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Reduction in Aggregation of Insulin Through Conformational Stabilization by Glucose

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Aggregation of insulin molecule remains a serious obstacle in the development of alternative methods of diabetes therapy. To reduce the insulin's aggregation process and increase in structural stability, the effects of glucose were studied by UV, fluorescence, and circular dichroism spectroscopy as well as by urea denaturation study in phosphate buffer, pH 7.5. The results show a decrease in aggregation of insulin and increase in midpoint of urea denaturation curve in case of incubation with glucose effectively at 3 fold concentrations relative to insulin ([glucose]/[insulin] = 3). Therefore the results indicate that incubation of insulin with glucose improve its physical and structural stability.

Key Words: Insulin, Glucose, Aggregation, Stabilization, Conformational change.

INTRODUCTION

Insulin has been in therapeutic use for more than 90 years, therefore it is supposed to be one of the most important and commonly used therapeutic proteins. Insulin molecule consists of 51 amino acid residues arranged in two polypeptide chains (A and B), linked by two disulfide bonds. The A chain forms nearly two α-helices and B chain forms a single α-helix followed by a turn and a β-strand. Insulin exists in monomer state at low concentrations (< 0.6 μg/mL) and dimerizes at higher ones; three dimers assemble into a hexamer that finally leads to aggregation and fibrilation[1-3]. Since the three-dimensional structure of proteins determine their function, aggregation of proteins poses a major problem in various fields of research and application. Aggregation of proteins generally culminates in loss of functional activity[4,5]. Aggregation is a mechanism resulting in physical instability of proteins and remarkably reduces the biological potency causing problems in drug delivery systems[6-8]. The self-association of insulin into dimers, hexamers, high molecular weight aggregations and insoluble

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fibrils has been recognized as a problem in the treatment of diabetes mellitus, especially in formulations intended for implantable insulin pumps[2,9,10]. Insulin aggregation is accompanied by drastic reduction of biological potency and delivery routes following serious problems for drug delivery systems and undermining the patients' abilities to control their blood glucose level[10-12]. It is therefore of utmost importance to devise ways for preventing insulin aggregation. The two insulin molecules in the dimer are held together by nonpolar forces (hydrophobic interactions)[1,7]. It is evident that the stabilization mechanism is consistent with the destabilizing role attributed to hydrophobic surfaces[7]. On the other hand, the removal of carbohydrate from naturally glycosylated proteins can cause a decrease in thermal stability of proteins. This is often accompanied by an increased tendency toward protein aggregation[13,14]. We hypothesized that addition of carbohydrates in the insulin environment should suppress self-aggregation tendency by decreasing the hydrophobic interactions. In this study the analyses of the effects of some sugars such as glucose on physical and structural stability of insulin showed that glucose is more effective and compatible when it is incubated with insulin for a week's time.

EXPERIMENTAL

Materials used were metal free bovine insulin (Novo Nordisk), D-glucose, D-mannitol (Sigma), sucrose, trehalose (Merck) and xylitol (Fulka). Dialysis tube cut off 1200 was purchased from Sigma. Other chemicals were of analytical grade and used without further purification.

Metal-free bovine insulin was purified by equilibrium dialysis in distilled water for removing *m*-cresol from the samples. The pH of each sample was adjusted at 8 using NaOH. Samples were freeze-dried in liquid nitrogen at -180ºC. To study the effects of various sugars, dried pure insulin was dissolved in phosphate buffer 0.2 M at pH 7.2 where its concentration was 1.5 mg/mL determined by UV spectrophotometer UV-Vis 3100 Shimadzu. The extinction coefficient[15] used was $\varepsilon_{280} = 5700\ M^{-1}\ cm^{-1}$.

Protein samples with different concentrations of glucose were incubated at 37ºC and structural changes of insulin were studied at different time intervals of incubation (1, 3 and 7 d incubation).

Aggregation studies: Insulin solution becomes turbid upon insulin aggregation, thus the turbidity changes can be monitored and detected *via* UV-vis spectrophotometer at 360 nm[16]. Aggregation studies were carried out at different incubation time intervals.

Fluorescence spectroscopy: The conformational study of insulin at different concentrations of sugars was studied by fluorescence spectrophotometer Hitachi MPF4. Excitation wavelength was 280 nm and λ_{max} emission was 320 nm.

Circular dichroism (CD) spectroscopy: Circular dichroism measurements were performed employing a Jasco-J715 spectropolarimeter at protein concentration of 1 mg/mL for Far UV-CD and 1.5 mg/mL for near UV-CD at room temperature. Results were reported as a mean residue ellipticity having units of $deg.cm^2.dmol^{-1}$ using a mean residue weight of 115 Da. The molar ellipticity was determined as $[\theta] = (100 \times (MRW) \times \theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentrations in mg/mL and l is the length of the light path in cm[17].

Denaturation study: Urea induced denaturation of protein was also studied through fluorescence spectrophotometry[18]. Here different concentrations of urea were used as a denaturant reagent to study insulin's structural stability in the presence of different concentrations of glucose by spectrofluorimetry.

RESULTS AND DISCUSSION

In the absence of additives, insulin solutions aggregate completely within 24 h. Attempts to stabilize insulin solutions have included the use of additives such as phenol's derivatives, nonionic and ionic surfactants, glycerol and metal ions such as calcium[7].

Aggregation studies showed no change in turbidity in case of different sugars such as mannitol, sucrose, xylitol, trehalose and even glucose (data not shown). But upon incubation of insulin with each of them only glucose was effective in reducing amount of aggregation. Glucose can autonomously react with a wide variety of proteins through Schiff base reactions as a reducing sugar. This non-enzymatic reaction, named glycation, occurs through a condensation reaction between carbohydrates (especially glucose) and free amino groups of NH_2-terminus or the susceptible ε-amino groups of lysine residues of proteins[19-21]. Historically, glycation of lysyl side chain and N-terminal amino groups by glucose was one of the major glycation process studies[22]. Three available amino groups of insulin (GlyA1, Phe B1 and Lys B29) can react with aldehyde groups of sugars through schiff-base mechanism. Glycation of protein leads to the formation of early glycation adducts (fructoseamine derivatives) and advanced glycation end products (AGEs)[22]. Therefore the incubation time increase proceeds the reaction and results in Amadori products (end products)[19,20], which induces the stable form of glycated protein. In other words, formation of Amadori products (stable form of glycated proteins) is a time dependent reaction. Therefore, insulin was incubated with glucose at different concentrations in 37ºC for 1 week (sufficient time of glycation reaction for AGEs production[23]) and resulting turbidity was measured in different time intervals.

Fig. 1 shows the intensity of 360 nm absorption *vs.* time of incubation for different ratios of glucose/insulin solution which is indicative of a decreasing trend of turbidity with increasing glucose concentration. Incubation of proteins with sugars, like insulin with glucose, can lead to modification of any available free amino groups. Here incubation of insulin with glucose can modify each of its three available free amino groups. It seems that with increasing the incubation time, the glycation reaction ends up and leads to irreversible reaction due to AGEs formation. Hence, increasing the time can cause immobilization of each modified group which results in glycation of some of the three free amino groups. In other words upon increasing of time, due to AGEs formation, reversion does not occur in all modified groups. Addition of polar groups on surface of insulin can increase its surface polarity and prevent aggregation. As a result, prevention of self association of insulin by covalently linked sulfated groups (as a polar group) has been reported[24]. Thus the modification of free amino groups by glucose upon incubation time can increase surface polarity of insulin and sugar residues (glycated residues) of insulin as interfaces, interact with solvent (water) and the polypeptide chain. This effect of glucose, as a reducing sugar, may cause the prevention of self-assembling of insulin molecules as well as formation of dimer and hexamer through non-polar interactions. This was confirmed by increasing glucose concentration (Fig. 1). The results show that increasing of glucose/insulin ratio from 1 to 3, decreases the amount of turbidity as a result of three free amino groups reaction with glucose which lead to suppression of surface hydrophobic interaction by increasing surface polarity. Hence, in this case the optimum concentration of glucose for decreasing aggregation of insulin is the ratio of 3:1 ([glucose]/[insulin] = 3) that can cause modifying all three amino groups. In this condition the surface polarity must be maximum and to cause minimum aggregation.

Conformational changes of insulin

It is important to know about insulin's structure if glucose prevents its self-association. In this case, addition of glucose at different concentration ratios (mentioned above) to insulin was studied by fluorescence technique. Results show that glucose acts as an anchor and causes an increase in insulin molecule rigidity (Fig. 2). The rigidity of protein tends to increase fluorescence intensity[25,26]. In other words, the compactness of molecule causes a decrease in the mobility and increase in intensity of aromatic residue responsible for fluorescence. Fig. 2 shows that fluorescence intensity has a direct relationship with glucose concentration ratio (apparently; fluorescence intensity increases in a range of concentration ratio of 1 to 3 of glucose). This means glucose induces an increase in insulin's rigidity and structural compactness. This phenomenon can cause reduction of surface

accessible area (SAA) of insulin molecules and leads to prevention of aggregation (Figs. 1 and 2). To confirm this we used far and near UV-CD techniques. The rigidity of the protein strongly affects the intensities of UV-CD bands (decreased rigidity of the protein, with more highly mobile side chains results in lower intensities)[17]. The far-UV CD was used to examine secondary structure of protein and near UV-CD experiments were used in case of tertiary structure measurements[17,27]. These experiments showed that insulin had more secondary and tertiary structures at higher glucose/insulin ratio (Figs. 3A and 3B). To conclude, glucose induces insulin's compactness and leads to suppression of aggregation phenomena.

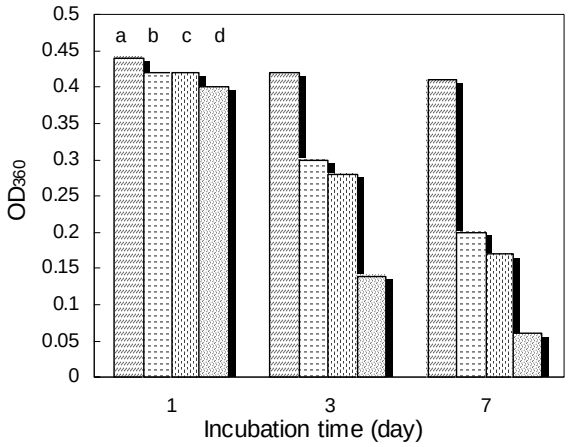

Fig. 1. Absorbance at λ_{max} = 360 nm as a good creation of aggregation measurment *vs.* time of incubation (d) with glucose at different glucose/insulin ratios; (a) 0:1, (b) 1:1, (c) 2:1, (d) 3:1

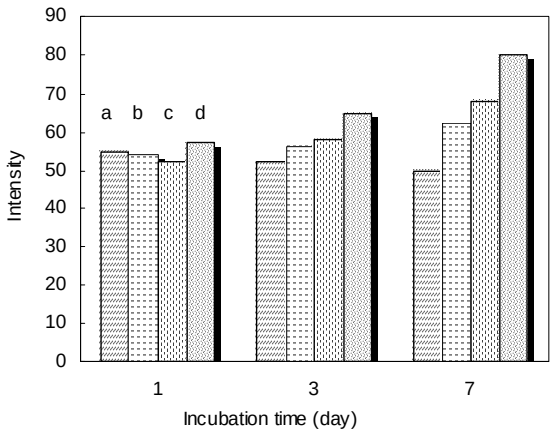

Fig. 2. Fluorescence intensity at λ_{max} = 320 nm *vs.* time incubation (days) with glucose at different glucose/insulin ratios; (a) 0:1, (b) 1:1, (c) 2:1, (d) 3:1

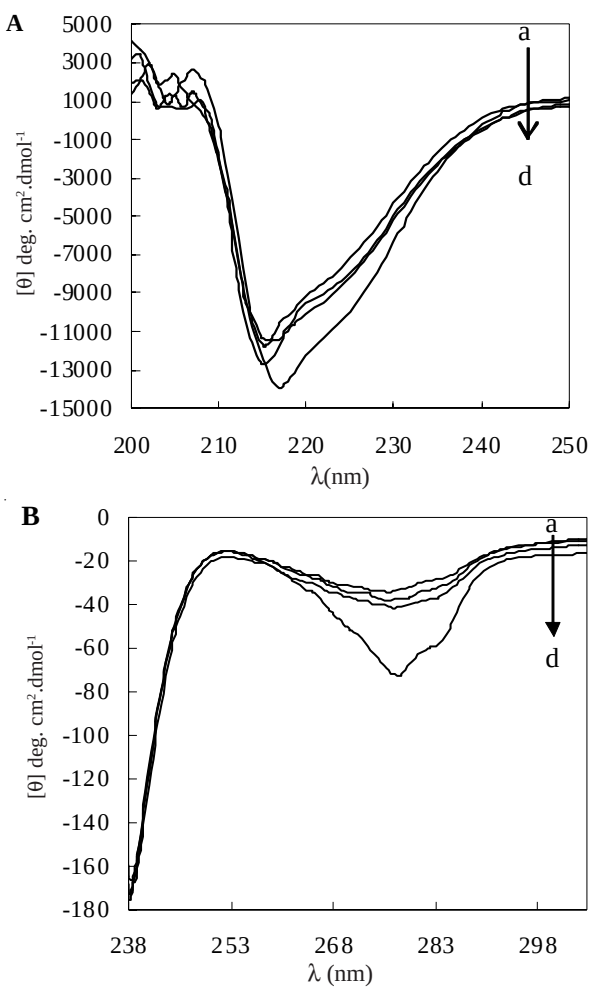

Fig. 3. (A) Far UV-CD and (B) Near UV-CD spectra of insulin molecule at different glucose/insulin ratios after 7 d incubation from a to d in order of 0:1, 1:1, 2:1and 3:1

The structural stability of insulin in the presence of glucose has also been investigated. Thus, its structural change has been studied in the presence of different concentrations of urea as a denaturing reagent *via* fluorescence technique. Fig. 4 shows the effect of urea as a strong denaturant on conformational stability of insulin in the presence of glucose. Fig. 4 shows that with increasing in glucose concentration, the midpoints of denaturation curves (minimum points) shift to right (Table-1). It is suggested that higher stability of insulin at high concentrations of glucose is related to increasing of compactness upon interaction with glucose (especially at higher concentration ratio). The total results support the relationship between the higher structural stability of insulin and the decrease in aggregation in the presence of glucose.

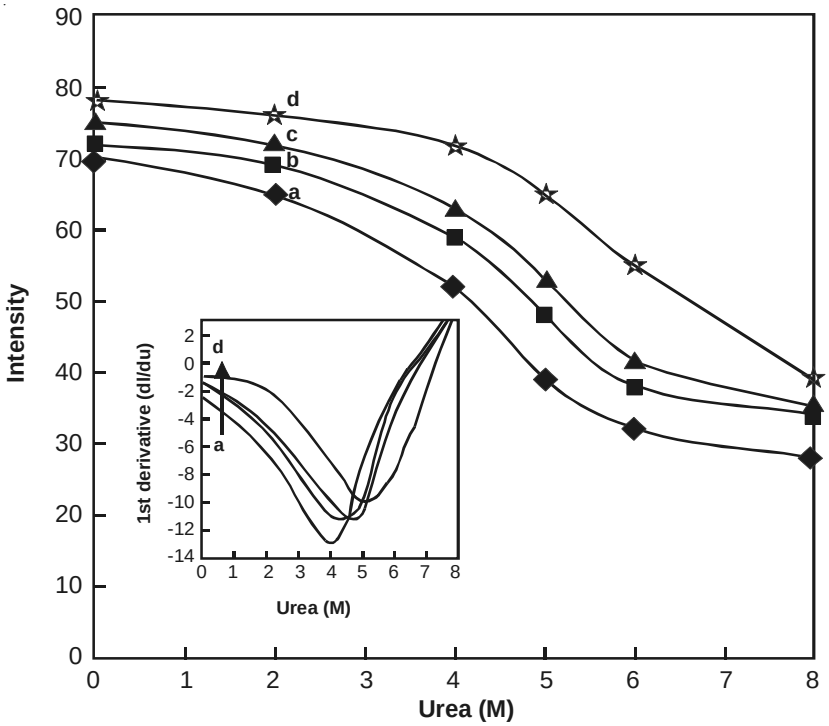

Fig. 4. Fluorescence intensity at λ_{max} = 320 nm *vs.* urea concentration (M) for 4 different glucose/insulin sample ratio after 7 d of incubation; (a) 0:1, (b) 1:1, (c) 2:1, (d) 3:1. Inset shows the first derivative of fluorescence intensity (dI/dU) *vs.* urea concentration

TABLE-1
MIDPOINT CONCENTRATION OF UREA ($U_{1/2}$) IN DIFFERENT GLUCOSE/INCLUSION RATIOS

Glucose/insulin ratio	Midpoint concentration of urea ($U_{1/2}$)
0.0	4.00 (M)
1.0	4.70 (M)
2.0	4.90 (M)
3.0	5.25 (M)

Finally, glucose as a reducing sugar may possibly prevent self-assembling of insulin molecules and formation of dimmer, hexamer through non-polar interactions. Moreover glucose can be considered as a better stabilizer of insulin, as compared with the other known bioprotectants such as trehalose.

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