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Conformational and bioactivity analysis of insulin: Freeze-drying TBA/water co-solvent system in the presence of surfactant and sugar

Zhang Yong[a,*], Deng Yingjie[a], Wang Xueli[b], Xu Jinghua[a], Li Zhengqiang[b,**]

[a] School of Pharmacy, Shenyang Pharmaceutical University, Wenhua Road No. 103, Shenhe District, Shenyang 110016, China
[b] Key Laboratory for Molecular Enzymology and Engineering, The Ministry of Education, Jilin University, Changchun, China

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ABSTRACT

Despite the extensive research into the freeze-drying of aqueous solutions of proteins, it remains unknown whether proteins can survive the lyophilization process in a water-organic co-solvent system and how the process and additives affect the structural stability and activity of the proteins. In the present study, a conformational analysis of insulin in the absence/presence of bile salt and trehalose was carried out, before and after freeze-drying of a tert-butyl alcohol (TBA)/water co-solvent system at volume ratios of TBA to water ranging from 50/50 to 0/100. The study involved the use of ultraviolet derivative and fluorescence spectroscopy, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy. Also the bioactivity of insulin was evaluated in vivo using the streptozotocin (STZ)-induced diabetic mice as an animal model. Initial investigations indicate that the extent of the structural change of insulin depends significantly both on the TBA content and on the concentration of additives, such as sodium deoxycholate, prior to lyophilization. This could be accounted for by the phase behavior properties of the TBA/water co-solvent system, surface denaturation together with the selective and/or forced dispersion of insulin during phase separation. Lyophilized insulin in the presence of bile salt and trehalose retained more of its bioactivity and native-like structure in the solid state compared with that in the absence of additives at various TBA/water ratios, although in all cases there was a major and reversible rearrangement of secondary structure after rehydration, except for insulin at 50% TBA (v/v). Furthermore, both lyophilization in non-eutectic systems and less structural changes in the formulation process lead to more bioactivity.

1. Introduction

Freeze-drying, or lyophilization, is the most commonly used method for dehydration in the pharmaceutical and food industries, and it has become even more important with the arrival of more protein products on the market. In addition, freeze-drying also offers the chance to develop protein delivery carriers accompanied by the self-assembly of protein and carriers (Morita et al., 2000; Oh et al., 2006). However, the structural stability and the retention of biological activity have been major concerns associated with the freeze-drying of protein formulations; there are also crucial properties of proteins which distinguish them from foods and small molecular weight drugs. In order to preserve the native structure and, therefore, the greatest long-term stability and biological activity, various methods have been proposed to mitigate or prevent protein denaturation induced by freezing and drying, and a number have been extensively and intensively studied (Allison et al., 1998, 1999; Luthra et al., 2007). Among these, one widely employed strategy is the use of stabilizing additives during the lyophilization process. These stabilizers include sugars, polyols, polymers, amino acids and surfactants, the possible stabilization mechanisms of which have been deeply discussed in detail in the above-mentioned studies and other publications (Carpenter and Crowe, 1988; Wang, 2000; Chang et al., 2005). For many proteins, a combination of these cryo- and lyo-protectants will confer suitable chemical and physical stability on the freeze-dried solid.

Although the vast majority of currently marketed pharmaceutical and biological products are freeze-dried from simple aqueous solutions, many mineral and organic solvents have been shown to possess similar properties to frozen water and can sublime under reduced pressures, and so could be used in freeze-drying (Rey, 1999). The potential advantages and disadvantages of the use of these organic or organic/water co-solvent systems in lyophilization have been fully summarized by Teagarden and Baker (2002). In the promising field of freeze-drying using non-aqueous co-solvent systems, tert-butyl alcohol (TBA)/water systems have been thoroughly evaluated, and used in the manufacture of a marketed pharmaceutical product to be administered by injection (Teagarden

* Corresponding author. Tel.: +86 24 23917603; fax: +86 24 23917603.
** Corresponding author. Tel.: +86 431 88499261; fax: +86 431 88980440.
E-mail addresses: zhangyongking1@gmail.com (Z. Yong), lzq@jlu.edu.cn (L. Zhengqiang).

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et al., 1998). The reason why TBA/water co-solvent systems have attracted so much attention from experts and pharmacists is that TBA exhibits many properties which can benefit both process optimization and product quality, such as a high vapor pressure, low toxicity, and marked solubilizing capability. In addition, TBA can freeze completely in most commercial freeze-dryers, readily accelerating mass transference during primary and secondary drying and, thereby, increase the sublimation rate and decrease the drying time (Kasraian and DeLuca, 1995a,b). However, until recently almost all studies published using TBA/water co-solvent systems in freeze-drying were performed on small molecular weight drugs which need not maintain a unique three-dimensional conformation for their biological activity and pharmaceutical functionality (Wittaya-Areekul et al., 2002; Van Drooge et al., 2004; Telang and Suryanarayanan, 2005). This is the first study of this co-solvent system in protein freeze-drying carried out to gain valuable information about the fundamental interrelationships between the protein and stabilizers, such as bile salt and trehalose, during lyophilization of the organic/water system. And this study is also a further sight into our previous work (Li and Deng, 2004).

It is well known that proteins adopt particular secondary and tertiary structures, which are extremely closely related to their biological activity. The non-native structures or partly unfolded states often result in protein aggregation, precipitation or accelerated chemical degradation and, thus, result in a short shelf life or harmful immunity reactions (Wang, 1999, 2000). In the past two decades, these have been the basis of studies involving formulation optimization, lyophilization and stability during long-term storage (Heller et al., 1999; Passot et al., 2005; Liu, 2006; Luthra et al., 2007). Thus, in this study, based on published information, protein was lyophilized in a TBA/water co-solvent system to obtain more valuable information. We took insulin as a model protein because its structural stability has been studied in detail (Wei et al., 1991; Vecchio et al., 1996). Trehalose, which is an excellent lyo-protectant with a clear stabilizing mechanism of hydrogen bond substitution during drying, was used as a stabilizer (Allison et al., 1999; Souillac et al., 2002; Liao et al., 2004; Chang et al., 2005). Another additive that we selected to study is bile salt, sodium deoxycholate which has been typically used as a safe penetration enhancer and powerful solubilization agent in pharmaceutics. However, there are few reports about its use as protective agent for freeze-drying, especially for lyophilization of non-aqueous co-solvent systems. Finally, the bioactivity of lyophilized insulin was evaluated in vivo using streptozotocin-induced diabetic mice as an animal model, in parallel with the investigation of conformational changes which was carried out using a combination of circular dichroism (CD) and Fourier transform infrared (FTIR) together with ultraviolet derivative and fluorescence spectroscopy.

2. Materials and methods

2.1. Materials

Bile salt, sodium deoxycholate was generously provided by ICE Industria Chimica Emiliana (Reggio Emilia, Italy). Tert-butanol was provided by Tedia Company Inc. (Fairfield, OH, USA). Dihydrate trehalose was obtained from Sinozyme Biotechnology Co. Ltd. (Nanning, China). Porcine zinc insulin (27.6 IU mg^{-1}) was purchased from Wanbang Biochemical Company (Xuzhou, China), and streptozotocin from Sigma Chemicals (St. Luois, MO, USA). These compounds and protein were used as received without further purification. Deionized double-distilled water was used throughout the study. All other chemicals were of analytical reagent grade or purer.

2.2. Preparation of co-solvent systems

The aqueous solution of insulin (25 mg/ml) was prepared by dissolving protein powder in double-distilled water into which concentrated NaOH was added dropwise with gently stirring until the pH reached 10.0, as monitored by a pH-meter. The resulting solution was passed through Millipore filter paper with a pore size of 220 nm to remove any insoluble substances. The sample solutions including bile salt and trehalose at various concentrations were prepared in the same way as insulin. Then 0.3 ml aliquots of these sample solutions were placed into 10 ml freeze-drying vials containing 0.5 ml TBA/water (pH 10.0) with various concentrations of TBA, and the mixture was gently shaken until the temperature reached room temperature. Subsequently, 0.2 ml aliquots of the aqueous solution of insulin were added and mixed homogenously with gentle shaking in an ice-water bath. This method eliminated most of the exothermic reaction during mixing that might result in structural changes in insulin. Thus, the resultant solutions to be lyophilized are uniform and optically clear with a pH of 10.0.

As a result, the co-solvent systems including insulin and additives were prepared with apparent volume at ratios of TBA to water of 50/50, 25/75, 10/90, and 0/100. Each vial with a fill volume of about 1 ml contained 5 mg insulin and a different weight of additives. The mole ratio of additive to protein was 100/1, 50/1 and 0/1 for bile salt, and 25/1 and 0/1 for trehalose, respectively. Control formulations contained the same components except for insulin.

2.3. Freeze-drying

The freeze-drying process was performed in a FDU-1100 lyophilizer (EYELA, Japan). The freeze-drying process was as follows: freezing at −80 °C for 24 h; primary drying at −35 °C for 20 h; secondary drying at 25 °C for 24 h. The chamber pressure was maintained at about 20 Pa during the drying process, and the condenser temperature was controlled at −60 °C throughout the whole freeze-drying cycle. After lyophilization, the vials were immediately filled with dry nitrogen gas, sealed with rubber stoppers, and then were kept in a desiccator over P_2O_5 at 4 °C until testing.

For each condition examined, three vials of sample were lyophilized during the same freeze-drying cycle. One vial was used for FTIR spectroscopy, and the second was rehydrated for CD and ultraviolet and fluorescence spectroscopy, and the third was for biological activity studies. In this manner any differences in the freeze-drying process that may lead to structural changes and loss of activity were avoided. The samples for all spectral and activity data reported here were prepared and freeze-dried under identical conditions.

2.4. Ultraviolet derivative and fluorescence spectroscopy

Ultraviolet spectra were taken using a UV-2550 spectrophotometer (Shimadzu, Japan). A slit width of 1 nm, a “slow” scan rate, a 0.1-nm sampling interval, and a scan range from 250 to 320 nm were selected. The first-derivative absorption spectra were obtained with an eleven-point Savitsky-Golay derivative function based on the average data of three measurements. The fluorescence measurements were performed with an RF-5301 fluorospectrophotometer (Shimadzu, Japan) using 278 nm excitation and monitoring the emission over the range 298–450 nm. The slits of excitation and emission were 5 and 3 nm, respectively. The protein concentration was 0.25 mg/ml in the ultraviolet experiment, and 0.1 mg/ml in the fluorescence experiment. All background effects were subtracted.

Unless otherwise stated, the spectra were obtained at 25 °C using a built-in temperature-controlled sample holder which in a thermostated water bath. The samples were prepared and run at least

in triplicate, without any significant differences. Finally, the spectra were presented as a result of the average of triplicate measurements.

2.5. CD spectroscopy

The circular dichroism spectra were measured using a Jasco J-810 CD spectropolarimeter (Jasco, Japan) with a 0.5 mm path cuvette for far-UV CD and a 1.0 cm path cuvette for near-UV CD at a constant temperature of 25 °C. The ranges of far/near-UV region CD spectra were 250–190 nm and 320–250 nm, respectively. All spectra were obtained using a step size of 1 nm, a bandwidth of 2 nm, and a response time of 4 s, and an accumulation of 4 scans with the lamp housing purged with pure nitrogen to remove oxygen. The protein concentration for CD was 0.25 mg/ml. The spectra were background corrected and converted to the mean residue ellipticity (deg. cm^2 $dmol^{-1}$) using a molecular mass of 5800 Da and a total number of 51 amino acids. For estimation of the secondary structural composition of the protein, the CD spectra were evaluated using the SOMCD method (Unneberg et al., 2001).

2.6. FTIR spectroscopy

FTIR studies were conducted on a Nicolet 5700 FT-IR spectrometer (Nicolet, USA) equipped with an MCT detector. The spectra of lyophilized protein powders were measured as KBr pellets using a total of 256 scans at 2 cm^{-1} resolution (Griebenow and Klibanov, 1995). Protein solutions were analyzed by a ZnSe horizontal attenuated total reflectance unit with 1024 scans at 2 cm^{-1} resolution (Dong et al., 1990; Oberg and Fink, 1998). All second-derivative spectra were obtained by the corresponding subtraction procedure followed by a seven-point Savitsky-Golay derivative function and a 3–4 point baseline adjustment. In addition, the spectra were area-normalized in the amide I region from 1710 to 1590 cm^{-1} using Igor Pro software for comparison and analysis. All samples were prepared and analyzed at least in duplicate.

2.7. Bioactivity analysis

8-Week-old male Kunming mice, provided by Shenyang Pharmaceutical University Experimental Animal Centre, were housed in an isolated caging system in an air-conditioned animal room at 23 ± 1 °C. The body weight of each mouse was 22–25 g. All experimental procedures followed the guidelines for laboratory animal care and were carried out according to a protocol approved by the local animal ethics committee.

Mice were fasted for 16 h before diabetes was induced with STZ. They received a single i.p. injection of 160 mg/kg STZ (Sigma, St. Louis, MO, USA) freshly dissolved in 0.1 M citrate buffer, pH 4.5. Normal mice were injected with the equivalent volume of citrate buffer. Blood samples for glucose measurements were taken from the tail vein 7 days after STZ injection and standard breeding. The mice with a blood glucose of 11.1–25.0 mM were considered diabetic.

The number of experimental animals per group was at least 6 and they were used only once. Lyophilized samples were rehydrated in deionized double-distilled water to give a dose of 5 ml/kg body weight subcutaneously. Mice received 2.5 IU/kg insulin, and control mice received an equivalent volume of insulin-free solution. The plasma glucose level at zero time was taken as the 100% glucose level. The plasma glucose level was determined using the commercial reagent Glucose GT-1810 (Kyoto, Japan). Data are represented as mean ± S.E. Statistical analysis was carried out by one-way ANOVA. In all cases, $P < 0.05$ was considered to be statistically significant.

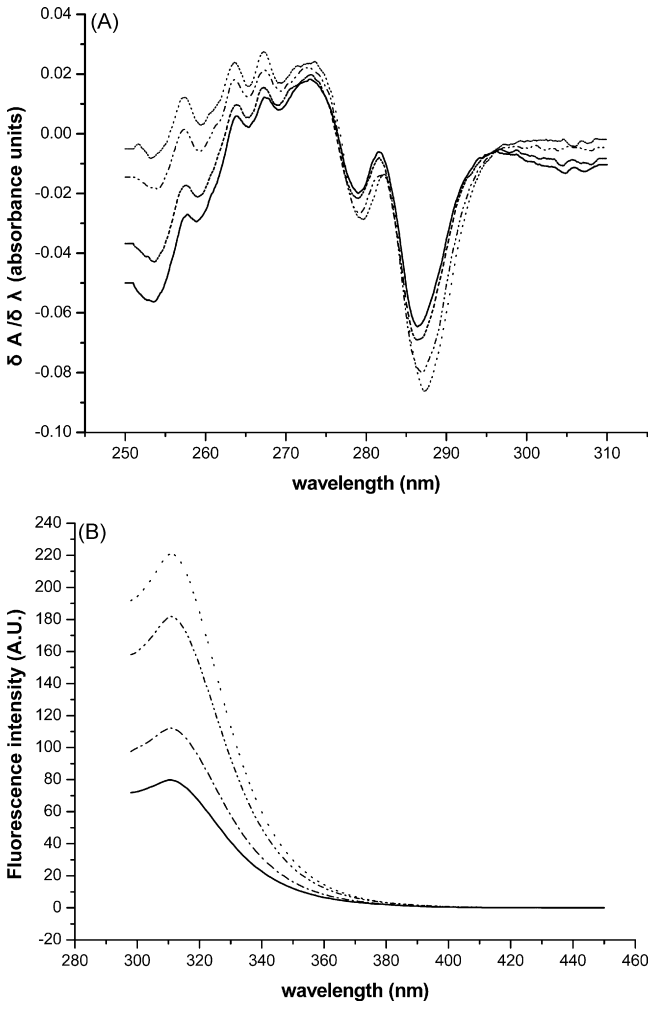

Fig. 1. (A) The corrected ultraviolet derivative spectra of insulin in the TBA/water co-solvent system, 0.75 mg/ml; (B) the corrected fluorescence spectra of insulin in the TBA/water co-solvent system, 0.1 mg/ml, pH 10.0 with a TBA apparent volume percent of 0% (solid curve), 10% (dash dot curve), 25% (dash dot dot curve) and 50% (dot curve).

3. Results

3.1. UV and fluorescence spectroscopy of insulin in the TBA/water co-solvent system

UV derivative and fluorescence spectroscopy of proteins are the most commonly used techniques for studying conformational transitions and for investigating the local microenvironment around aromatic chromophores, such as tyrosine and tryptophan residues (Brandts and Kaplan, 1973; Rock, 1983). Insulin is a 51-amino-acid polypeptide which possesses a unique tertiary and secondary structure. In the region from 260 to 300 nm, its major ultraviolet absorbance comes from four tyrosine residues (A14, A19, B16 and B26) because it lacks tryptophan residues and the contributions from phenylalanine residues and three disulfide bonds are negligible compared with that of the tyrosine residues, whereas insulin fluorescence completely depends on its four tyrosine residues. Thus, the spectral comparison could shed light on the structural changes and the effects of bile salt and trehalose occurring in the TBA/water co-solvent system.

The corrected derivative spectrum and fluorescence spectrum for insulin are shown in Fig. 1A and B, respectively. The UV derivative spectrum shows that the increase in TBA content results in a marked enhancement of the amplitude and a light shift of the

derivative bands to longer wavelengths. The bathochromicity and hyperchromicity indicate that the whole microenvironment around the four tyrosine residues in insulin are become more and more hydrophobic with the increasing TBA content, which agrees closely with the reduction in the solvent polarity (Brandts and Kaplan, 1973).

Unexpectedly, the fluorescence spectrum shows that the maximum emission wavelength of insulin is always 311 nm with a slight change of less than 1 nm, although it displays an increasing fluorescent intensity with the increase in the percentage of TBA. Generally, a more hydrophobic environment around chromophores corresponds to a shorter maximum emission. Thus, this suggests a weak change in the tyrosine residues microenvironment which is not enough to produce a marked spectral shift, but an increase in the fluorescence quantum ratio. This reasonable explanation has been proved by performing an N-acetyl-L-tyr-ethyl ester experiment under the same conditions (data not shown).

In addition, in all cases, sodium deoxycholate and trehalose did not have any significant effect on the spectra of insulin (data not shown), which showed that the interaction between insulin and TBA was much more important than that between insulin and sodium deoxycholate/trehalose.

3.2. CD spectroscopy of insulin in the TBA/water co-solvent system

In order to further investigate the effects of co-solvent system and additives on the insulin integral structure of insulin, a CD experiment was carried out. All far-UV CD spectra of insulin in the co-solvent system showed two minima at 208 and 222 nm, which are typical of the predominant α-helix structure proteins (see Fig. 2A). This is in close agreement with the spectra obtained by others (Pocker and Biswas, 1980; Kim and Shields, 1992). Also the secondary structure of insulin in solution without additives and TBA, estimated by the SOMCD method, agreed well with that obtained by others (Melberg and Johnson, 1990). However, on increasing TBA in the co-solvent, the absolute intensities at 208 and 222 nm displayed successive enhancement. The result of the secondary structure estimation indicates a marked increase in the α content up to 79.5% with a simultaneously distinct reduction in the β-sheet and random coil content, which most likely results from some of the micelle-like properties of TBA or its aggregates (Zana and Eljebari, 1993). The effect of helix induction is also very similar to those of other alcohols (Jayaraman et al., 1996; Arunkumar et al., 1997), and may be exerted by: (a) reduction of hydrophobic interactions; (b) strengthening intra-protein hydrogen bonding; (c) weaker shielding from electrostatic interactions; (d) directly preferential binding to the surface of insulin with simultaneous dehydration (Thomas and Dill, 1993; Hirota et al., 1998; Fioroni et al., 2002). In addition, the near-UV CD spectra of insulin show a synchronous and notable loss in tertiary structure, although we cannot neglect the contribution of intramolecular disulfide bonds to the near CD spectra (see Fig. 2B).

Sodium deoxycholate displays slight cooperative and concentration-dependant effects of TBA on the secondary structure of insulin (see Fig. 3) when the TBA percent is less than 25%. However, no apparent difference was observed in insulin spectra at a TBA content of increasing more than 25%, which indicates that TBA has a marked effect on insulin structure compared with that of sodium deoxycholate. Compared with sodium deoxycholate, trehalose has less effect on the secondary structure of insulin under our experimental conditions and this might be due to its low concentration and the result of non-specific and weak preferential exclusions (Kaushik and Bhat, 2003). These conclusions agreed with those obtained from the UV derivative and fluorescence spectra.

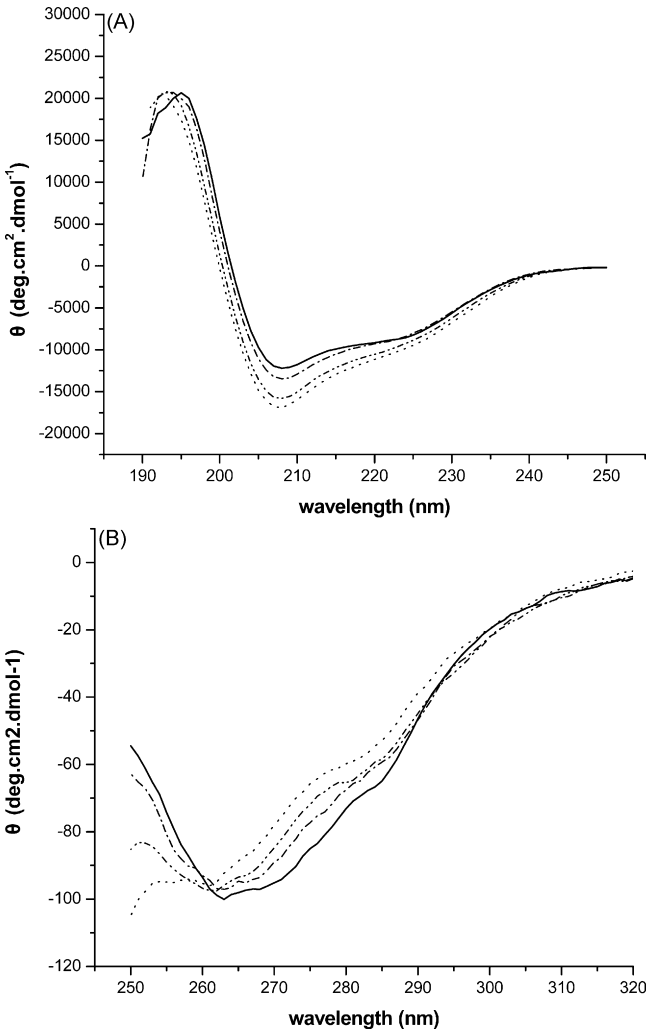

Fig. 2. (A) Far-UV CD and (B) near-UV CD spectra of insulin in the pH 10.0 TBA/water co-solvent system with a TBA apparent volume percent of 0% (solid curve), 10% (dash dot curve), 25% (dash dot dot curve) and 50% (dot curve).

3.3. FTIR spectroscopy of insulin before and after lyophilization

The second-derivative FTIR spectra of insulin, in aqueous and lyophilized states, without additive added, are shown in Fig. 4A. The aqueous spectrum is closely similar to the reported one (Sarmento et al., 2007). The strong bands near 1654 and 1639 cm^{-1} arise from α-helical and β-sheet structures, respectively (Byler and Susi, 1986; Dong et al., 1990). The weaker band around 1683 cm^{-1} comes from the contribution of β-turn (Dong et al., 1990; Chang et al., 1996). The broad bands in the spectrum of aqueous protein solution might be due to a relatively mobile conformation, where the A and B chains are packed into a volume larger than the minimum. However, the spectra of all four formulations lyophilized with no additive show broader bands than those of the aqueous spectrum. Besides, in contrast with the spectrum of aqueous insulin solution, all of them show an observable increase in intensity at 1618 cm^{-1} accompanied simultaneously with the appearance of a new peak at wave numbers above 1690 cm^{-1} which hint at the formation of an intermolecular β-sheet in the dried solid (Chang et al., 1996; Souillac et al., 2002). These notable changes indicate that the process of freeze-drying in the TBA/water system induces marked structural changes. Interestingly, the spectra of formulations 1 and 3 display similar band shape and intensity at 1656 cm^{-1}, whereas formulations 2 and 4 at 1658 cm^{-1} and near 1645 cm^{-1}, which could not be the result

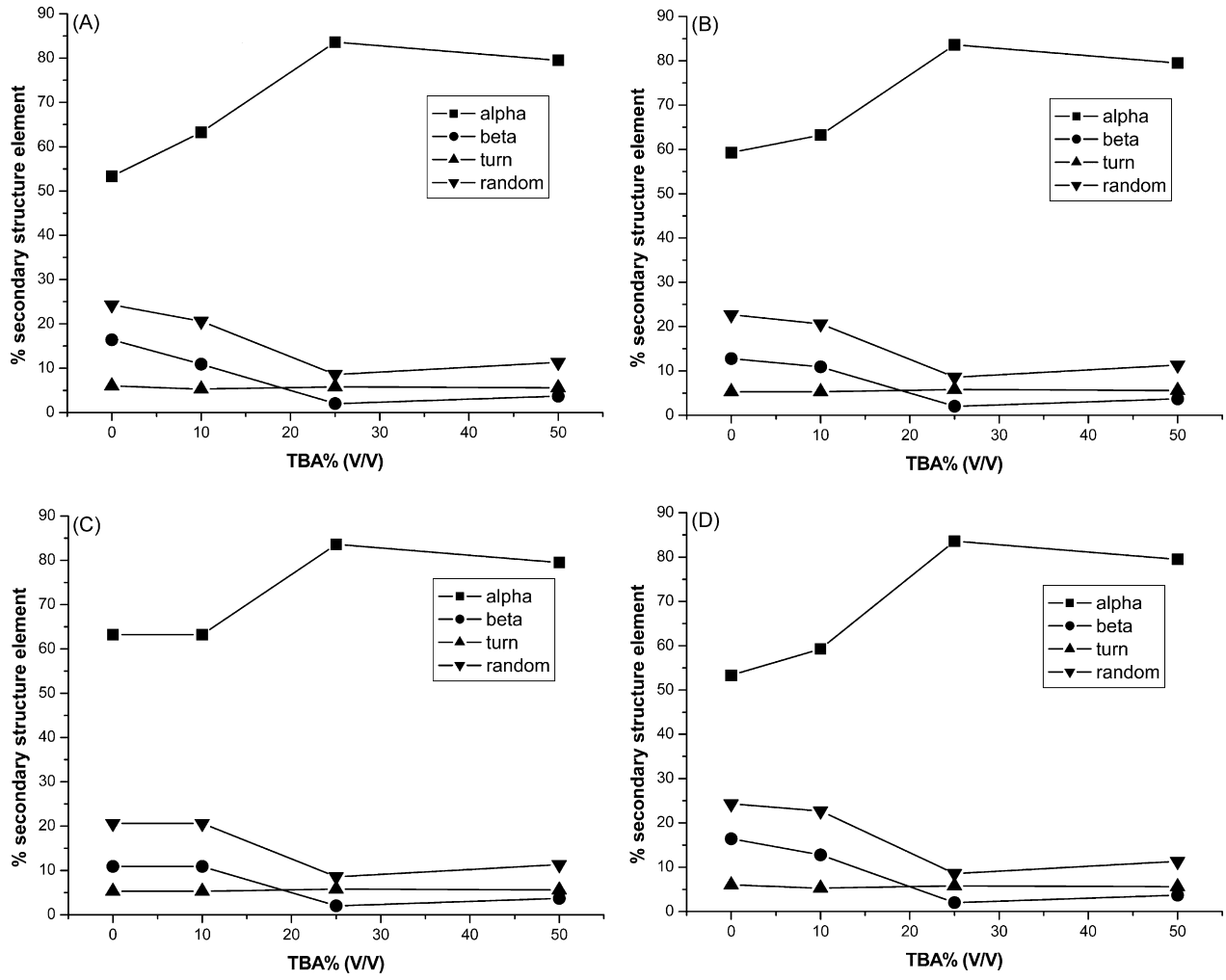

Fig. 3. Secondary structure composition of insulin (0.75 mg/ml) with increasing TBA concentration, pH 10.0. (A) Insulin; (B) with an NaDC:insulin mole ratio of 50:1; (C) with an NaDC:insulin mole ratio of 100:1; (D) with trehalose:insulin mole ratio of 25:1. The data were determined from SOMCD deconvolution of the far-UV CD spectra of the protein.

of differences in measurement due to the good reproducibility in the multiple experiments. The strong bands at 1656 and 1658 cm^{-1} represent the α-helical fraction, and the bands at 1645 cm^{-1} might arise from the non-ordered structures (Byler and Susi, 1986). These phenomena could be explained by the different properties of the phase behavior in the phase diagram of the TBA–water system, and surface denaturation together with the selective and/or forced dispersion of insulin during phase separation.

Fig. 4B shows the spectra of insulin, in the aqueous and lyophilized states, in the presence of trehalose with a mole ratio of trehalose to insulin: 25/1. When compared with those of Fig. 4A, the spectra are clearly less altered and very closely resemble the corresponding spectrum of aqueous insulin, except for formulation 3. However, they all display somewhat broader bands than the aqueous insulin spectrum. In particular, the new band near 1691 cm^{-1} is still present in these spectra. Thus, the addition of trehalose at a low concentration appears to attenuate the unfolding of insulin observed on lyophilization, but it does not maintain an entirely native structure in the dried states following freeze-drying in the TBA/water co-solvent system. The phenomenon observed for the formulation 3 and the similarities observed in Fig. 4A and B will be discussed in detail in the next part.

The influence of sodium deoxycholate on insulin structure was also determined and the results are shown in Fig. 5. In contrast to the spectra of insulin lyophilized with no additive or trehalose, these spectra resemble the spectrum of aqueous insulin in Fig. 4A, whatever the original compositions of the TBA/water co-solvent system are used. In addition, they all display narrower bands compared with those of the aqueous insulin spectrum. However, notable spectral differences near 1691 cm^{-1} were found between the groups with a mole ratio of 50/1 and 100/1 sodium deoxycholate to insulin. For the group with a mole ratio of 50/1, the band above 1690 cm^{-1} was observed in all spectra, although its intensity is markedly weaker than those of Fig. 4A and B. In the spectra of insulin lyophilized with a mole ratio of 100/1, the band which suggests the formation of an intermolecular β-sheet completely disappeared, accompanied by an increased intensity near 1641 cm^{-1} which suggests an increasing content of intramolecular β-sheet. Thus, the ability of sodium deoxycholate to maintain the native structure of insulin in dried state by freeze-drying in the TBA/water system is concentration-dependant.

3.4. Spectroscopic investigations of rehydrated lyophilized insulin

Despite the fact that the insulin structure in lyophilized solid is strongly affected by the initial composition of the co-solvent systems and the addition of bile salt and sugar, whether or not these structural rearrangements are reversible after rehydration is still unknown. A great deal of research has concentrated on structural change reversibility of lyophilized proteins after rehydration

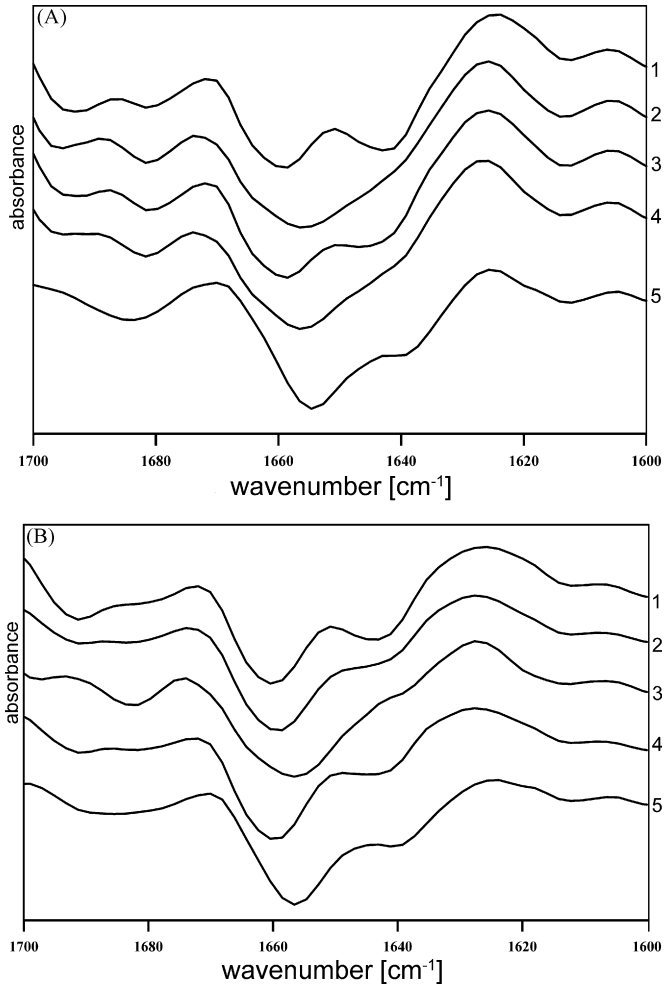

Fig. 4. FTIR second-derivative spectra of insulin in the aqueous and lyophilized states. (A) Without additive; (B) with a trehalose:insulin mole ratio of 25:1. (1) Lyophilized in water; (2) lyophilized in 10% (v/v) TBA; (3) lyophilized in 25% (v/v) TBA; (4) lyophilized in 50% (v/v) TBA; (5) insulin solution.

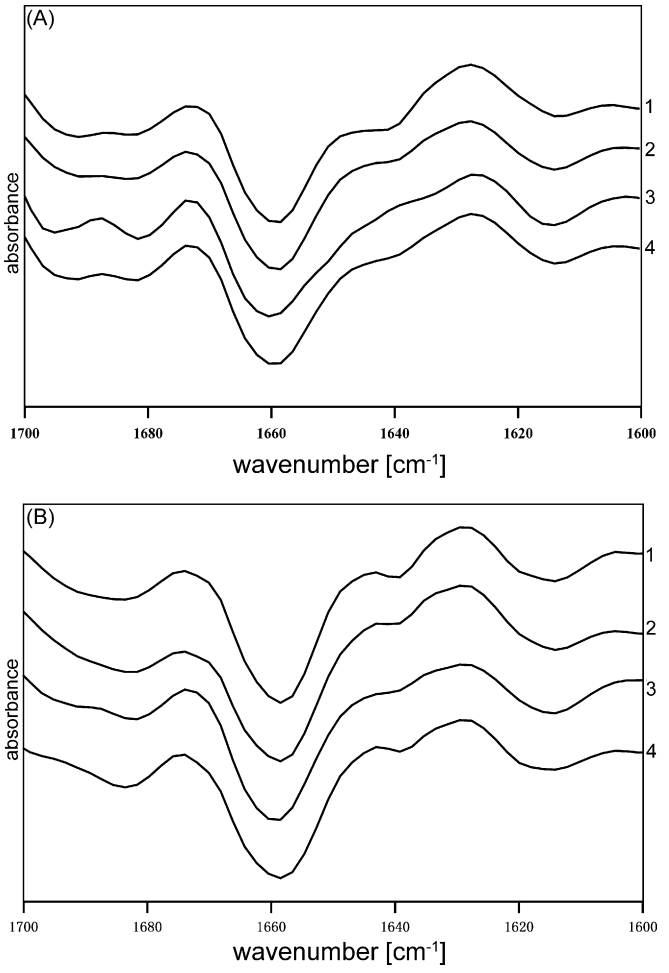

Fig. 5. FTIR second-derivative spectra of insulin in the aqueous and lyophilized states with an NaDC:insulin mole ratio of (A) 50:1 and (B) 100:1. (1) Lyophilized in water; (2) lyophilized in 10% (v/v) TBA; (3) lyophilized in 25% (v/v) TBA; (4) lyophilized in 50% (v/v) TBA.

(Prestrelski et al., 1993; Costantino et al., 1995; Souillac et al., 2002). In the present study, we investigated the insulin structure of all formulations, lyophilized in varying co-solvent compositions and in the absence or presence of additives, after rehydration in deionized double-distilled water using various spectroscopic methods.

After rehydration, all formulations immediately dissolve and exhibit a transparent appearance without any precipitate. The CD spectra are shown in Fig. 6. In group 1 (see Fig. 6A), three insulin formulations exhibit the same reversible change in secondary structure, whereas formulation 4 exhibits a markedly disordered structure after rehydration which may be due to the disruption produced by TBA in the original solution, and interface denaturation together with disrupted hydrogen bonds due to the removal of water when drying. However, after reconstruction, groups 3 and group 4 (see Fig. 6C and D) exhibit the same secondary structure as the original in aqueous solution, regardless of the sodium deoxycholate concentration and the original composition of the co-solvent, indicating the protection offered by sodium deoxycholate to the insulin structure during freeze-drying, especially at high concentrations of TBA. In the case of group 2 (see Fig. 6B) containing trehalose, only formulation 3 is slightly different from others after rehydration, and the original structure could not be recovered completely. The reason for this is still unclear and further research is needed. Compared with the results of the CD spectra, the UV derivative spectra and fluorescence spectra did not display any notable differences which is probably due to the properties of the four tyrosine chromophores of insulin in bulk solvent (Menendez and Herskovits, 1969; Menendez et al., 1969). This also indicates that CD spectroscopy is more powerful and sensitive than UV derivative and fluorescence spectroscopy as far as the investigation of insulin structure is concerned.

3.5. Pharmacological investigations of rehydrated lyophilized insulin

Due to the possible effect of trehalose metabolism on blood glucose levels, we did not examine the bioactivity of formulations containing trehalose (Demelier et al., 1975). The hypoglycemic effect of other formulations was tested and the results are shown in Figs. 7 and 8. In the control experiment without insulin in all groups, there is no modification in blood glucose level, which indicates that the additive has no observable effect on blood glucose levels of diabetic mice under our experiment conditions. However, both Figs. 7 and 8 show the order of the hypoglycemic-enhancing effect: formulation 1 > formulation 2 ≅ formulation 4 > formulation 3. The results will be discussed in detail in next part.

In order to further investigate the effect of sodium deoxycholate on insulin bioactivity in such complicated systems and formulation processes, we also compared the efficacy of formulations with the same co-solvent composition but different contents of sodium deoxycholate. The representative results are shown in

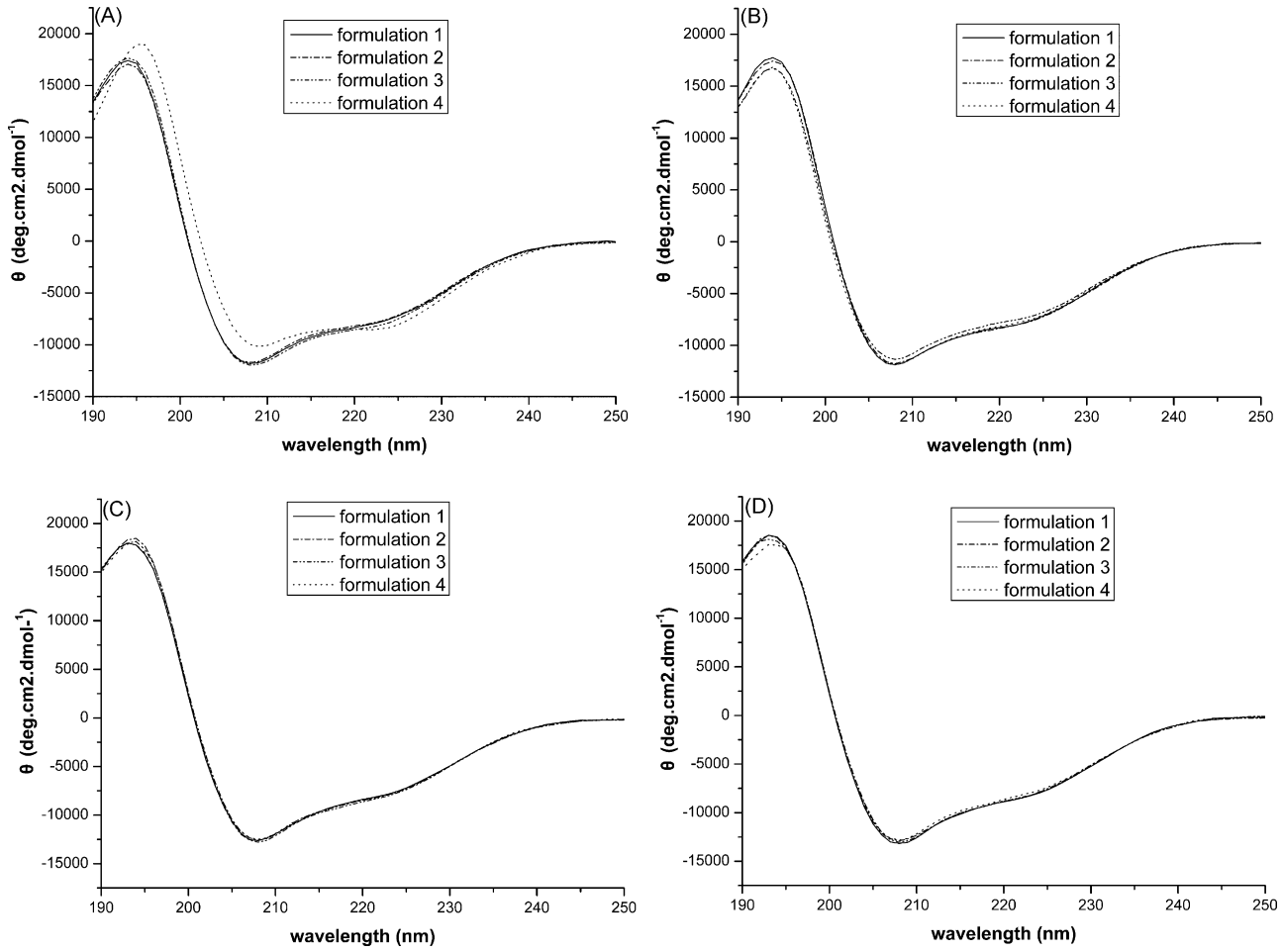

Fig. 6. Far-UV CD spectra of insulin rehydrated in deionized double-distilled water after lyophilization. (A) In the absence of any additive; (B) with a trehalose:insulin mole ratio of 25:1; (C) with an NaDC:insulin mole ratio of 50:1; (D) with an NaDC:insulin mole ratio of 100:1.

Fig. 9A and B where the order of the hypoglycemic effect is group 2 (NaDC/insulin, 50:1 in mole ratio) ≅ group 3 (NaDC/insulin, 100:1 in mole ratio) > group 1 (insulin), which indicates the protective effect of sodium deoxycholate on the bioactivity of insulin during freeze-drying.

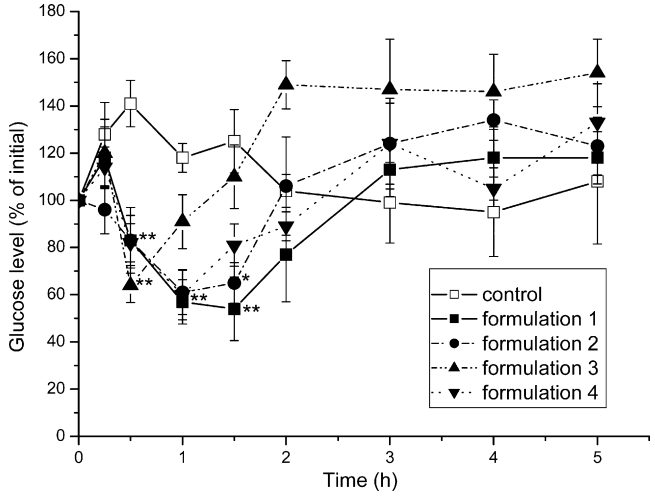

Fig. 7. Plasma glucose levels obtained in mice following subcutaneous injection of control solution or formulations 1, 2, 3 and 4 lyophilized in the absence of any additive (mean ± S.E., $n = 6$); $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control.

4. Discussion

4.1. Effect of co-solvent composition on insulin structure during freeze-drying

Like other alcohol/water systems, the TBA/water co-solvent system exhibits incomplete mixing at a molecular level (Dixit et al., 2002). In dilute TBA aqueous solution, TBA molecules could cluster around the hydrophobic methyl groups, and help the surrounding water molecules form a clathrate-like network by hydrogen bonding, whereas they associate into hydrogen bonded chains together with water molecules at a high concentration (Head-Gordon, 1995; Sinibaldi et al., 2006). When frozen, this system produce one pure hydrate containing 70% TBA by weight, and two kinds of eutectics with 20 and 90% TBA, respectively (Kasraian and DeLuca, 1995a,b). Thus, at a microcosmic level, both the solution and frozen solid of the TBA/water co-solvent are phase separation system which could be complicated further by the addition of other agents. At high concentrations (>90%, w/w) of TBA, the co-solvent system would produce a strong salt-out phenomena in the presence of insulin or other additives, although it is apparently miscible over the entire range without agents.

In this study, we investigated four representative compositions in the TBA/water phase diagram containing 0, 10, 25 and 50% by apparent volume, corresponding to four systems containing 0, 7.94, 20.6 and 43.7% by weight of TBA, separately. The phase state changes when cooling are presented in Table 1, according to the TBA/water phase diagram. All formulations in Fig. 4A underwent similar

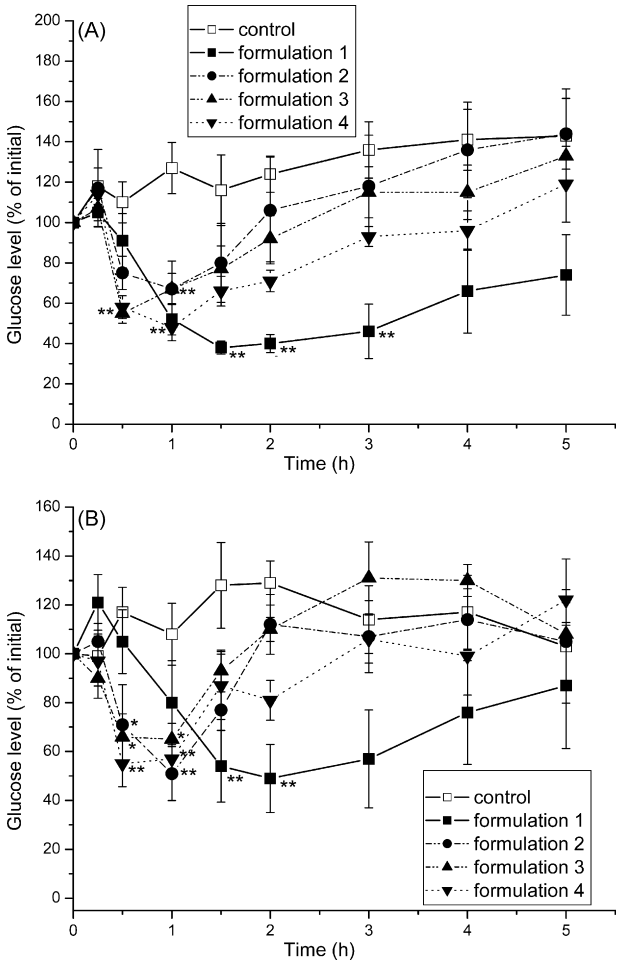

Fig. 8. Plasma glucose levels obtained in mice following subcutaneous injection of control solution or formulations 1, 2, 3 and 4 lyophilized with (A) an NaDC:insulin mole ratio of 50:1 and (B) with an NaDC:insulin mole ratio of 100:1 (mean ± S.E., $n = 6$) .*$P < 0.05$ vs. control; **$P < 0.01$ vs. control.

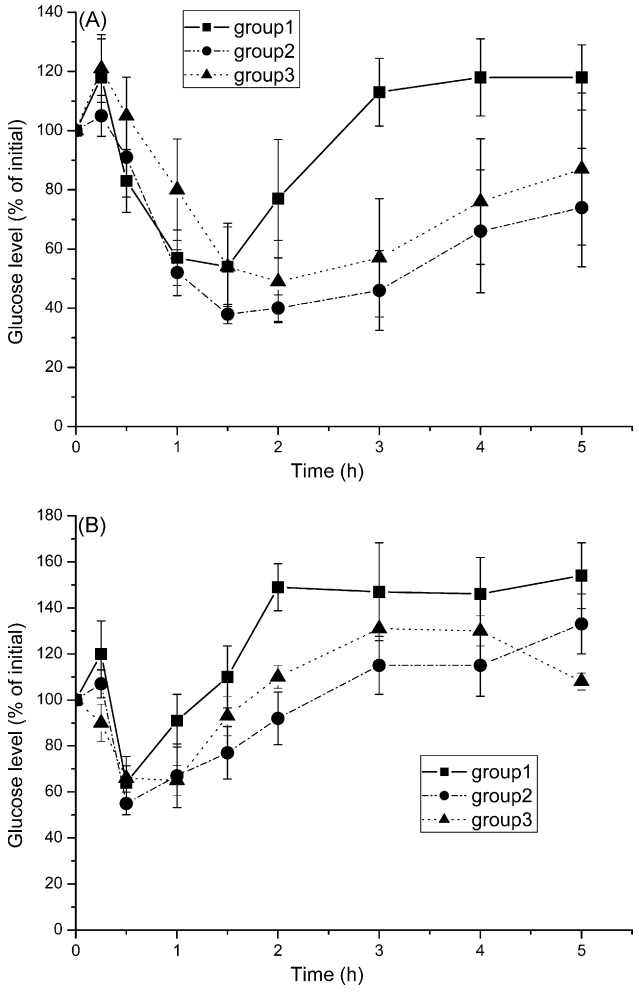

Fig. 9. Plasma glucose levels obtained in mice following subcutaneous injection of (A) lyophilized in water and (B) lyophilized in 25% (v/v) TBA. Group 1 (solid square) in the absence of any additive; group 2 (solid circle) with an NaDC:insulin mole ratio of 50:1; group 3 (solid triangle) with an NaDC:insulin mole ratio of 100:1 (mean ± S.E., $n = 6$).

stresses during freeze-drying, such as phase separation, interface denaturation, frozen concentration, selective and/or forced dispersion of insulin and dehydration to a varying extent. Phase diagram analysis together with spectra comparison could shed some light on the major factors involved. As we can see in Table 1, when cooling the last three formulations, some compositions always continuously freeze and form solids until the liquid composition is close to that of the eutectic A containing 20% (w/w) TBA. Although we could not provide direct evidence due to the requirement of extreme experiment conditions, it is very likely that the insulin structure would be partly unitary in the last three formulations before drying with the help of the slow cooling rate and long-time freezing process. Thus, the great similarity of formulations 2 and 4 could be at least partly accounted for by the enforced dispersion of insulin in the eutectic A solid due to the frozen concentration which result in severe structural disarrangement during freeze-drying.

Table 1
Phase state changes of TBA/water co-solvent system in formulations when cooling.

Formulation	Composition (l)	Medium state	Terminal state (s)
Formulation 1	Water	Water (l) + ice (s)	Ice
Formulation 2	10% TBA	Eutectic A (l) + ice (s)	Ice + eutectic A
Formulation 3	25% TBA	Eutectic A (l) + eutectic A (s)	Eutectic A
Formulation 4	50% TBA	Eutectic A (l) + Hyd. B (s)	Eutectic A + Hyd. B

l, liquid state; s, solid state.

Compared with the spectra of formulations 2 and 4, the spectrum of formulation 3 originally containing 20.6% (w/w) TBA shows a notable difference. This is because that its composition is very close to that of eutectic A (20%, w/w TBA), which could form smaller eutectic ice crystals during freezing and, therefore, produce more interface denaturation. In other words, the frozen concentration accompanied by a strong interface denaturation would have a more disruptive effect on protein structure. This reasonable explanation has been indirectly proved by scanning electronic microscope characterization of the dried sample, and agrees very well with the work of Kasraian (Kasraian and DeLuca, 1995a,b). In addition, all the spectra of the formulations suggest that the frozen concentration should be responsible for increasing the intermolecular β-sheet and reducing the α-helix content of insulin, whether it happens in ice or in eutectic A.

4.2. Effect of additives on insulin structure during freeze-drying

In the case of trehalose, the disordered states in the spectra of formulations 1, 2 and 4, are partly counteracted, but are very similar to those in absence of trehalose, which may result from insufficient trehalose molecules being present to protect the protein secondary structure during drying. The marked differences in the spectrum of formulation 3 is perhaps due to the effect of the above-mentioned

interface denaturation, although we could not exclude the possibility that trehalose disrupts eutectic A or new phase formation during freeze-drying. To sum up, the preserved structures suggest that trehalose, at a low concentration, still provides observable protection of the structure of insulin during freeze-drying of the co-solvent system, probably via the substitution of hydrogen bonds due to its insolubility in TBA.

In order to investigate further effects of the frozen concentration and interface denaturation on the structure of insulin, we added sodium deoxycholate to the TBA/water co-solvent system. Bile salts are mild amphiphilic molecules, with a rigid structure distinctly different from those of conventional ionic and non-ionic surfactants. Sodium deoxycholate has a flattened ellipsoid shape with dissimilar sides: the concave side with two OH groups is hydrophilic, whereas the convex one is hydrophobic and is in contact with that of another bile salt molecule in the micelle formation (Sobhan Sen et al., 2002). Bile salt aggregation is proposed for the primary–secondary micelle model based on a stepwise aggregation mechanism, which occurs over a relatively broad concentration range (Small, 1971). Due to high steric hindrance and limited number of hydroxyl groups, sodium deoxycholate is less able to protect the protein structure by hydrogen bonds substitutions compared with trehalose. Thus, we could easily differentiate the effect of the interface denaturation and frozen concentration from that of hydrogen bond substitution on protein structure during lyophilization. Because sodium deoxycholate is highly soluble in both TBA and alkali solution, we can increase the molar ratio of sodium deoxycholate to insulin in the co-solvent system compared with that of trehalose to insulin. Unexpectedly, when the molar ratio reached 50/1, twice that of trehalose to insulin, the spectra of formulations 1, 2 and 4 displayed narrow bands and a similar band shape at the same wavelengths. This suggests that the substitution of hydrogen bonds during drying does not play a major role in the protection of protein structure during lyophilization. Besides, the marked recovery in the native-like structure of formulation 3 in Fig. 5B hints at the great influence of interface denaturation on structural rearrangement. In addition, the difference between Fig. 5A and B, especially the disappearance of peaks at wave numbers above 1690 cm^{-1} in Fig. 5B, indicates that a high concentration sodium deoxycholate could counteract the effect of the frozen concentration and, therefore, reduce the formation of the intermolecular β-sheet, which agrees very well with other studies (Costantino et al., 1998). Unfortunately, it is very difficult to obtain more valuable information from formulations with 20% (w/w) TBA due to the complex factors involved, such as interface denaturation, possible changes in composition or the position of eutectic A solid or new phase formation during the cooling process.

4.3. Effect of additive and co-solvent composition on biological activity of insulin

In the group without any additives, Fig. 7 shows the order of the hypoglycemic-enhancing effect: formulation 1 > formulation 2 ≅ formulation 4 > formulation 3; this does not agree with the structure investigation after rehydration, but with the structure investigation of insulin prior to freeze-drying and in the solid state. Similar phenomena are also shown in Fig. 8, which could not result from the residual TBA content because there is no marked difference between the efficacy of insulin aqueous solutions even at a high TBA concentration (10%, w/w) (data not shown). The high hypoglycemic efficacy of formulation 1 in all cases could be accounted for by the lack of disruption of TBA during freeze-drying and the complete return to a native structure, while the low efficacy of formulation 3 is probably due to the effect of the strong interface denaturation together with a change in the nature of the active site during lyophilization. As we have proved, with regard to the disrupted structure of insulin in the co-solvent system prior to lyophilization, formulation 3 produces the most marked changes compared with the other formulations in all groups, while formulation 1 retains the initial structure. This is because the insulin structure in formulation 3 of all groups would be disrupted to a greater degree during freeze-drying due to the strong interface denaturation coming from the smaller ice crystals of eutectic A. Although there is a closely similar secondary structure after rehydration in all formulations except for formulation 4 of group 1, the greater the structural disruption the more possibilities there are for a mistake in the structure of the active site which strongly resembles the process of protein renaturation after expression (Paris et al., 1990; Han et al., 1997; Tran-Moseman et al., 1999). The inconsistent correlation, between the activity and either the final structure when resolved again or the structures obtained in the dried powders, agrees with other publications (Dong et al., 1996). Thus, avoiding great structural disruption during the protein formulation process is probably more important than preserving the final native-like structure, although we cannot neglect the directive function of the native structure with regard to the physical and chemical stability of the protein.

In the further investigation, we found the efficacy of sodium deoxycholate-contained formulations lyophilized in the same co-solvent system always show higher hypoglycemic effect, compared with those without any additives. The representative results are shown in Fig. 9A and B where the order of the hypoglycemic effect is group 2 (NaDC/insulin, 50:1 in mole ratio) ≅ group 3 (NaDC/insulin, 100:1 in mole ratio) > group 1 (insulin). This indicates that the presence of sodium deoxycholate provides a protective effect on insulin bioactivity, which should contribute to the possible mechanisms of structural protection discussed earlier during the freeze-drying process.

5. Conclusions

In this study, the effect of the TBA content of the co-solvent system on insulin structure prior to lyophilization is concentration-dependent, while the phase behavior properties of this system during freeze-drying are major factors responsible for protein structure changes and loss of bioactivity. The system with the composition of eutectic A containing 20% TBA (w/w) exhibits a stronger effect on insulin structure and bioactivity than the other systems. The disruptive effects could be counteracted to a great extent by the addition of trehalose and sodium deoxycholate. Trehalose has a protective effect on insulin structure, probably via substitution of hydrogen bonds, while the mild surfactant, sodium deoxycholate, is more protective on the native structure of insulin and, therefore, results in high bioactivity mainly due to resistance to the frozen concentration and interface denaturation in a concentration-dependent manner. However, further optimization and combination of co-solvent system and cryo-/lyo-protectant are required to preserve completely the intrinsic structure and bioactivity of the protein.

In conclusion, insulin as well as other potentially therapeutic proteins can be successfully freeze-dried in a TBA/water co-solvent system in the presence of cryo- and lyo-protectant additives, such as trehalose and sodium deoxycholate. However, the long-term stability of protein formulations needs to be further investigated. The present work illustrates that it is necessary and crucial to analyze the phase behavior of the co-solvent system before lyophilization in order to protect the protein structure and retain the bioactivity. Furthermore, both lyophilization in non-eutectic systems and avoiding great structural disruption during the protein formulation process appear to be very important in order to retain protein bioactivity.

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