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Effects of Sugar Additives on Protein Stability of Recombinant Human Serum Albumin during Lyophilization and Storage

Yongmoon Han, Byung-Seok Jin[1], Sang-Bong Lee[2], Yeowon Sohn[3], Jee-Won Joung[3], and Jue-Hee Lee

Department of ImmunoMicrobiology, College of Pharmacy, Dongduk Women's University, Seoul 136-714, Korea, [1]Department of Applied Chemistry, College of Natural Sciences, Dongduk Women's University, Seoul 136-714, Korea, [2]Korea Institute of Industrial Technology, Chonan-Si 330-825, Korea, and [3]Biologics Headquarters, Korea Food & Drug Administration, Seoul 122-704, Korea

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Few researches on the protein stabilization of recombinant human serum albumin (rHSA) have been done. In the present study, we assessed the impact of sugar lyoprotectants on the protein stability of lyophilized rHSA (65 KDa) in the solid state. For the assessment, rHSA was formulated with sucrose and trehalose, respectively, alone or in combination with mannitol, which were lyophilized and stored at 35°C. Degradation and aggregation of the resulting lyophilized formulations was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Induction of amorphous state by the lyophilactants with rHSA was determined by differential scanning calorimetry (DSC). The protein secondary structure of the rHSA in the formulations was analyzed by Fourier transform infrared spectroscopy (FT-IR). Results from SDS-PAGE analysis displayed that mannitol formulation caused aggregation resulting in a few bands that were greater than 65 KDa, whereas sucrose and trehalose formulations revealed no such aggregation. However, the aggregation of the protein decreased when mannitol was combined with sucrose or trehalose. DSC measurement supported the electrophoresis data showing that sucrose and trehalose formed complete amorphous state, but mannitol induced a partial amorphous state. These data indicate during lyophilization the most effective protein protection against aggregation was provided by sucrose and trehalose. The protection lasted during 4 months storage at 35°C. FT-IR analysis displayed that the sucrose formulation inhibited deamidation. In conclusion, our data suggest that sucrose and trehalose as additives seems to be sufficient to protect from lyophilization of rHSA protein and also maintain its stability in the solid state during storage.

Key words: rHSA, Sugar additives, Lyophilization, Stability, SDS-PAGE, DSC, FT-IR

INTRODUCTION

Drugs are mainly transported by human serum albumin (HSA) that is the most abundant protein accounting for approximately 60% of the total protein in the blood (Peters, 1996). As one of the most extensively studied proteins, HSA has many important pharmacological activities (He and Carter, 1992; Takakura *et al*., 2006). For instance, this protein functions to regulate plasma osmotic pressure between tissues and the blood, and it also works as a depot and transport protein (Kragh-Hansen, 1981; Benet *et al*., 1996). In clinical situations, hypoalbuminaemia (low serum albumin) is seen in various disease state (Offringa, 1998; MacAulay *et al*., 2006), and the serum albumin concentration seems to be inversely related to mortality (Cochrance, 1998). In recent, many scientists put tremendous endeavor for finding an efficient method to obtain massive amount of HSA, a medically important protein.

In the last few decades, recombinant technology has been utilized to massively produce protein drugs, known as recombinant protein medicines, and improve their functions. In case of recombinant human serum albumin (rHSA), for instance, dimer form of rHSA made by recombinant skill is known to have high blood circulation and low vascular permeability in comparison naïve form of HSA (Matsushita *et al*., 2006). In the development and utilization of such medicines, a major problem is the

Correspondence to: Yongmoon Han, College of Pharmacy, Dongduk Women's University, 23-1 Wolgok-Dong, Sungbuk-Gu, Seoul, 136-714, Korea
Tel: 82-2-940-4521, Fax: 82-2-940-4195
E-mail: ymhan@dongduk.ac.kr

stability of proteins (Arakawa, 1993; Chi *et al.*, 2003). In order to prevent this problem, it is suggested to prepare protein drugs in the dried solid state by lyophilization (freeze-drying). However, dehydration by freeze-drying can also cause unacceptable protein denaturation and aggregation (Dong *et al.*, 1995; Prestrelski *et al.*, 1993b), which result in the loss of biological activity upon rehydration (Prestrelski *et al.*, 1993a). Some reports (Franks, 1990; Kim *et al.*, 2003) indicate that additives like sucrose and trehalose can protect proteins during both lyophilization and storage in the dried form. Nevertheless, there is much controversy over the mechanisms by which additives stabilize proteins, but it seems that protein dehydration dictates the recovery of protein activity upon subsequent rehydration. Theories supporting concepts of protections during freezing and drying involve the Timasheff preferential exclusion mechanism (Arakawa, 1993; Carpenter *et al.*, 1996; Kim *et al.*, 2003) and the glassy state hypothesis in which a stabilizing additive forms an amorphous phase that inhibits protein unfolding by preventing aggregation (Franks, 1990, Slade *et al.*, 1991). In addition, the water replacement hypothesis supports that saccharide addition to protein provide hydrogen bonding with the protein in place of the lost water molecule due to dehydration, thus preventing unfolding of the protein (Carpenter and Crowe, 1989; Prestrelski *et al.*, 1993). For storage stability, a sample should be stored below its characteristic glass transition temperature (Tg) (Roy *et al.*, 1992; Duddu *et al.*, 1997) and an additive causing crystallization should be avoided during lyophilization (Izutsu *et al.*, 1994; Seefeldt *et al.*, 2005). However, a report (Johnson *et al.*, 2002) indicates that crystalline excipient itself must not necessarily damage for a protein. In any case, it should be reminded that all these aspects are minimal criteria required for the protein stability of few protein drugs, and thus the criteria are not applicable for the all proteins.

Based on those concepts and the consideration, we investigated protein stability of a recombinant HSA by formulating the protein with sugar additives such as sucrose, trehalose, and mannitol, alone or in combined with the disaccharide. In this work, selection of the additives was based on other's reports (Cleland *et al.*, 2001; Patro *et al.*, 2002). The samples formulated were stored at 4°C and 35°C for 4 months, and their protein stabilities were compared to each other. For the protein-stability analyses, degradation and aggregation were visualized by electrophoresis of the formulations; Tg values and amorphous development were measured by differential scanning calorimetry; and the protein secondary structure was characterizes by Fourier transformed infrared spectroscopy.

MATERIALS AND METHODS

Preparation of rHSA

The rHSA produced in yeast (*Saccharomyces cerevisiae*) was purchased from ProSpec TechnoGene (Rehovot, Israel). This product is a single and non-glycosylated polypeptide having a molecular weight of apprixmately 65 KDa. Purity of the protein is 99.98% by determining with high-performance liquid chromatography.

Preparation of formulation

The purchased rHSA was dialyzed against ice-cold sterile Dulbecco's phosphated saline solution (DPBS; Sigma, St. Louis, MO) using dialysis membrane (MWCO = 12~14 KDa; Sigma) for 24 h prior to use in experiments to remove sodium octanoate contained in the purchased protein. The protein concentration was determined spectrophotometrically (Biomate 3, Thermo Electron Co., Madison, WI) at 280 nm (Chung *et al.*, 1974). The dialyzed rHSA was mixed 1:1 by v/v with various additives that were prepared at twice the desired concentration using DPBS as a diluent. The additives used were sucrose, trehalose, and mannitol that were all purchased from Sigma. The rHSA was formulated with sucrose or trehalose, alone or in combination with mannitol. That is, rHSA (1 mM) was mixed with sucrose (20 mM or 60 mM), trehalose (20 mM or 60 mM), or mannitol (40 mM) dissolved in DPBS, respectively. In case of combination, rHSA (1 mM) was mixed with 40 mM mannitol and sucrose (20 mM) or trehalose (20 mM), respectively. A control rHSA received no additive. The concentrations of the additives were based on Capenter's work (Cleland *et al.*, 2001). Another set of the formulations identically prepared as above was stored at 4°C.

Lyophilization

Formulations were pipetted into 1.5 mL microcentrifuge tubes and lyophilized in a freeze-drier (Eyela, Japan). The formulations contained in tubes were frozen by placing the tubes on pre-cooled shelves (4°C) and cooling the shelves to -50°C. The formulations were maintained at this temperature for 3 h before drying started at a condenser temperature of -50°C for primary drying and a chamber pressure of 60 μm Hg for 48 h. Secondary drying was performed with shelf temperatures at -10°C for 8 h, at 20°C for 6 h and at 25°C for 4 h. Chamber pressure was maintained at the same condition as the above. Samples were rehydrated by pipetting 1 mL of ice-cold DPBS into the dried powder contained in the designated tubes. The samples were divided into two sets. One set was stored at -4°C, and the other set was kept at 35°C for 4 months prior to use in the experiments.

Protein stability analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The samples were prepared with 10% SDS in presence or absence 5% β-mercaptoethanol, respectively, corresponding to reducing and non-reducing conditions of gel-electrophoresis. Electrophoresis of the samples (1.5 mg of a sample per lane) was done as previously described (Laemuli, 1970; Han *et al.*, 1999). Protein-bands were visualized by staining with Coomassie Blue and destaining with a mixture of acetic acid and methanol. For the evaluation of protein sizes produced, a standard molecular weight marker (SDS6H, Sigma) was run on the gel with the samples to evaluate molecular sizes of test samples. The molecular markers were 205-, 116-, 97-, 66-, 45-, and 29-KDa molecules corresponding to myosin, β-galactosidase, phophorylase B, albumin (bovine plasma), albumin (egg), and carbonic anhydrase, respectively. Density of the bands was measured by a gel-documentation system (UVI Platinum Tech. U.K.).

Measurements by differential scanning calorimetry (DSC)

Approximately 3~10 mg of lyophilized formulations were sealed in an aluminum pan and equilibrated at 30°C for 5 min in the DSC (Q-10, TA, DuPont) under the N_2-gas injection. The samples were heated at 10 °C/min to a final temperature of 220°C. Minimum point in melting endothermal peak was specified as melting temperature. Comparative reduction of crystallinity due to each formulation was estimated from fusion enthalpy (per gram) values. The values were obtained by integrating melting peak area of the respective DSC curve.

Determination of the secondary structure change by FT-IR (Fourier transform infrared spectroscopy)

The FT-IR measurement was done at room temperature on a Nicolet Nexus 670 FT-IR spectrometer (Madison, WI, U.S.A.) equipped with a KBr detector, and a KBr beam splitter. All FT-IR spectra were recorded using a resolution of 4 cm^{-1} and 60 scans. A reference spectrum was recorded prior to each experiment to correct for background effects all the spectra recorded. The instrument was purged with dry nitrogen gas to reduce the interference of waster vapor and CO_2 in all the FT-IR measurement. A rehydrated protein formulation at a concentration of 10 mg/mL was dried in the silicate-enriched chamber at room temperature after putting the formulation on a KBr pellet. The pellet was placed within the FT-IR apparatus, and spectrum of the formulation was recorded after equilibrium. The spectrum of diluent (DPBS) collected at the same condition as described above was subtracted from spectra of samples solutions to obtain actual protein spectrum. The subtraction criterion for assessment of the protein secondary structure was based on others' FT-IR analytical method (Dong *et al.*, 1990; Wi *et al.*, 1998).

RESULTS

Determination of protein stability during lyophilization and rehydration by SDS-PAGE

The initial experiment determined effects of the formulations during lyophilization and rehydration on rHSA under the non-reducing and reducing conditions of SDS-PAGE. The gel-patterns obtained from rehydrated samples after lyophilization were compared with gel-patterns from samples in aqueous state before the lyophilzation. Data showed that the mannitol formulation stored at either 4°C or 35°C for 4 months resulted in several bands (* areas; ranged from greater than 205 KDa to 97 KDa) above the 65 KDa-band on the gel, whereas below the 65 KDa-band no fragmented band was observed (Fig. IA; Lane 3). In the both temperatures, the lyophilized rHSA alone as a control displayed a band pattern similar to bands formed by the mannitol formulation, and in both cases band-density at the position of 65 KDa was reduced (Fig. 1A; Lane 8). The reduction of the mannitol formulation was greater than reduction of the rHSA alone, revealing that there were 14% and 58% reductions, respectively, corresponding to the mannitol formulation and unformulated rHSA (rHSA alone) as measured by gel-documentation system. However, in case of rHSA containing mannitol mixed with sucrose or trehalose, there was a single band identical to those of the sucrose only and trehalose only formulation (Fig. 1A; Lanes 4 & 5). The gel-pattern of these lyophilized formulations after the 5 months storage at 35°C developed the almost same as pattern as shown in Fig. 1 (data not shown). In contrast to the results with the lyophilized formulations, formulation of non-lyophilized mannitol formulation in aqueous state demonstrated a single band as did with all other formations regardless of the temperature difference (Fig. 1B). In addition, the formulations were not affected by temperature, demonstrating that the entire gel patterns at the two different temperatures in the non-lyophilized samples were almost same as compared to each other (Fig. 1A & B).

In another experiment to determine degree of denaturation, the formulations were analyzed under the reducing condition. Results showed that all the formulations that were stored at 35°C displayed a single 65 KDa-band without any degraded band of smaller molecular size than the 65 KDa-band, regardless of lyophilization (Fig. 2). These results were the same as compared with formulations stored at 4°C (data not shown).

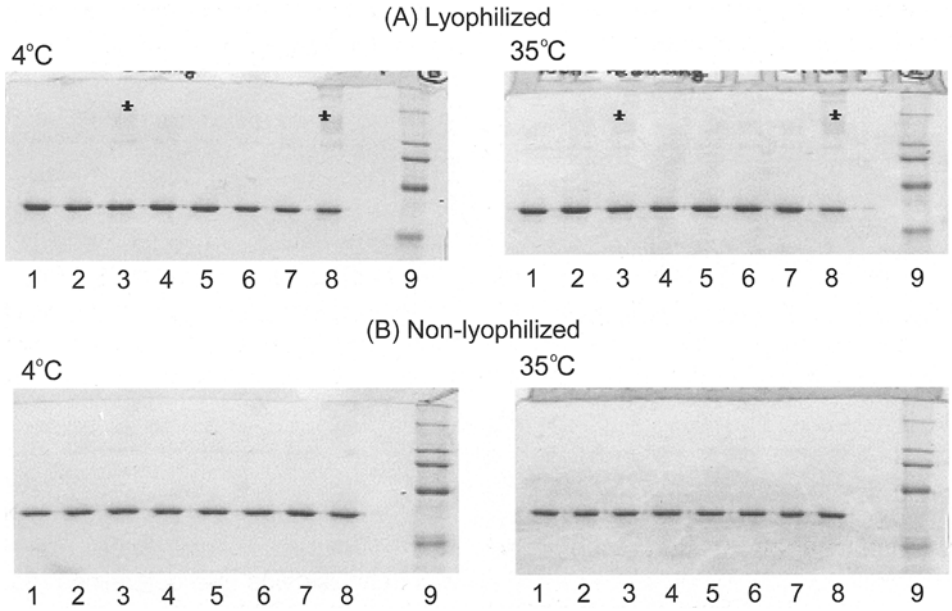

Fig. 1. Analysis of lyophilized (A) and non-lyophilized (B) formulations under the non-reducing condition of SDS-PAGE. The gel-electrophoresis revealed that after lyophilization, the mannitol formulation that had been stored for 4 months caused aggregation of rHSA, which resulted in bands bigger than 65 KDa rHSA (* areas; ranged from greater than 205 KDa to 97 KDa). With non-lyophilized samples, these observations were not detectable. In both panels (unit: mM): lanes 1, sucrose 60; 2, trehalose 60; 3, mannitol 40; 4, sucrose 20 plus mannitol 40; 5, trehalose 20 plus mannitol 40; 6, sucrose 60; 7, trehalose 60; 8, no additive (rHSA alone); 9, molecular markers. The markers were 205-, 116-, 97-, 66-, and 45- 29-KDa molecules, respectively.

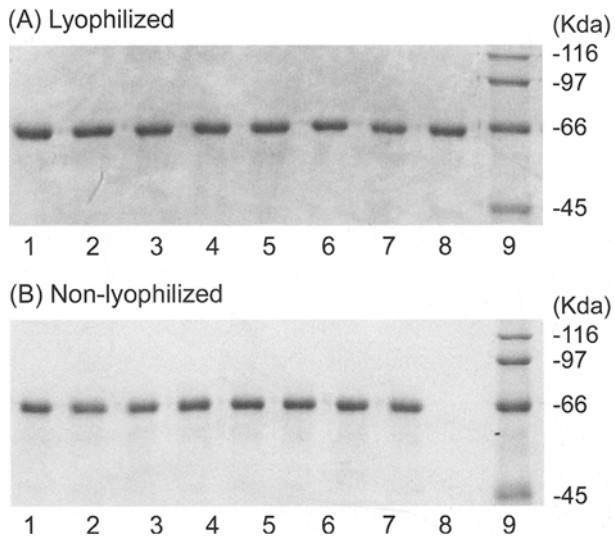

Fig. 2. Analysis of the lyophilized formulations under the reducing condition of SDS-PAGE. In both panels (unit: mM): lanes 1, sucrose 60; 2, trehalose 60; 3, mannitol 40; 4, sucrose 20 plus mannitol 40; 5, trehalose 20 plus mannitol 40; 6, sucrose 60; 7, trehalose 60; 8, no additive (rHSA alone); 9, molecular weight.

DSC results indicate retardation of rHSA crystallization

The DSC analysis was carried out to verify the influence of sucrose and mannitol formulation on the crystallization of rHSA. The melting points of sucrose alone and mannitol alone were 192°C and 167°C, respectively (Fig. 3A), and melting point of rHSA alone was 123°C (Fig. 3B). When rHSA was formulated with sucrose, the melting point peak of rHSA was almost disappeared, demonstrating that sucrose inhibited the crystallization of the protein by possibly inducing an amorphous structure (Fig. 3B). By mathematical calculation of fusion enthalpy of each of the combinations, the fusion enthalpy of rHSA was 177 J/g, which was reduced to 62 J/g by formulating the protein with sucrose, thus indicating that the formulation with sucrose reduced crystallinity of the protein. On the other hand, in case of the mannitol formulation, there was a broad peak at 140°C besides another peak at 110°C, resulting in that mannitol did not completely inhibit the crystallization of rHSA during lyophilization (Fig. 3A). The latter result was confirmed because fusion enthalpy of mannitol alone (320 J/g) was much greater than that of sucrose alone (129 J/g). In addition, according these data, addition of sucrose at 20 mM to the mannitol formation was sufficient to remove the entire aggregation. These results reflected data that were obtained from the non-reducing SDS-PAGE (Fig 1A) showing that aggregation due to crystallization by mannitol developed several bands above the 65 KDa-band, which was similar to the band pattern displayed by the rHSA alone.

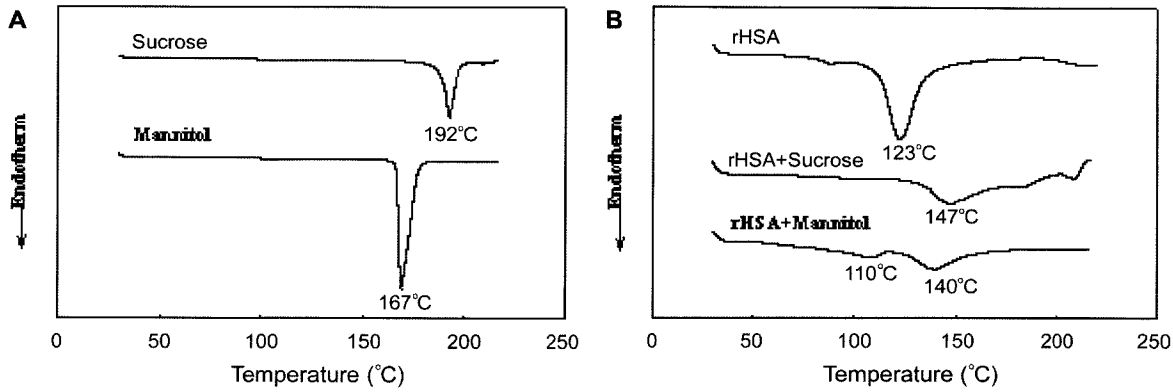

Fig. 3. Thermograms of sucrose alone or mannitol aone (A) and rHSA formulated with sucrose or mannitol (B) measured by DSC. Addition of sucrose to rHSA induced complete amorphous sate, whereas mannitol did not protect the protein stability.

FT-IR analysis of change of protein secondary structure

The selected transmission spectra measured by FT-IR showed few changes over the entire range of curves without the application of curve-fitting (Fig. 4). That is, as compared to the control curve from rHSA with no additive (Fig. 4A), the curve from the sucrose formulation (Fig. 4B) resulted in a somewhat flat plateau. The similar observation with trehalose was also determined (data not shown). In case of the mannitol formulation, there was a peak located at around 2300 cm^{-1} on the curve (Fig. 4C), which possibly was caused by CO_2. Except the CO_2-caused peak, pattern of the mannitol plus sucrose formulation (Fig. 4D) was very similar to the pattern from the mannitol

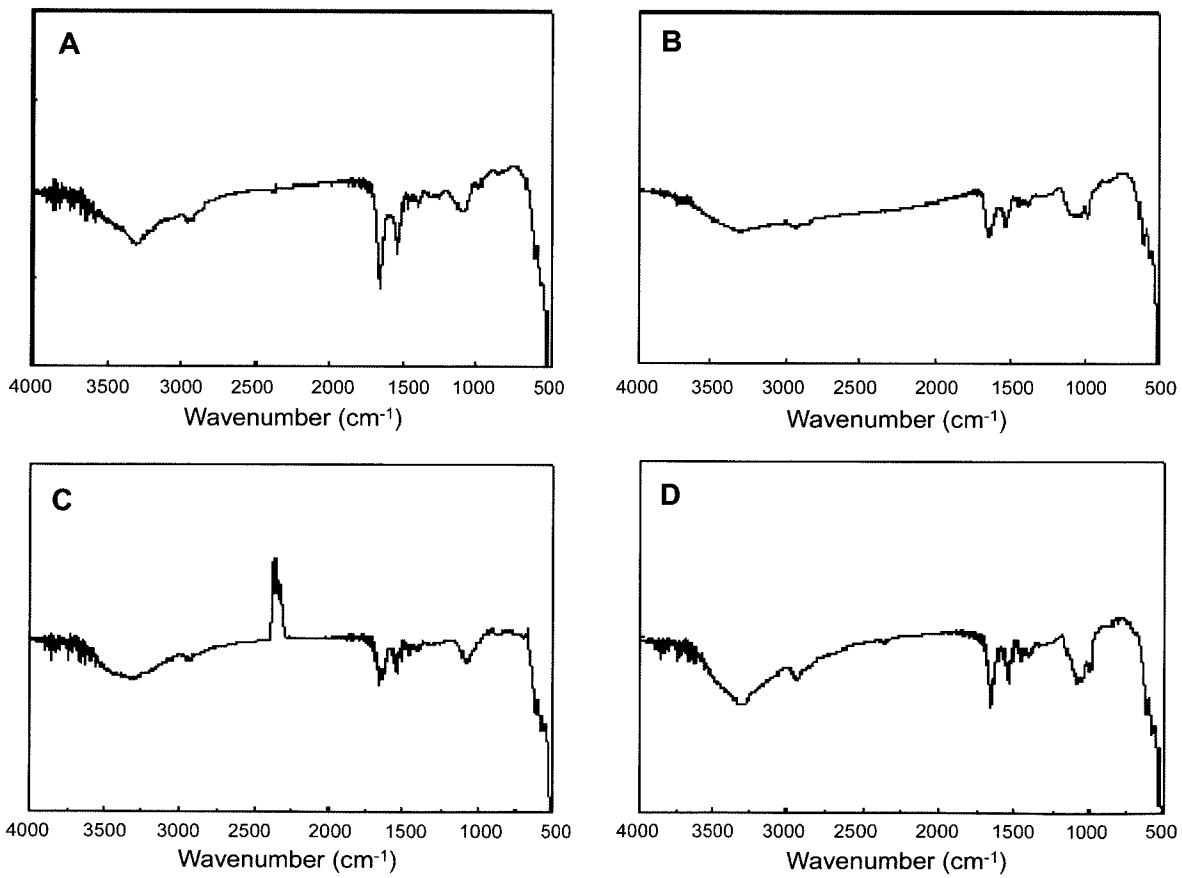

Fig. 4. Pattern of transmission spectra of the formulations measured by the FT-IR. The pattern showed an entire region of spectrum of rHSA formulated with no additive (A), sucrose (20 mM) (B), mannitol (40 mM) (C), and sucrose/mannitol (D) formulations, respectively, and lyophilized.

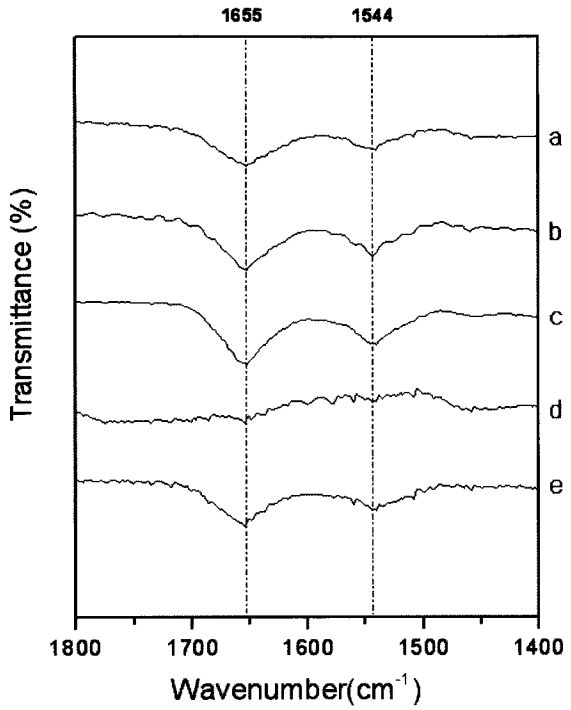

Fig. 5. Determination of protein secondary structure by FT-IR analysis. Formulations mentioned in Fig. 4 were further analyzed for alteration of amide (1655 cm^{-1}) and carboxyl (1544 cm^{-1}) bonds. No protein denaturation was observed if rHSA was formulated with the respective additive. In the spectrogram, rHSA formulated with sucrose (a), mannitol (b), sucrose plus mmanitol (c), and no additive (d). The (e) displays FT-IR analysis of non-lyophilized intact rHSA without addition of any additive.

formulation. These FT-IR data were further analyzed to determine the protein secondary structure. In this analysis, the baseline passing through the ordinates at 2000 and 1000 cm^{-1} was subtracted after the curve-fitting was utilized, and the curves were analyzed by determining the spectra shapes for the carboxyl and amide bonds that are indicative of protein denaturation located at approximately 1640~1540 cm^{-1} (Dong *et al.*, 1990). Results showed that basically there was no change of amide and carboxyl bands of the rHSA if the protein was formulated with the sucrose additive (Fig. 5). However, the lyophilization of rHSA alone resulted in no significant peaks at the two positions on the spectrum, possibly due to protein denaturation (Fig. 5d) as compared to fresh rHSA without addition of sucrose additive before lyophilization (Fig. 5e). Spectra from sucrose, mannitol, and sucrose plus manntiol formulations had clear peaks at the same positions like the control spectrum (Fig. 5).

DISCUSSION

In this work, we observed that protection of rHSA during freeze-drying was dependent on an amorphous phase induced by sucrose and trehalose added to the protein. That is, upon subsequent rehydration of rHSA that was formulated with the respective disaccharide additives, the protein stability was analyzed. By electrophoresis analysis, mannitol formulation caused aggregation of the protein, which was easily detectable. This observation was further confirmed by the DSC analysis that demonstrated sucrose formulation formed complete glassy state, but the mannitol formulation partially induced complete amorphous state. The observation was not a surprise because fusion heat of mannitol was almost a 3 folds-greater than fusion heat of sucrose. In fact, it is well known that mannitol causes aggregation during lyophilization. However, there has been reported that crystalline excipient itself must not necessarily be damaging for a protein (Johnson *et al.*, 2002). Our purpose of testing mannitol was to determine the importance of an amorphous phase formation for stability of rHSA. Thus, in this work we used mannitol as a control for the comparison with the disaccharide additives. The combination with sucrose or trehalose blocked the formation of aggregation providing an increased amorphous state. In that case, the molar ration of 2:1 to mannitol and sucrose seemed to be sufficient for the blockage. These formulations of sucrose only, trehalose only, and combination with mannitol were not affected by temperature, at least, for the entire duration of 4 months storage.

By FT-IR analysis, the protein secondary structure was determined. Data obtained from the FT-IR analysis also reflected the data obtained from the SDS-PAGE and DSC showing that aggregation due to crystallization by mannitol caused some alteration in the secondary structure where the amide linkage was changed. In case of rHSA without any additive added, there were no significant peaks at the points determining the amide and carboxyl groups, which indicates occurrence of denaturation. The mannitol formulation resulted in partial denaturation. Therefore, an additive like mannitol that substantially causes aggregation during lyophilization did not protect the protein, but the combination with sucrose or trehalose recovered the protein stability. However, obtaining an amorphous condition seems to be insufficient for protein protection. For example, dextran that readily forms a glass has no protection of recombinant human Factor XIII (Kreilgaard *et al.*, 1998). This suggests that inhibition of aggregation is not sufficient to protect protein.

In our work, the best protection for rHSA was provided by sucrose and trehalose forming a complete glassy condition during lyophilization. Our data seems to be consistent with data resulted from other proteins (Carpenter *et al.*, 1996; Izutsu *et al.*, 1994; Prestrelski *et al.*, 1995). From all of the data, this protective mechanism would be explained by the capacity of the additives to hydrogen bond in place of water with the protein in dried solid. The

presence of an amorphous phase containing protein and additive also appeared to be a major factor for maintaining a long-term storage of the protein. In case of sucrose only or combination with mannitol, we stored the formulations in the dried solid at 35°C, which was far below their fusion enthalpy (per gram) values of melting-point peaks. Four months after the storage, degree of the protein stability was the same as non-lyophilized native rHSA in aqueous state. These data are supported by other researchers' suggestions (Arakawa *et al.*, 1993; Franks, 1990; Chang *et al.*, 1995; Krishnan, 2002) that long-term storage stability of a dried protein depends on formation maintaining an amorphous phases by additive and storage at temperatures below the melting points of the formulations. In conclusion, sucrose and trehalose appeared to be sufficient for providing stability of rHSA that lead the full recovery of native protein after rehydration and storage.

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