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Inhibitory Effects of β-Cyclodextrin and Trehalose on Nanofibril and AGE Formation During Glycation of Human Serum Albumin

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Abstract: The effects of β-cyclodextrin (β-CyD) and trehalose on glycation of human serum albumin (HSA) were studied. These additives reduced AGEs and nanofibril formation of HSA under *in vitro* glycation conditions and improved its helical structure. These were accomplished through direct interactions of them with HSA and alterations in solute-protein interactions.

Keywords: Diabetes, trehalose, β-cyclodextrin, glycation, HSA, glucose.

1. INTRODUCTION

The spontaneous non-enzymatic glycation of proteins is a major contributing factor to complications of diabetes. In this reaction reducing carbohydrates bind covalently to the free amino groups of proteins and ultimately lead to the formation of irreversible, heterogeneous and toxic advanced glycation end products (AGEs) [1, 2]. During AGE-formation the lysine and arginine side chains are targets of modification, with lysine residues being more reactive [3]. Albumin represents approximately 80% of the circulating glycated proteins [4]. It is an abundant protein in serum (40 mg/ml) [5] and functions as a carrier of a very wide variety of compounds. Human serum albumin (HSA) is also a major contributor to the oncotic blood pressure [6] and may play a protective role as an antioxidant *in vivo* [7]. Glycation reactions are associated with severe alterations in structural, biological, and functional properties of many proteins including albumin [8]. In spite of insulin therapy glycation of albumin remains a serious long-term complication of diabetes. Thus, treatment of hyperglycemia may not be sufficient to prevent complications of diabetes [8, 9]. In addition, glycation is one of the modification reactions that spontaneously occur during a number of industrial food processing [10]. Therefore, understanding how these reactions are mediated and developing strategies to block them will be beneficial.

Several methods have been proposed for inhibition of glycation reactions [11], including the use of water soluble polysaccharides [12].The natural water-soluble polysaccharide β-cyclodextrin (β-CyD; Fig. **1A**) is a α-1, 4-linked cyclic oligomers of seven D-glucose. It possesses remarkable properties in drug formulation, delivery and other technologies as enzyme mimics, drug carriers, odor and taste-masking compounds, and also in insulin injections for modifying its pharmaceutical properties. β-CyD forms inclusion complexes in which CyDs serve as the host providing a hydrophobic cavity for small molecules such as fatty acids and amino acids with suitable size, shape, and polarity [13-15].

Trehalose Fig. (**1B**), a non-reducing disaccharide of α-D-glucopyranosyl and α-D-glucopyranoside, is one of the most prevalent compatible solutes. Trehalose has potential applications in the areas of protein stabilization, vaccine development, liposome formation, organ preservation, therapeutics for diseases caused by protein aggregation, dry skin, dental caries, and others [16]. Its strong stability is the result of the very low energy glycoside oxygen bond joining the two hexose rings [17]. Xie and Timasheff proposed that protein stabilization by trehalose at room temperature is determined by an increase in the preferential exclusion of trehalose upon unfolding [18].

We recently reported studies on the structural properties of glycated HSA (GHSA) [19-21] and introduced alginate, a polymer polysaccharide, as an antiglycating agent for HSA [22]. Here we determined the structural effects of β-CyD and trehalose on glycation of HSA and protein structure.

2. EXPERIMENTAL

2.1. Materials

Human serum albumin (≥ 96%, free fatty acid), β-cyclodextrin, β -D (+) glucose, Ethyleneamide-Tetraacetic Acid (EDTA), nitroblue tetrazolium (NBT) and sodium bicinchoninate (BCA) were from Sigma. The membrane filters (0.2 μm pore size, 25 mm in diameter) and dialysis tubing (cut off 10,000 MW) were from Whatman (UK). The Treha-

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lose, L-valine, 9, 10 -phenanthrenequinone and sodium azide were from Merck (Germany). Thioflavin T (ThT) and 2,4,6-trinitrobenzene sulfonic acid (TNBSA) were from Fluka. All other materials were of analytical grade. All solutions were prepared with deionized water.

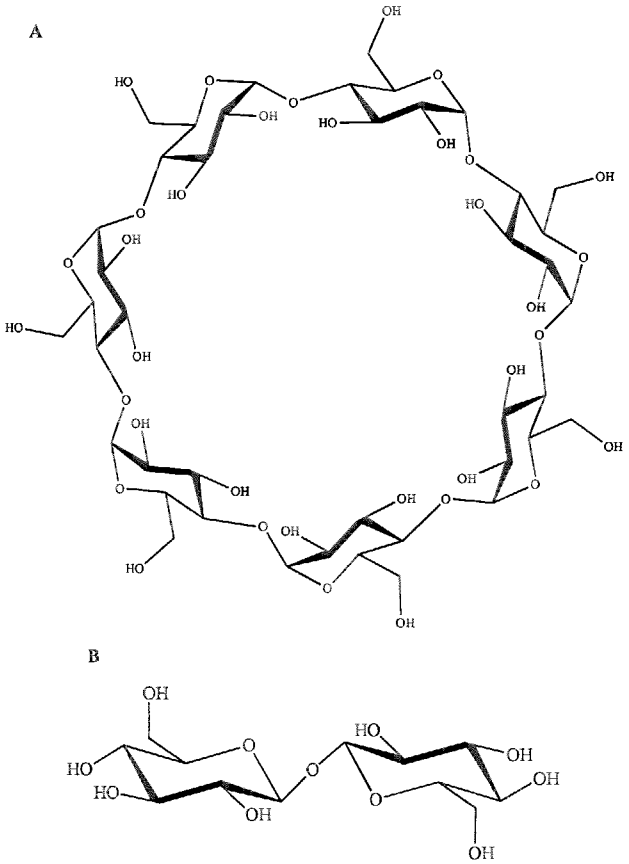

Figure 1. The chemical structures of non-reducing carbohydartes. A) β-CyD, composed of seven glucose residues and, B) trehaolse composed of a-D-glucopyranosyl-(1→1)-a-D-glucopyranoside with a particular glycosidic linkage.

2.2. Methods

2.2.1. In-Vitro Glycation of HSA

Samples of HSA were diluted to 10 mg/ml in sodium phosphate buffer (50 mM Na_2HPO_4/NaH_2PO_4, EDTA 1 mM and 0.1 mM sodium azide at pH 7.4) in capped vials under sterile conditions, which contained glucose (40 mM), glucose (40 mM) with β-CyD (10 mM) or trehalose (100 mM), β-CyD (10 mM) or trehalose (50 mM), or no additive as a control. Protein concentration was determined spectrophotometrically with an extinction coefficient (E1%) of 5.30 at 280 nm [23] using a Shimadzu spectrophotometer model UV-3100.

All dishes were autoclaved prior to use in order to inactivate proteases, and all solutions were filter-sterilized (0.2 μm). The capped vials were protected from light and incubated at 37°C for 42 days. Samples were then dialyzed against sodium phosphate buffer at 4°C for 48 h. Following dialysis, the samples were aliquotted and stored at -20°C until analyzed [3]. After dialysis, the concentration of protein samples was determined in triplicates by bicinchoninic acid protein assay (BCA assay).

2.2.2. UV-Visible Analysis

A Shimadzu UV-3100 spectrophotometer, equipped with a water bath (±0.5 °C), was used for recording the spectra of the HSA samples (1 mg/ml in 50 mM sodium phosphate buffer, pH 7.4, at 25°C) in a wavelength range of 200–550 nm.

2.2.3. Circular Dichroism Analysis

Far-UV CD was used to measure changes in the secondary structure of HSA (0.2 mg/ml), using an Aviv circular dichroism spectropolarimeter model 215 with a path length of 0.1 cm, at a range of 190-260 nm, using ammonium d-10-camphorsulfonic acid for calibration. The results are expressed as the molar ellipticity [θ], which is defined as [θ] = θ millidegrees / {10*number of amino acid (lc)}, where c is the molar concentration of sample, and l is the length of the light path in Cm.

2.2.4. Trp Fluorescence Measurements

The fluorescence of tryptophan residue in diluted samples (0.2 mg/ml) in the presence of 50 mM sodium phosphate buffer (pH 7.4), was monitored with excitation wavelength at 290 nm at 25°C in a Cary Eclipse fluorescence spectrophotometer.

2.2.5. Determination of Fibrillar State with ThT

The fibrillar state of the incubated HSA was determined via ThT [3]. We measured the fluorescence of HSA (0.2 mg/ml) and 10 μM ThT reagent in 50 mM phosphate buffer, pH 7.4 at the excitation/emission wavelengths of 450/490 nm at 25°C in a Cary Eclipse fluorescence spectrophotometer.

2.2.6. AGE Formation Analysis

The emission intensity of the samples (1 mg/ml) at 370 nm excitation was obtained in a Cary Eclipse fluorescence spectrophotometer for detection of AGE. Each point represents the mean of three independent experiments.

2.2.7. Determination of Free Lysine and Arginine Residues

The determination of the level of free lysine residues was carried out using 2,4,6-trinitrobenzene sulfonic acid (TNBSA), which reacts specifically under mild conditions with free amino groups to form trinitrophenyl derivatives as described by Sharma *et al.* [24]. The number of free arginine residues in the protein was determined using 9, 10-phenanthrenequinone reagent as described by Schmitt *et al.* [3]. The amounts of free lysine and arginine in each sample were obtained from the mean of three independent experiments.

2.2.8. Determination of Amadori Products

Samples were assayed for Amadori products based on the nitroblue tetrazolium (NBT) reaction with ketoamines. 0.18mg/ml sample of protein was mixed with 0.03 mM NBT reagent (in carbonate buffer, pH 10.35) and incubated for 1 h at 37°C and then absorbance was measured at 530 nm. Each point represents the mean of three independent experiments.

3. RESULTS

The major aim of this study was to provide direct evidence for the effects of natural non-reducing carbohydrates, β-CyD and trehalose, on HSA-glycation. This information was attained by detection of AGE-absorbance, Amadori product quantification, the quantification of the reacted lysine and arginine side-chains, and the study of HSA structure incubated with glucose in the presence or absence of β-CyD or trehalose. The far-UV CD spectrum of HSA was characterized by the presence of two strong negative bands at 208 and 222 nm, which represent the helical characteristics of HSA. Fig. (**2**) shows loss of helical structure as demonstrated by a decrease in the negative ellipticity at 208 and 222 nm following incubation of HSA with glucose for 42 days compared to the control (HSA incubated without any additive). HSA in the presence of β-CyD or trehalose showed more negative ellipticity values at 208 and 222 nm after 42 days of incubation (Fig. **2**). However, the far-UV CD data for HSA incubated with a mixture of glucose and β-CyD for 42 days showed an increase in negative ellipticity compared to HSA control (Fig. **2A**). The sample of HSA incubated with glucose and trehalose showed a similar spectrum as HSA control (Fig. **2B**).

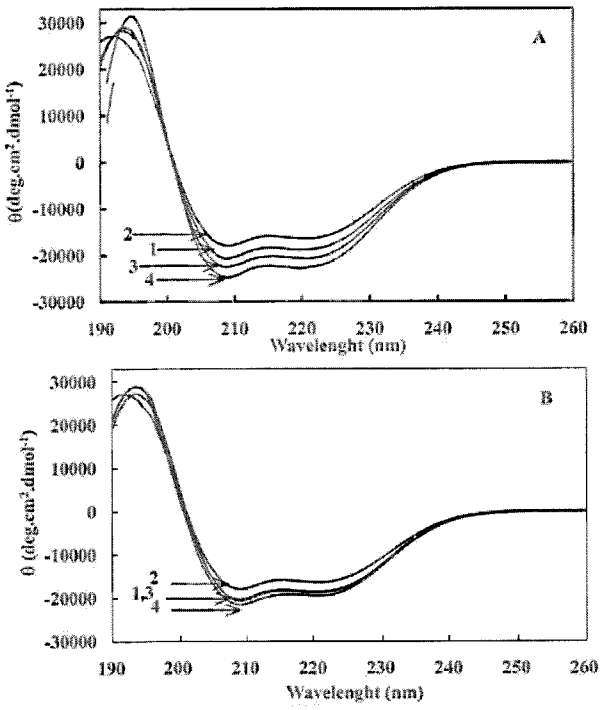

Figure 2. Circular dichroism (CD) spectra of HSA samples incubated for 42 days in 50 mM phosphate buffer, pH 7.4 at 37°C with 1) no additives (control), 2) 40 mM glucose, 3) A: 40 mM glucose and 10 mM β-CyD; B: 40 mM glucose and 100 mM trehalose, and 4) A: 10 mM β-CyD; B: 100 mM trehalose.

ThT is a dye that interacts with the fibrillar structure of proteins inducing the enhancement of fluorescence intensity. This quality of ThT has been employed in the detection of amyloid fibril structures in proteins [3]. The fibrillar state of GHSA was enhanced compared to HSA control (Fig. 3). The intensity of ThT fluorescence of HSA with trehalose in the presence or absence of glucose was equal to that of the HSA control. The intensity of ThT fluorescence of HSA with β-CyD in the absence of glucose was equal to that of the HSA control. However, the intensity of ThT fluorescence of HSA with β-CyD in the presence of glucose was less than GHSA.

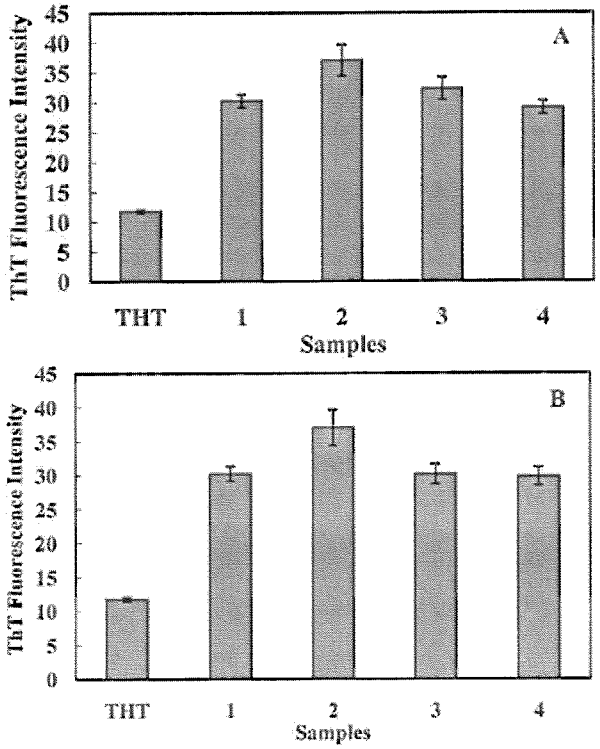

Figure 3. Thioflavin T fluorescence intensity after excitation at 450 nm for HSA samples incubated for 42 days in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (control), 2) 40 mM glucose, 3) A: 40 mM glucose and 10 mM β-CyD; B: 40 mM glucose and 100 mM trehalose, and 4) A: 10 mM β-CyD; B: 100 mM trehalose.

The AGEs showed an increase in fluorescence intensity emission after excitation at 370 nm compared to control HSA. Fig. (**4**) shows the enhancement in fluorescence intensity for GHSA. However, the presence of β-CyD or trehalose with glucose decreased the fluorescence intensity of HSA compared to GHSA. HSA incubated with β-CyD or trehalose in the absence of glucose showed lower fluorescence intensity, which was significantly lower than the samples in the presence of glucose. Fig. (**5**) shows the increase in absorbance for GHSA, a criteria indicating AGE formation [3]. The presence of β-CyD or trehalose alone induced an increase in the absorbance of HSA compared to the control HSA.

The fluorescence intensities of samples at excitation of 290 nm showed decreased intensity for GHSA (Fig. **6**). A

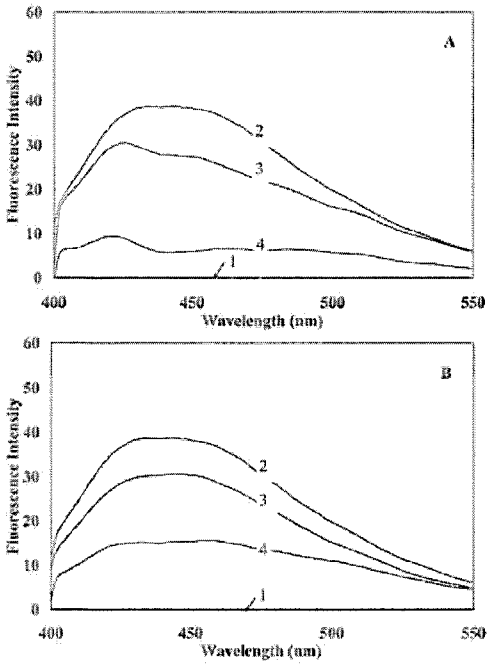

Figure 4. Fluorescence intensity after excitation at 370 nm of HSA samples incubated for 42 days in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (control), 2) 40 mM glucose, 3) A: 40 mM glucose and 10 mM β-CyD; B: 40 mM glucose and 100 mM trehalose, and 4) A: 10 mM β-CyD; B: 100 mM trehalose.

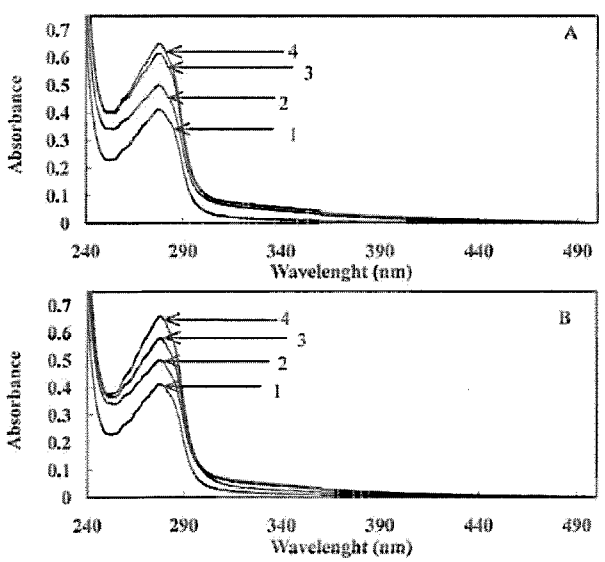

Figure 5. UV-vis spectra of HSA samples incubated for 42 days in 50 mM phosphate buffer, pH 7.4 at 37°C with 1) no additives (control), 2) 40 mM glucose, 3) A: 40 mM glucose and 10 mM β-CyD; B: 40 mM glucose and 100 mM trehalose, and 4) A: 10 mM β-CyD; B: 100 mM trehalose.

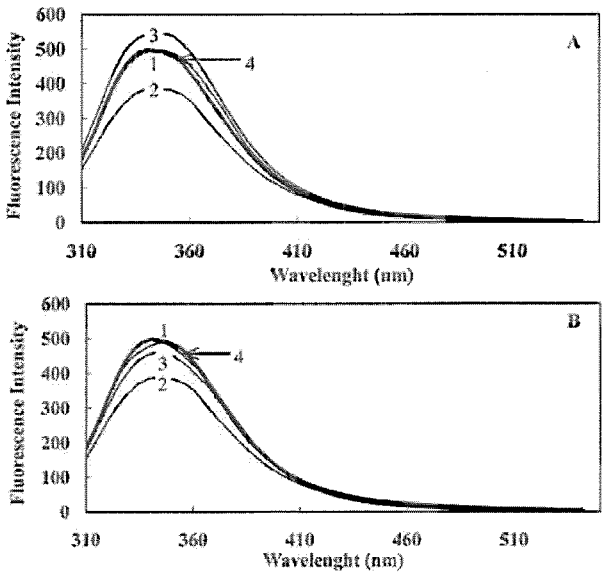

Figure 6. Trp fluorescence intensity after excitation at 290 nm of HSA samples incubated for 42 days in 50 mM phosphate buffer, pH 7.4 at 37 °C in 1) no additives (control), 2) 40 mM glucose, 3) A: 40 mM glucose and 10 mM β-CyD; B: 40 mM glucose and 100 mM trehalose, and 4) A: 10 mM β-CyD; B: 100 mM trehalose.

slight red shift in HSA fluorescence was observed in the presence of β-CyD alone, while the presence of β-CyD and glucose induced an increase in the fluorescence intensity. The HSA fluorescence in the presence of trehalose was similar to control HSA. However, in the presence of glucose and trehalose the HSA solution exhibited a slight decrease in fluorescence intensity and a red shift in fluorescence.

AGE formation was also determined using the quantification of the reacted lysine and arginine side-chains. Free lysine and arginine residues were measured by TNBSA and 9, 10-phenanthrenequinone, respectively. In control HSA sample all of the 58 lysine and 18 arginine residues were free of glucose. However, the number of free lysine and arginine residues decreased for the HSA samples incubated with Glucose. Fig. (7) shows the number of lysine residues that were not bound to glucose in each sample. The number of involved lysine residues in GHSA was 6, whereas in the presence of β-CyD or trhalose and glucose the number of lysine residues involved was 5 compared to the control. Arginine residues reacted much less with glucose in glycated samples. However, in the presence of β-CyD or trehalose, arginine residues did not react with glucose (data not shown).

Amadori products can reduce NBT reagent and then produce colored Formazan dye with an absorption maximum at 530 nm. Fig. (8) compares formation of this dye in GHSA in the presence and absence of trehalose or β-CyD reflecting the generation of Amadori products. Our results showed that the extent of Amadori product formation was increased in GHSA. However, the presence of trehalose or β-CyD re-

sulted in a significant decrease in formation of Amadori products in GHSA.

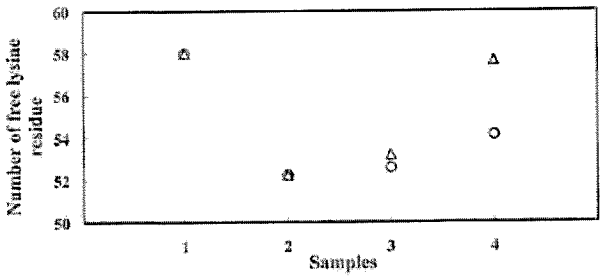

Figure 7. TNBSA absorbance at 340 nm of HSA samples incubated for 42 days in 50 mM phosphate buffer, pH 7.4 at 37 °C with 1) no additives (control; ○ or Δ), 2) 40 mM glucose (○ or Δ), 3) 40 mM glucose and 10 mM β-CD (○) or 40 mM glucose and 100 mM trehalose (Δ), and 4) 10 mM β-CyD (○) or 100 mM trehalose (Δ).

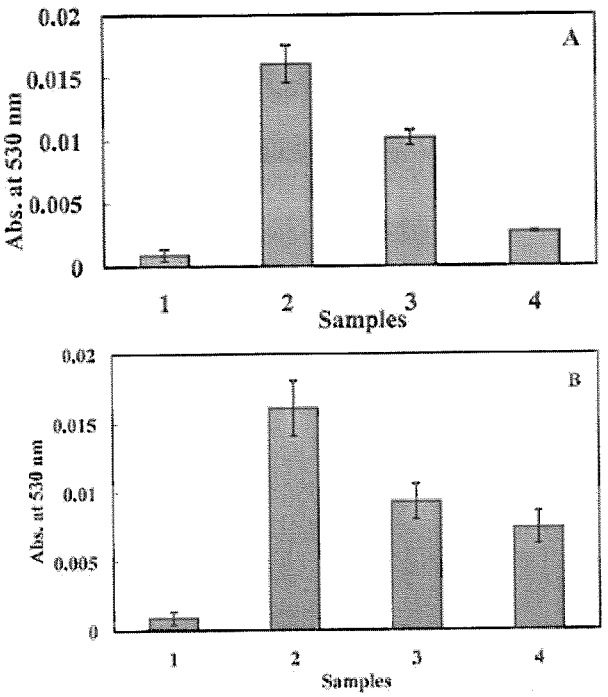

Figure 8. NBT absorbance at 530 nm of HSA samples incubated for 42 days in 50 mM phosphate buffer, pH 7.4 at 37 °C in: 1) no additives (control), 2) 40 mM glucose, 3) A: 40 mM glucose and 10 mM β-CyD; B: 40 mM glucose and 100 mM trehalose, and 4) A: 10 mM β-CyD; B: 100 mM trehalose.

4. DISCUSSION

CyDs have received considerable attention because of their various usages in drug formulation and drug delivery systems [15]. Trehalose is also used in substantial quantities in modern food sources such as honey, baker's yeasts and commercially grown mushrooms [25]. Trehalose has also been isolated from various seed plants, including sunflowers [26]. Ancient man used insects and fungi for diet containing more concentration of trehalose compared to modern Western diet [27]. However, the health consequences of such diets require further elucidation.

The accumulation of AGEs is a major contributing factor to complications of diabetes. Thus, prevention and improvement of such complications may be possible by inhibiting AGEs formation. Here we show that the presence of β-CyD or trehalose stabilized HSA and GHSA as determined by enhancement of CD helicity through their interactions with HSA. These interactions weakened the HSA hydration and induced self-association within the chain of HSA. Thus, a much tighter secondary structure of α-helix becomes dominant in the protein.

AGEs formation induced aggregation and nanofibril formation in proteins are the cause of several conformational diseases in organs and circulation including diabetic vasculopathies and Alzheimer's disease [28]. The presence of aggregation and nanofibril in prolonged GHSA is generally confirmed by a number of analysis including ThT interactions and electron microscopy [18]. ThT measurements showed the presence and increased formation of fibril structures in the GHSA. However, the intensity of fluorescence of ThT decreased in the presence of β-CyD or trehalose. Thus, the presence of β-CyD or trehalose diminished secondary structural changes and transition from a helical to a β-sheet structure of GHSA, and thus decreasing the formation of nanofibril assembly during HSA glycation.

Another criteria indicating AGE formation is an increase in the absorbance [3]. However, we observed that the presence of β-CyD or trehalose affected HSA absorbance in the absence of glucose. Thus, the use of absorbance changes to judge the onset of AGE formation may not be suitable. A previous study confirmed that the absorbance of typical UV spectrum for albumin increases in the presence of CyDs [29]. It was also indicated that the solvation shell and the intramolecular or intermolecular association of the chromophoric groups on albumin may be partly or totally destroyed in the presence of CyDs. Thus, the induced enhancement of absorbance upon the interaction of β-CyD with HSA is not due to AGE formation but it is mediated through its association with the chromophoric groups on HSA or altered solute–environment interactions. Trehalose, as co-solvent, may also change absorbance of typical UV spectrum of albumin in similar manner [30].

The Trp residue fluorescence is used as a detector of conformational changes in ternary protein structure and polarity of Trp local environment [30-32]. HSA has a single Trp (Trp 214) near the protein surface in helix 2 of subdomain IIA [33, 34]. The presence of β-CyD induced a slight red shift in HSA fluorescence, thus suggesting that the binding of β-CyD to HSA is accompanied by changes in the dielectric environment of the indole ring of Trp [31]. A red shift always indicates that the Trp residues are, on average, more exposed to the solvent, whereas a blue shift is a consequence of transferring the Trp residues into a more hydrophobic environment. The fluorescence intensity decreased with glycation of HSA, while the presence of β-CyD induced an increase in fluorescence intensity. Thus, the interaction between β-CyD and glucose with HSA contributed to the al-

teration in ternary structure of HSA near the binding site. Furthermore, the increased α-helix content indicates a tightening of secondary structure, and hence the ternary structure of HSA may be disrupted to some extent.

A marginal change in the fluorescence spectrum and emission maxima for HSA incubated with trehalose, as compared to the spectrum of control HSA, indicated the retention of local environment around the Trp residue in the presence of trehalose. In the presence of glucose and trehalose a slight decrease in fluorescence intensity and a red shift in fluorescence were observed. Trehalose reduced the mobility of HSA by either forming hydrogen bonds with HSA or by forming a glassy matrix around the protein molecule [30]. Due to this reduced flexibility, unfolding and exposure of hydrophobic core and, thus, aggregation are prevented. In addition, the specific high viscosity of trehalose solution [30] may be responsible for the reduced mobility of the protein molecules. The trehalose also has a large hydration volume (capacity to absorb water) [30]. 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 [30]. Thus, the protein becomes preferentially hydrated, but the radius of the solvation layer and the apparent volume of protein decreases, in a phenomenon that leads to a more stable protein conformation. Recently, Kaushik and Bhat showed that the interaction of trehalose with various side chains of proteins contribute to its stability effect [35].

The interactions between aromatic amino acids of HSA and β-CyD stabilized HSA; such interactions possibly affect the overall three-dimensional structure of the protein. In addition, the spectrum changes of CD contribute to the backbone conformation of HSA. CyDs are cyclic oligosaccharides consisting of at least six D-glucose units. The oligosaccharides form a truncated cone in which the primary hydroxyl groups are directed to the narrow side and the secondary hydroxyl groups are on the wide side of the torus. Because of the arrangement of the functional groups, the interior of the torus is hydrophobic whereas the outer surface is hydrophilic. The hydrophilic surface allows β-CyD interaction with polar groups such as lysine residues in the outer side of the cone. However, trehalose is a non-reducing disaccharide and can not react with amino groups on lysine residues as β-CyD and/or glucose do.

5. CONCLUSION

The results of this paper confirm that the presence of β-CyD or trehalose inhibit the pathway of AGE formation by interfering with the glycation of HSA. In this way, β-CyD and trehalose are capable of improving stability via interaction with HSA and alter solute-protein interactions. Hydrophobic side chains of the protein interact with the cavity of β-CyD leading to a hydrophilic covering layer which increases protein stability. However, such interactions may affect the overall three-dimensional structure of the protein. The interactions of trehalose with various side chains of HSA and hydrogen bond formation contributed to its stability effects. Trehalose reduced mobility of protein and, therefore, prevented the glycation and unfolding of HSA. Thus, the presence of trehalose and CyDs in foods may inhibit glycation reactions under hyperglycemia providing protection against pathogenic effects of AGEs during diabetes and consumption of processed foods.

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LIST OF ABBREVIATIONS

HSA = Human Serum Albumin

GHSA = Glycated Human Serum Albumin

β-CyD = β-Cyclodextrin

AGEs = Advanced glycation end products

ThT = Thioflavin T

NBT = Nitroblue tetrazolium

TNBSA = 2,4,6-Trinitrobenzene sulfonic acid

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