabilization behavior of a number of pharmaceutically important proteins and antigens (toxoids) [19–23]. Serum albumins have been used the most whenever studies regarding stability of protein drugs have been reported [4,6,20,21,23,24]. The aggregation of solid serum albumin formulations, following exposure to moisture, has also been reported [2,4,6,7,21]. Other pharmaceutically relevant proteins, which have been reported to degrade when exposed to humid conditions, include human insulin, recombinant human albumin and tetanus toxoid [20,21,25].

We report here for the first time that trehalose, a non-reducing disaccharide which has found widespread use as a stabilizer of biomolecules, is able to prevent the destabilization of BSA caused by moisture-induced aggregation. We have investigated the mechanism of the protective action of trehalose using denaturing and non-denaturing PAGE as well as tryptophan intrinsic fluorescence. The nature of the aggregates has been examined using two dyes, Thioflavin T and CongoRed.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA, fraction V), standard protein markers (29–200 kDa), brilliant blue R (CAS No. 6104-59-2), Bradford reagent, CongoRed (CAS No. 573-58-0), Thioflavin T (CAS No. 2390-54-7) and silver staining kit were purchased from Sigma–Aldrich, Bangalore, India. Trehalose was a product of Fluka and was purchased from Sigma–Aldrich, Bangalore, India. All other reagents and chemicals used were of analytical grade or better.

2.2. Methods

2.2.1. Lyophilization of BSA

Lyophilization of BSA was performed using a freeze dryer (FD 3, Allied Frost, New Delhi, India) attached with a pre-freezer. Round bottom flasks containing 5 ml of BSA solution at 100 mg/ml (in water whose pH had been adjusted to 7.3) were pre-frozen in a shell at −38 °C for 1 h. Frozen BSA was lyophilized. Prior to lyophilization, BSA was dialyzed against distilled water, pH 7.3, for 5 h with five changes to remove the excipients present in the commercial sample. For moisture-induced aggregation studies of BSA in the presence of trehalose, BSA (dialyzed) and trehalose were mixed in variable ratios, rest of the conditions were the same as for BSA alone. The purpose of dissolution prior to co-lyophilization was to ensure uniform distribution of trehalose in the lyophilized BSA powder. Unless otherwise stated, this lyophilized BSA (referred to further as 'BSA'), with and without trehalose, was used throughout the study.

2.2.2. Moisture-induced aggregation of BSA

BSA (25 mg) was placed in a 15 ml stoppered glass vial, and a small volume (5–50 μl) of PBS (5 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.3) was added. The resultant sample was thoroughly mixed using a micro pipette tip, which was left in the vial [4]. The vial was then stoppered, sealed with parafilm, wrapped with aluminum foil, and placed in a controlled temperature incubator maintained at 55 °C. After 48 h, the vial was opened, 10 ml of PBS added and the resultant suspension was stirred at room temperature for 2 h. The pipette tip was discarded and the undissolved powder was subsequently removed by centrifugation (23,000*g* for 1 h). The amount of protein precipitated was determined by subtracting the amount of protein present in the supernatant from the starting protein, using the dye binding method [26]. For the moisture-induced aggregation of BSA in the presence of trehalose, the same procedure was adopted except that the starting solid mass was taken in a manner so that the amount of protein in the final mixture remained 25 mg.

2.2.3. Electrophoresis

SDS–PAGE and non-denaturing PAGE were carried out on 12% gel using standard protein markers. Electrophoresis was carried out in a miniVE electrophoresis unit (GE Healthcare, Uppsala, Sweden) under conditions of constant voltage. Proteins were stained with 0.1% Coomassie brilliant blue R-250 (in 45% methanol, 10% acetic acid) and destained in the same solvent.

2.2.4. Tryptophan intrinsic fluorescence

Aggregated protein was diluted to a final concentration of 0.1 mg/ml in PBS (50 mM, pH 7.4). Fluorescence emission spectra were acquired on a luminescence spectrometer (LS 50B, Perkin Elmer, Beaconsfield, England) using an excitation wavelength of 280 nm (slit width 5 nm) and emission wavelength range of 290–450 nm (slit width 10 nm). The spectrum of sample with no protein was subtracted from the spectra of the corresponding samples containing protein.

2.2.5. Thioflavin T spectrofluorimetric assay

Thioflavin T stock solution (2.5 mM) was prepared by dissolving Thioflavin T in PBS (50 mM, pH 7.4). The undissolved dye was removed by centrifugation (10,000*g*, 10 min). The aggregated protein was diluted to 1 ml in PBS (50 mM, pH 7.4) and incubated with Thioflavin T

using a slight modification of the published protocol [27–29]. The molar ratio of protein to dye was 1:5. After 1 h, the excitation and fluorescence emission spectra were acquired on a luminescence spectrometer (LS 50B, Perkin Elmer, Beaconsfield, England). The excitation spectra of all the samples were recorded in the wavelength range of 360–480 nm. For recording the emission spectra, an excitation wavelength of 440 nm (slit width 5 nm) and emission wavelength range of 450–570 nm (slit width 10 nm) were used. The spectrum of sample with no protein was subtracted from the spectra of the corresponding samples containing protein.

2.2.6. CongoRed spectrophotometric assay

A 300 mM stock solution of CongoRed was prepared in PBS (50 mM, pH 7.4) containing 10% ethanol (v/v). The undissolved dye was removed by centrifugation (10,000*g*, 10 min). The aggregated protein was diluted to 1 ml in PBS (50 mM, pH 7.4) and incubated for 30 min with CongoRed using a slight modification of the published protocol [29]. The molar ratio of protein to dye was 1:5. Spectra were recorded between 400 and 700 nm on UV–Vis spectrophotometer (DU 7400, Beckman Coulter, Palo Alto, USA). The spectrum of CongoRed alone was subtracted from the spectra of the corresponding samples containing protein.

2.2.7. CongoRed birefringence assay [27]

The solution of CongoRed was prepared by dissolving a saturated amount of CongoRed in 80% ethanol which was pre-saturated with sodium chloride. Protein samples (10 μl) were air dried on a glass slide. The resulting sample was incubated with 200 μl of CongoRed solution. After 2 min, the extra solution was removed by blotting with lint free filter paper. Specimens were viewed at 500× magnification with a polarized light microscope (Leika DMLP light microscope, Leika Microsystems Wetzlar GmbH, Wetzlar, Germany) under crossed polarized and optical light. Images were acquired by Leika DC 300 camera and analyzed by Leika IM 50 version 1.2 software.

3. Results

3.1. Moisture-induced aggregation of BSA

Moisture-induced aggregation of BSA was performed in the presence and absence of trehalose. The results obtained are shown in Fig. 1. Maximum aggregation was shown by BSA that was incubated with 20 μl of PBS for 48 h under elevated humidity at 55 °C (about 50% insoluble protein was precipitated upon reconstitution in PBS). Further addition of moisture led to an increase in the amount of protein detected in the supernatant, indicating decreased precipitation due to aggregation. BSA was then incubated under the same conditions but in the presence of different concentrations of trehalose (Fig. 2). Addition of even a small amount of trehalose (BSA:trehalose::1:0.25, w/w) led to an improvement in the aggregation properties of BSA. At the ratio of trehalose to protein approaching 1:1 (w/w), there was a significant reduction in the amount of BSA precipitated by moisture which could not be surpassed even after incubation with a higher amount of trehalose (Fig. 2). A control was run where BSA was incubated in the presence and absence of trehalose but without any added moisture. No aggregation could be detected in this case (Fig. 3).

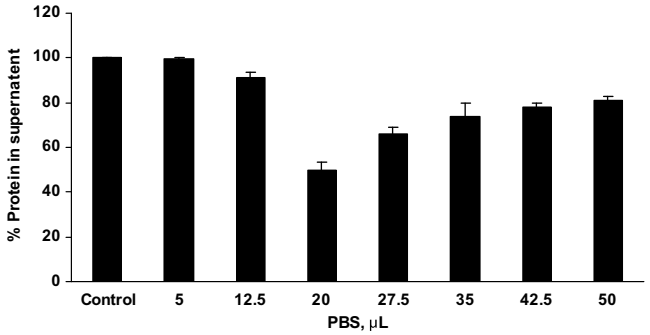

Fig. 1. Moisture-induced aggregation of BSA in the presence of variable amount of PBS. Different aliquots of PBS (5–50 μl) were added to lyophilized BSA (25 mg) and the samples were incubated for 48 h at 55 °C. The amount of protein precipitated was calculated by subtracting the amount of protein detected in the supernatant from the amount of protein initially taken for the study, which is designated as 100%. Lyophilized BSA incubated for 48 h at 55 °C without added PBS is taken as control. Values represent means of two independent experiments and error bars represent the standard deviation.

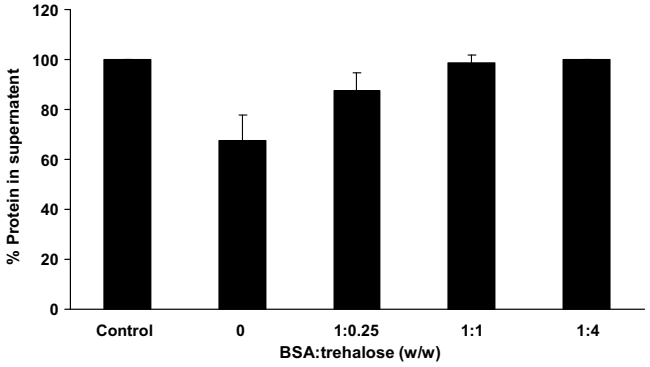

Fig. 2. Effect of trehalose on moisture-induced aggregation of BSA. Trehalose was co-lyophilized with BSA in different ratios (1:0.25–1:4). The amount of protein precipitated was calculated by subtracting the amount of protein detected in the supernatant from the amount of protein initially taken for the study, which is taken as 100%. Lyophilized trehalose BSA incubated for 48 h at 55 °C without added PBS was taken as control. BSA:trehalose 0 indicates BSA lyophilized without trehalose. Values represent means of two independent experiments and error bars represent the standard deviation.

3.2. Electrophoresis

Aggregates of BSA formed during moisture-induced aggregation were run on SDS–PAGE (denaturing PAGE) and native PAGE (non-denaturing PAGE). Fig. 4 shows

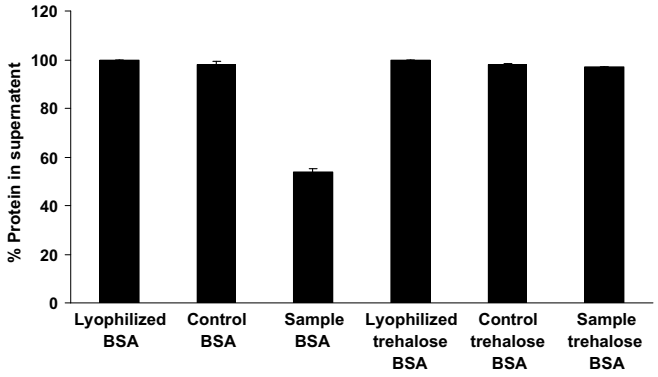

Fig. 3. Moisture-induced aggregation of BSA in the presence and absence of trehalose. BSA (25 mg) lyophilized with and without trehalose were incubated with (sample) and without (control) PBS (20 μl) for 48 h at 55 °C. The amount of protein precipitated was calculated by subtracting the amount of protein detected in the supernatant from the amount of protein initially taken for the study, which is taken as 100%. Values represent means of two independent experiments and error bars represent the standard deviation.

the results of denaturing (Fig. 4A) and non-denaturing (Fig. 4B) PAGE for all the samples and controls incubated for 48 h at 55 °C. As shown in Fig. 4A and B, no higher molecular weight bands could be detected after lyophilization of dialyzed BSA (lane 2), which confirmed that under the conditions employed, lyophilization did not cause aggregation of BSA. This was also confirmed by carrying out solid-state FTIR analysis of the BSA samples before and after lyophilization, which did not show any change in the pattern of the peaks (data not shown). Incubation of BSA for 48 h at 55 °C without PBS (in the absence of moisture) (lane 3), co-lyophilization of BSA with trehalose (without moisture without incubation for 48 h at 55 °C) (lane 6) and incubation of co-lyophilized trehalose BSA powder for 48 h at 55 °C without moisture (lane 7) also showed the absence of any aggregates. Thus, these were used as controls for monitoring moisture-induced aggregation of BSA. Incubation of BSA for 48 h at 55 °C resulted in aggregates which were insoluble in PBS upon reconstitution. Lane 5 in Fig. 4A shows streaking along with the presence of some higher molecular weight bands for this reconstituted sample. Non-denaturing PAGE also indicated the presence of higher molecular weight aggregates near the loading well (Fig. 4B, lane 5). Lane 2 in Fig. 4A and B shows a similar pattern as the lanes for commercial BSA (lane 1) or co-lyophilized trehalose BSA (co-lyophilized trehalose BSA without any PBS added but with incubation for 48 h at 55 °C) (lane 7). The supernatant obtained after centrifuging off the aggregated BSA (Fig. 4A and B, lane 4) did not show the presence of any soluble aggregates. This was also confirmed by size exclusion chromatography (SEC-HPLC) of the supernatant (data not shown), which showed only a single peak at the retention time of the monomeric protein, confirming the absence of soluble aggregates.

SDS–PAGE and native PAGE of various BSA samples were also carried out in the presence of urea and DTT. For this, aggregates were dissolved in 1 mM EDTA or 1 mM EDTA containing 10 mM DTT or 1 mM EDTA containing 10 mM DTT and 6 M urea. Concurrently, control sets (without any denaturant/reducing agent) were also run. The presence of higher molecular weight bands for the sample suspended in 1 mM EDTA showed that the aggregates were insoluble in 1 mM EDTA (Fig. 5A and B, lane 5). As compared to lane 6 (Fig. 5A and B) which showed the presence of a few bands at higher molecular weight showing that DTT alone could not solubilize the aggregates completely, DTT (10 mM), in the presence of urea (6 M), was able to dissolve the aggregated BSA completely

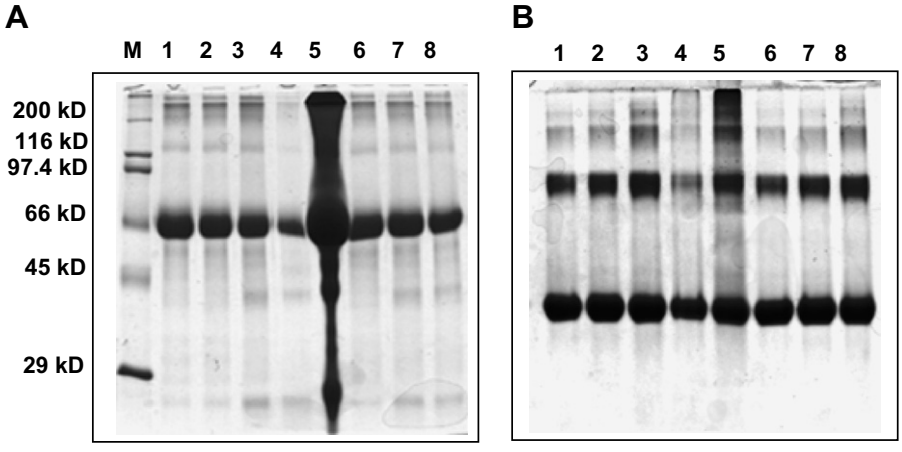

Fig. 4. SDS–PAGE (A) and native PAGE (B) for moisture-induced aggregation of BSA. Electrophoresis of the various samples and controls was carried out to monitor the moisture-induced aggregation of BSA in the presence and absence of trehalose on 12% gel. The amount of protein loaded in each lane is 20 μg. Lane M, molecular mass standard; lane 1, commercial BSA; lane 2, lyophilized BSA (without incubation at 55 °C for 48 h); lane 3, BSA control (BSA incubated at 55 °C for 48 h without any PBS added); lane 4, BSA supernatant; lane 5, BSA aggregates; lane 6, trehalose BSA lyophilized powder (without incubation at 55 °C for 48 h); lane 7, trehalose BSA control (incubated at 55 °C for 48 h without any PBS added); lane 8, trehalose BSA sample (incubated with PBS at 55 °C for 48 h). Gels were stained with Coomassie blue.

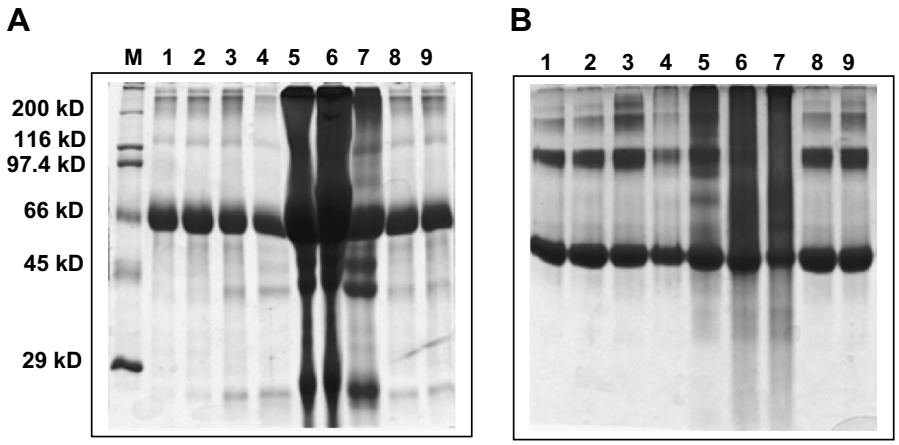

Fig. 5. SDS–PAGE (A) and native PAGE (B) of BSA aggregates dissolved in different reagents. Aggregated BSA was dissolved in EDTA, DTT and urea in different combinations as indicated in the text. Electrophoresis of aggregated BSA in different reagents was carried out. The amount of protein loaded in each lane is 20 μg. Lane M, molecular mass standard; lane 1, commercial BSA; lane 2, lyophilized BSA (without incubation at 55 °C for 48 h); lane 3, BSA control (incubated at 55 °C for 48 h without any PBS added); lane 4, BSA supernatant; lane 5, BSA aggregates in 1 mM EDTA; lane 6, aggregated BSA in 1 mM EDTA and 10 mM DTT; lane 7, aggregated BSA in 1 mM EDTA, 10 mM DTT and 6 M urea; lane 8, trehalose BSA control (incubated at 55 °C for 48 h without any PBS added); lane 9, trehalose BSA sample (incubated with PBS at 55 °C for 48 h). Gels were stained with Coomassie blue.

(Fig. 5A and B, lane 7). This is evident from the significant decrease in the number of higher molecular weight bands (as compared to lanes 5 and 6). 1 mM EDTA was used to prevent the autoxidation of DTT [4]. As mentioned above, the aggregates were partially soluble in EDTA–DTT and completely soluble in EDTA–DTT–urea. Thus, it was thought prudent to determine the effect of EDTA alone. Hence, aggregates were dissolved in 1 mM EDTA where they remained insoluble (Fig. 5A and B, lane 5).

3.3. Tryptophan intrinsic fluorescence

Tryptophan intrinsic fluorescence showed a marked decrease in the intensity and a small shift in the emission maxima for aggregated BSA (Fig. 6, Curve II). When incubated with trehalose, there was only a slight decrease in the fluorescence intensity and no change in the emission maxima (Fig. 6, Curve III) as compared to BSA alone (Fig. 6, Curve I).

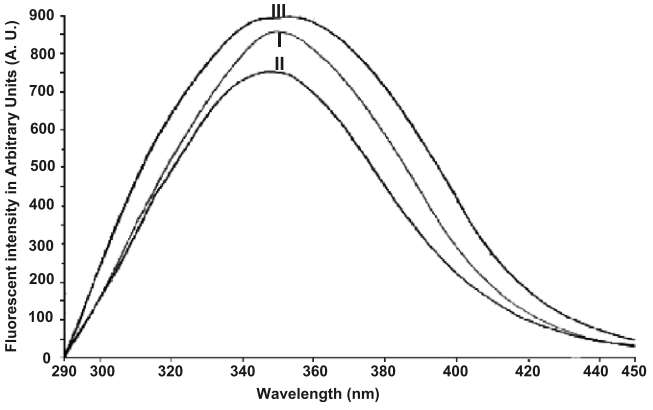

Fig. 6. Intrinsic fluorescence spectra of BSA. The fluorescence was measured after excitation at 280 nm in 50 mM PBS at a protein concentration of 0.1 mg/ml. Lyophilized BSA (I); aggregated BSA (II) and aggregation of BSA in the presence of trehalose (III). Spectra were subtracted from respective blanks and smoothened. The concentration of protein in all the cases is the same.

3.4. Thioflavin T spectrofluorimetric assay

The excitation spectra of native and aggregated BSA in the presence of ThT are shown in Fig. 7A. There is no change in the wavelength of maximum excitation even though the value of absorption is significantly higher in the case of the dye being incubated with the aggregated BSA. For Thioflavin T enhanced fluorescence assay, 1 ml solution of 75 μM Thioflavin T (in 50 mM PBS, pH 7.4) was used with 15 μM BSA (dye to BSA molar ratio 5:1). The result showed a striking enhancement in the fluorescence intensity of the dye in the presence of aggregated BSA as compared to that with native BSA (Fig. 7B). BSA incubated with 1:1 (w/w) trehalose for 48 h at 55 °C under elevated humidity conditions showed approximately twofold decrease in the fluorescence intensity of Thioflavin T as compared with aggregated BSA and showed greater resemblance to the spectrum of the dye with native BSA (Fig. 7B).

3.5. CongoRed spectrophotometric assay

The results of spectroscopic assays of CongoRed with BSA, aggregated BSA and BSA incubated with trehalose are shown in Fig. 8. The spectrum of BSA showed a red shift of 9 nm as compared to the spectrum of CongoRed alone while this shift was 15 nm for aggregated and trehalose protected BSA (Fig. 8A, inset). CongoRed incubated with aggregated BSA showed higher absorbance than native BSA and trehalose protected BSA. CongoRed sub-

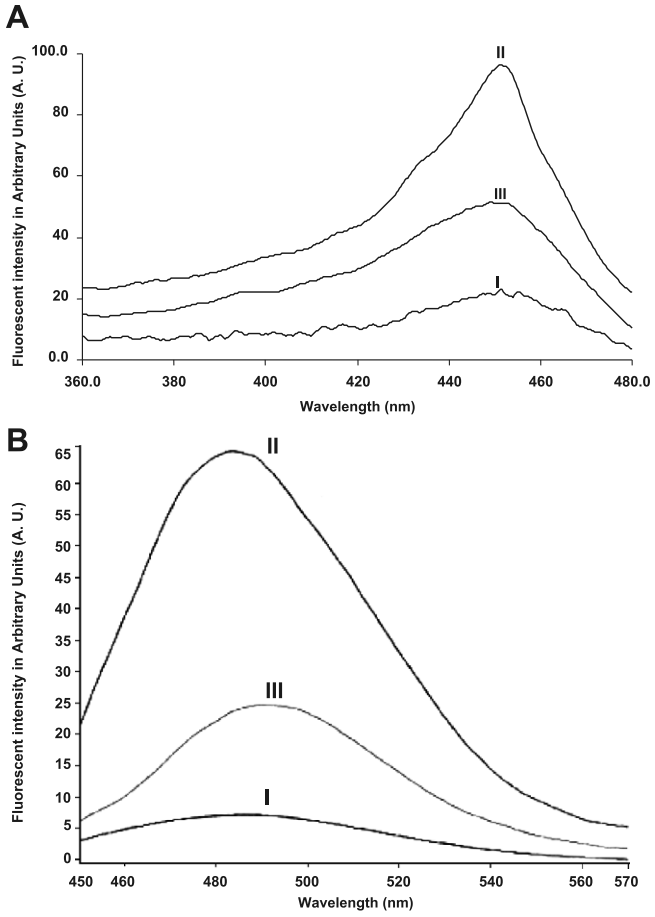

Fig. 7. Thioflavin T (ThT) excitation and fluorescence enhancement spectra. (A) The excitation spectra of ThT solution with lyophilized BSA (I), aggregated BSA (II) and aggregation of BSA in the presence of trehalose (III). Spectrum for Thioflavin T was subtracted from the spectra of ThT with protein and smoothened. Molar ratio of protein to Thioflavin T was 1:5. The concentration of protein in all the cases is the same. (B) The emission spectra of ThT solution with lyophilized BSA (I), aggregated BSA (II) and aggregation of BSA in the presence of trehalose (III). Spectrum for Thioflavin T was subtracted from the spectra of ThT with protein and smoothened. Molar ratio of protein to Thioflavin T was 1:5. Excitation wavelength was set at 480 nm, slit width was kept 5 and 10 nm for excitation and emission, respectively. The concentration of protein in all the cases is the same.

tracted spectrum (Fig. 8B) also showed a considerable difference in absorbance values among BSA, aggregated BSA and BSA incubated with trehalose.

3.6. CongoRed birefringence assay

CongoRed birefringence studies were done for different BSA samples. Under bright field, all the BSA samples treated with CongoRed showed red color (Fig. 9A, C and E) which indicated that the dye bound to BSA in a non-specific manner irrespective of whether aggregation occurred or not. However, under polarized light, only aggregated BSA exhibited apple green birefringence that is characteristic of amyloid type of structures [28] (Fig. 9D), while native BSA (Fig. 9B) or trehalose BSA sample (trehalose BSA incubated with PBS for 48 h at 55 °C) (Fig. 9F) did not show any birefringence.

4. Discussion

A variety of additives/excipients are available for prevention of protein denaturation, among which sugars like trehalose, sucrose, maltose, etc., are used more commonly [1,12,30]. Our studies have focused on the protective effect of trehalose on moisture-induced aggregation of BSA. In particular, we characterized the aggregation pattern of BSA in the presence and absence of trehalose by different techniques. The conditions used to induce aggregation in BSA resemble the accelerated stress conditions that are commonly used in the pharmaceutical industry to estimate the storage stability of a product, especially for small molecule drugs [5,31]. Enhanced temperature and humidity mimic the conditions that a protein drug would encounter over an extended period of time, especially in tropical countries, and which will decide its storage stability [5,10,31,32]. As indicated in Results section, about 50% of BSA aggregated under optimized conditions. The trend of the graph (Fig. 1) follows the generally reported observation that the amount of precipitated protein increases with the amount of added moisture and then decreases upon further increase in moisture content [4,6,21]. The reduced amount of aggregates formed at higher moisture content probably reflects the dissolution of the aggregates formed because of the presence of increased amount of water. The amount of moisture required to maximally precipitate BSA (8 μl/10 mg) in this case is higher than what is reported in the literature [4,6]. This amount depends on the initial water content of the dried protein and it has been shown that reducing the water content of the starting preparation (by employing stronger lyophilization conditions) leads to an increase in the amount of moisture required for causing aggregation [7,11,33]. When BSA was incubated under the same conditions but in the presence of 1:1 (w/w) trehalose, no aggregation was observed. This indicates that trehalose, at the ratio of 1:1 (w/w), was effective in protecting BSA against moisture-induced aggregation.

The effect of chaotropic agents like urea and reducing agents like 1,4-dithio-DL-threitol (DTT), β-mercaptoethanol, etc., on denaturation and renaturation of different proteins is well established [4,34–36]. Urea is well-known to disrupt non-covalent bonds between proteins [34,35]. It is used quite commonly to dissolve inclusion bodies during recombinant protein production [36]. DTT and β-mercaptoethanol are used to break covalent, mainly disulfide, bonds in proteins. The results in Fig. 5 show the presence of several types of interactions in aggregated BSA. DTT alone is ineffective in solubilizing aggregates completely while in urea–DTT, aggregates could be solubilized. This indicates the presence of covalent linkages which are partially accessible to DTT alone and become completely accessible to DTT in the presence of the denaturating

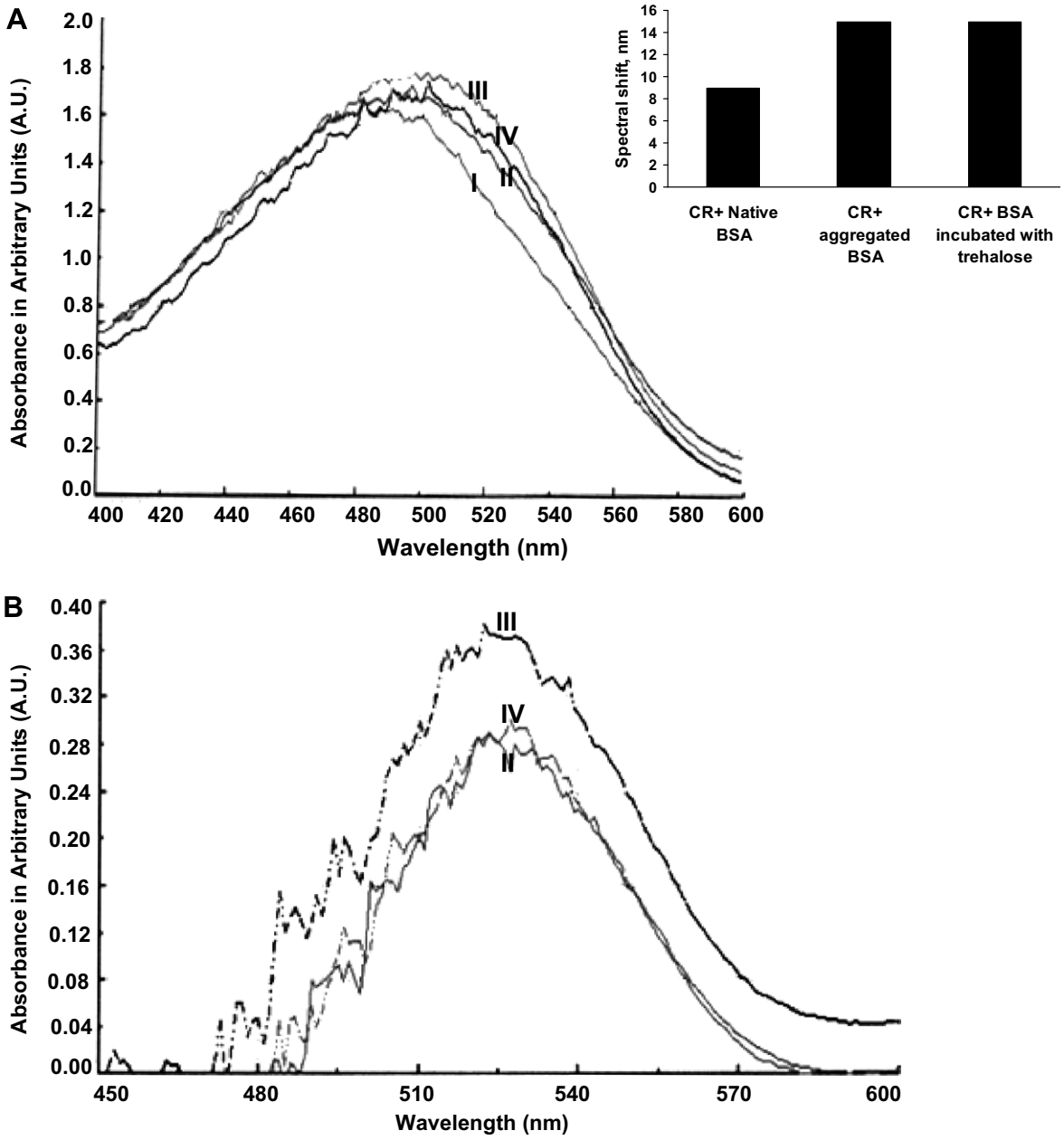

Fig. 8. CongoRed binding spectrum for BSA. CongoRed absorption spectra were acquired in the visible region of light for CongoRed alone (I); BSA (II); aggregated BSA (III) and BSA incubated with trehalose (IV). Both non-subtracted (A) and subtracted (B) spectra are shown. For (B), the spectrum of CongoRed was subtracted from that of CongoRed plus protein. The concentration of protein in all the cases is the same. Inset shows the shift in maxima for different samples from the maxima of CongoRed.

agent, urea. Thus, physical interactions within the aggregates are destroyed by urea. Many studies on aggregation of proteins have shown conformational changes upon aggregation [2,37]. Due to such type of changes, the hydrophobic core of the protein becomes exposed. Therefore, hydrophobic–hydrophobic interaction could be the reason for inaccessibility of some of these bonds to DTT alone. These interactions are disrupted by urea (which is known to interfere with non-covalent interactions) and the aggregates become completely soluble in DTT–urea. The amino acid sequence of BSA has 35 Cys residues which form 17 disulfide bonds in the folded native BSA [37]. The remaining unpaired Cys residue may be involved in disulfide bond formation between monomers during the aggregation of BSA, which is in agreement with the hypotheses reported in the literature [4,5].

The fluorescence of Trp residues in the protein molecule has long been known to be sensitive to the polarity of their local environment [38,39]. The intensity of fluorescence emission and the position of the emission peak exhibit a change when the immediate environment around the fluorescing residues is altered [38]. One mol-

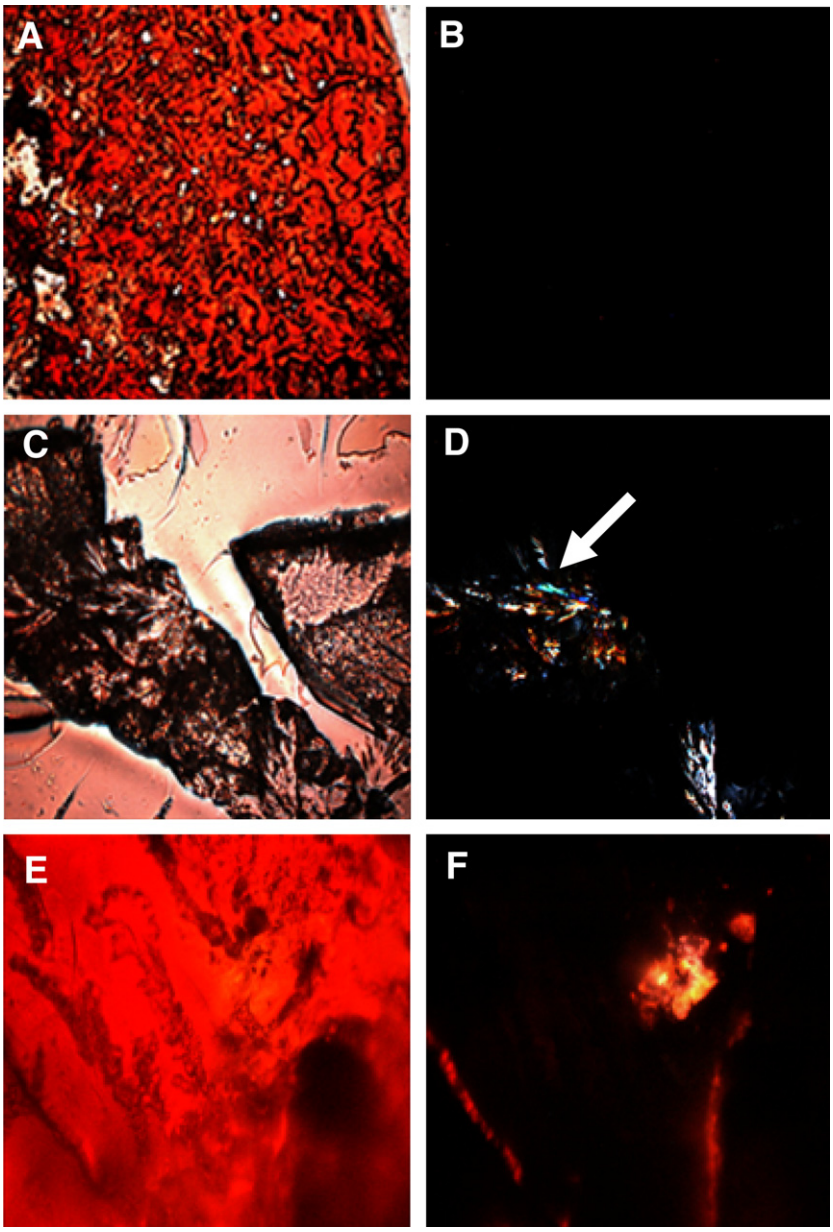

Fig. 9. CongoRed birefringence of BSA. Samples were visualized under optical (A, C and E) and polarized (B, D and F) light at 500× magnification. BSA (A and B), BSA aggregates (C and D) and BSA incubated with trehalose (E and F). (For interpretation to colours in this figure, the reader is referred to the web version of this paper).

ecule of BSA has two Trp residues located in two different domains. Trp-134 is situated in the eighth helix of D129-R144 in domain I. It is near the protein surface but is buried in a hydrophobic pocket of the domain. Trp-214 is in the second helix of E206-F221, in an internal part of domain II [37,40,41]. Thus, Trp fluorescence can be used as a sensitive method for monitoring conformational changes in BSA during moisture-induced aggregation. In native BSA, the fluorescence emission band is attributed to both these Trp residues but the larger contribution is from Trp-134 [42]. Militello and coworkers have shown the possible role of free Cys-34 in the aggregation pathways of this molecule [37]. It is noteworthy that this Cys-34 is also present in domain I where Trp-134 is located [37]. As reported in the literature [4,5] and indicated by our electrophoresis results, the disulfide linkage through the free Cys residue is the main pathway of moisture-induced aggregation in BSA. Therefore, conformational changes and rearrangement of the local environment upon aggregation probably cause the quenching in fluorescence of Trp-134. As shown in Fig. 6, Curve II, such changes cause a marked decrease in the emission intensity of the molecule, reflecting a change in the polarity of the local environment around the fluorescent species. The theory of exposure of hydrophobic core during the aggregation of proteins is well known [2]. As discussed above, in the case of BSA, Trp-134 is buried in a hydrophobic pocket of domain I. It is probable that

upon unfolding of the protein during moisture-induced aggregation process, the local environment of Trp-134 changes. Since Cys-34 is also in the same domain where Trp-134 is situated, this residue becomes more prone to disulfide cross linking with Cys residues of adjacent molecules. Hence disulfide bond formation between unpaired Cys residues might be the cause for aggregation. A change in the environment of Trp residues in BSA molecules might result in the subsequently observed quenching of tryptophan fluorescence. Fig. 6, Curve III shows only a marginal change in the fluorescence intensity and emission maxima for BSA incubated with trehalose as compared to the spectrum of native BSA (Fig. 6, Curve I), which indicates the retention of local environment around the Trp residue in the presence of trehalose. Thus, trehalose stabilizes the tertiary structure of BSA against aggregation as compared to native BSA, and does not allow the unpaired Cys residue to come in contact with another unpaired Cys moiety. This may explain the role that trehalose plays in protecting BSA against moisture-induced aggregation.

We next attempted to look at the nature of the aggregates formed by the interaction of BSA with moisture. Thioflavin T, a benzothiazole dye and CongoRed, a diazobenzidine sulfonate dye, are dyes that are known to interact with amyloid type of aggregates [27]. Stathopulos and coworkers have shown the efficacy of these dyes in the characterization of sonication-induced aggregates of proteins like BSA, myoglobin, lysozyme, etc., as amyloids [28]. In our studies, the aggregates caused marked enhancement in the fluorescence of Thioflavin T, exhibited apple green birefringence upon binding to CongoRed and caused changes in the absorption spectrum of the latter. Thus, the possibility of amyloid type of character in BSA aggregated by moisture cannot be ruled out. The mechanism of interaction of CongoRed and Thioflavin T with amyloid fibrils is not well understood. CongoRed and Thioflavin T are charged molecules; therefore, it is generally believed that a combination of hydrogen bonding, hydrophobic and electrostatic interactions between the dye molecule and the protein fibrils are responsible for the interaction [27]. A steric intercalation of CongoRed molecules between β-sheets of amyloid fibrils has also been proposed [27,43]. Many studies on the aggregation of BSA under different conditions have shown changes in the secondary structure of the protein [2,37,44]. The tryptophan intrinsic fluorescence data indicate the exposure of hydrophobic core during aggregation of BSA and possible changes in the tertiary structure of the protein. Thus, these dyes might be using any one of the above means to interact with the aggregates. Furthermore, these dyes did not show any significant interaction with trehalose protected BSA. It should be pointed out that even though CongoRed interacts non-specifically with the native protein as well, which is in agreement with reported observations [29], this does not translate to apple green birefringence under polarized light which is a characteristic of amyloid type of structures and is seen only in the case of aggregates.

Trehalose is well-known in protecting biomolecules under stress conditions, in vitro as well as in vivo [13–15,30]. Several hypotheses reported in the literature can be invoked to justify the observations of this study. Firstly, trehalose might be reducing the mobility of BSA by either forming hydrogen bonds with the protein [12,45,46] or by forming a glassy matrix around the protein molecule [13,47] during incubation with moisture at elevated temperature (55 °C for 48 h). Due to this reduced flexibility, unfolding and exposure of hydrophobic core and thus aggregation are prevented. It should be recalled that the specific viscosity of trehalose solution is about 2.5 times more than that of sucrose and maltose at the same concentration and is in a similar range as glycerol, another frequently used preservative [16]. The increased viscosity may be responsible for the reduced mobility of protein molecules. Secondly, trehalose has a large hydration volume (capacity to absorb water) [16]. Thus under moist conditions, trehalose can absorb water and leave BSA unaffected by the presence of moisture. Lastly, preferential hydration theory states that in a triphasic system of water, protein and a stabilizer like trehalose, the stabilizer is excluded from the solvation layer of protein [16,46]. As a result, the protein becomes preferentially hydrated, but the radius of the solvation layer and the apparent volume of protein decrease, in a phenomenon that leads to a more stable protein conformation. In the absence of trehalose, added moisture causes an increase in the apparent volume of BSA, making it unstable and prone to aggregation. The exact mechanism at work may be a concession to all these theories.

5. Conclusion

Storage instability of protein drugs is a frequently encountered problem in the pharmaceutical industry. The results presented above show that the non-reducing disaccharide trehalose can act as a useful excipient in the prevention of aggregation of BSA induced by moisture and may thus improve the storage stability of protein drugs. The nature of these aggregates has been judged to be 'amyloid-like', as probed using various spectrometric and electrophoretic techniques. It has been speculated that trehalose interferes with the interaction of Trp-134 with Cys-34 and does not allow disulfide-bridged aggregates to be formed.

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