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International Journal of Pharmaceutics 301 (2005) 161–169

international journal of pharmaceutics

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Effect of counterions on the physical properties of L-arginine in frozen solutions and freeze-dried solids

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Received 24 January 2005; received in revised form 27 April 2005; accepted 17 May 2005
Available online 18 July 2005

Abstract

The objective of this study was to elucidate the physical properties of L-arginine and various counterion combinations in frozen aqueous solutions and in freeze-dried solids. L-Arginine remains amorphous in the highly concentrated non-ice phase in frozen solutions with a T_g' (glass transition temperature of maximally freeze-concentrated solutes) of −41.4 °C. Some acids and salts (e.g., H_3PO_4, H_2SO_4, HNO_3, and NaH_2PO_4) raised the T_g', whereas others (e.g., HCl, CH_3COOH, HCOOH, Na_2HPO_4, and NaCl) had little effect or lowered the L-arginine T_g'. Co-lyophilization with phosphoric acid also raised the glass transition temperature (T_g) of amorphous freeze-dried L-arginine solids. Arginine–H_3PO_4 combinations exhibited properties that led to either the stabilization or destabilization of a model protein (lactate dehydrogenase: LDH) during freeze-drying, depending on their concentration ratios. Fourier-transform infrared (FT-IR) and diffusion reflectance near-infrared (NIR) spectra indicated the presence of interactions between the amino and/or guanidyl groups of L-arginine and phosphate ions in the amorphous freeze-dried cakes. It was postulated that the interaction between L-arginine and the multivalent counterions, as well as an increase in hydrogen bonding network, reduced the mobility of molecules in the frozen solutions and freeze-dried solids.
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Keywords: Freeze-drying; Near-infrared Spectroscopy; Amorphous solid; Glass transition

1. Introduction

Amorphous solids of active ingredients and/or excipients obtained by freeze-drying or rapid cooling of hot-melt liquids provide various characteristics of interest for pharmaceutical formulations (Hancock and Zografi, 1997; Craig et al., 1999). Obtaining practically stable amorphous solids is one of the major challenges to achieve the advances, such as higher dissolution speed and protein stabilization during freeze-drying, because amorphous solids exists in metastable states with greater molecular mobility than that of the corresponding crystallized forms. Optimizing the composition of components that exhibits a sufficiently high glass transition temperature (T_g) is one means of improving the stability of an amorphous solid and the

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doi:10.1016/j.ijpharm.2005.05.019

molecules embedded therein, as the molecular mobility increases significantly above the T_g (Akers et al., 1995; Nail et al., 2002). High-T_g amorphous solids are often achieved by lyophilizing a major ingredient with excipients that have a high intrinsic T_g (e.g., polymers). Molecular-level mixing of the components results in a T_g that follows the Gordon–Taylor equation (Shamblin et al., 1998). Solute complexation (e.g., saccharide–borate) and salt formation (e.g., sodium indomethacin) also affects the physical properties (e.g., T_g) of amorphous freeze-dried solids, whereas such effects are difficult to predict (Miller et al., 1999; Tong et al., 2002; Izutsu et al., 2003).

Disaccharides (e.g., sucrose, trehalose) are often added to lyophilized protein formulations to protect proteins from inactivation during processing and subsequent storage (Carpenter et al., 1997; Franks, 1998). Similar well-known cryo- and lyoprotective effects of various amino acids and their salts are attracting increased attention, because the chemical and physical diversity of these molecules can provide more choices in the design of formulations. The feasibility of various L-arginine salts for serving as stabilizing agents in freeze-dried protein formulations is of particular interest due to their potential protein structure-stabilizing effects, which help avoid dehydration-induced conformation changes and also due to their aggregation-preventing effects in the re-hydrated solutions (Zhang et al., 1996). Various amino acids and their salts, including L-arginine HCl, protect proteins from inactivation in frozen solutions, during freeze-drying (Seguro et al., 1990; Izutsu et al., 1991), during spray-drying (Mumenthaler et al., 1994), and in the storage of lyophilized solids (Hsu et al., 1995). An L-arginine and phosphoric acid combination is used in the lyophilized formulation of therapeutic proteins, including, for example, tissue plasminogen activator (rt-PA) (PDR 2003). The effective amino acids remain in an amorphous state during the freeze-drying process, suggesting that stabilization takes place in a similar manner as that associated with saccharides, namely, via the protection of the protein conformation by substitution of the surrounding water molecules and reduction of the chemical reaction in the glass-state solid (Izutsu et al., 1993; Carpenter et al., 1997; Franks, 1998). L-Arginine and acid combinations have been extensively used in the present decade to assist in the recovery of chemically unfolded proteins and recombinant proteins expressed in inclusion bodies (Buchner and Rudolph, 1991; Shiraki et al., 2002; Arakawa and Tsumoto, 2003). These agents reduce the interaction between structurally altered protein molecules that leads to aggregation. The addition of acids to a particular active ingredient or excipient solution not only shifts the pH, but also alters the physico-chemical properties of frozen solutions and freeze-dried solids (Akers et al., 1995; Tong et al., 2002). Various acids are known to affect the crystallinity and the glass transition temperature of vacuum-dried or freeze-dried L-arginine (Mattern et al., 1999). The objective of the present study was thus to elucidate how various acids affect the physical properties of L-arginine and L-lysine in frozen aqueous solutions and in freeze-dried solids. The effect of the excipients on the stability of a model protein (LDH: lactate dehydrogenase) during the process of freeze-drying was also studied.

2. Materials and methods

2.1. Materials

LDH (rabbit muscle lactate dehydrogenase) was obtained from Sigma Chemical Co. (St. Louis, MO). The enzyme was dialyzed against sodium phosphate buffer (20 mM, pH 7.0) before the experiments. L-Arginine, L-arginine hydrochloride, DL-arginine, and other chemicals used here were purchased from Wako Pure Chemical Co. (Osaka, Japan). L-Arginine phosphate monohydrate (LAP) crystal was prepared from aqueous solutions containing L-arginine and H_3PO_4 (pH 5.0) by evaporation at ambient temperature (Mazumder et al., 1995).

2.2. Freeze-drying

The freeze-drying of aqueous L-arginine and that of the protein solutions were performed using a Freezevac 1FCS freeze-drier (Tozai Tsusho, Tokyo, Japan). Aqueous solutions (200 μl each) in flat-bottom glass vials (diameter: 10 mm) were frozen by immersion in liquid nitrogen. Some of the solutions (3 ml) were freeze-dried in larger vials (diameter: 20 mm) for the near-infrared study. The tubes were placed on polytetrafluoroethylene film (thickness: 60 μm) on a shelf of the freeze-drier in order to slower the heat transfer.

The samples were freeze-dried under a vacuum without shelf temperature control for 24 h and then at 35 °C for 12 h. The freeze-dried solids were re-hydrated with distilled water. The pH of the aqueous solutions was measured using a pH meter (HM-60G, TOA, Tokyo) with a glass electrode (GST-5721T).

2.3. Thermal analysis

The thermal properties of the frozen aqueous solutions and the freeze-dried solids were analyzed using a differential scanning calorimeter (DSC Q-10, TA Instruments, New Castle, DE) with an electric cooling system and associated software (Universal Analysis 2000). Indium and cyclohexane were used for the DSC calibration. An aliquot (10 μl) of aqueous solution in a hermetic aluminum cell was cooled at 20 °C/min, and was scanned from −85 °C at 5 °C/min. A baseline shift in the thermogram, which was observed as a peak in the derivative thermogram, was assigned to be the glass transition temperature of the maximally freeze-concentrated phase (T_g') of the frozen solution. Freeze-dried solids (1.5–2.0 mg) in hermetic aluminum cells were initially cooled to −20 °C at −10 °C/min, and then were scanned at 10 °C/min.

2.4. Aggregation of lyophilized LDH

The effect of the co-solutes on the freeze-drying of a model protein (rabbit muscle LDH) was studied by size-exclusion HPLC analysis using a Shimadzu LC-10 system (Kyoto, Japan) with a TSK gel super SW 3000 column (35 °C, Tosoh Co., Tokyo, Japan). The freeze-dried solids from solutions (200 μl) containing LDH (0.1 mg/ml), 20 mM sodium phosphate buffer (pH 7.0), and various co-solutes were re-hydrated with distilled water for the HPLC analysis. The protein solutions were filtrated through 0.45-μm PVDF filters (Millipore, Bedford, MA) before injection. The solutions were eluted at 0.2 ml/min, with an aqueous solution containing 100 mM sodium phosphate buffer and 100 mM Na_2SO_4 (pH 6.7). Absorbance at 280 nm was monitored.

2.5. FT-IR and NIR analysis of freeze-dried solids

An FT-IR system (MB104, ABB Bomen, Quebec, Canada) with a dry gas generator (Balston, Haverhill, MA) and GRAMS/32 software (Galactic Ind., Salem NH) were used for the spectroscopic analysis. An aliquot of the freeze-dried solid was mixed with approximately 250 mg of KBr to create the tablet. The absorbance of each KBr tablet sample was collected in 128 scans at a resolution of 4 cm^{-1} in the range of 4000–400 cm^{-1}.

Near-infrared spectroscopy was performed using a Bruker MPA system with a diffuse-reflectance integrating-sphere probe (PbS detector) and OPUS software (Ettlingen, Germany). Near-IR light was directed upward from the bottom of the glass vials containing freeze-dried solids or crystal powder (2 g) to obtain the reflected signal over a range of 12,000–4000 cm^{-1} with a resolution of 8 cm^{-1} in 32 scans. The freeze-dried solids were measured twice by rotating the sample vials between measurements. L-Arginine phosphate monohydrate crystal was ground to make fine powder for the NIR study.

3. Results

Fig. 1 shows the thermograms of frozen aqueous L-arginine, L-lysine, and some acid solutions. Frozen L-arginine solution (500 mM) showed a thermal shift in the baseline (T_g': glass transition of maximally freeze-concentrated solutes) at −41.4 °C, indicating that L-arginine remained amorphous in the freeze-concentrated phase. These transitions have been defined as other thermophysical changes (e.g., the onset of ice melting endotherm, softening of the concentrated phase) (Chang and Randall, 1992; Shalaev and Franks, 1995). The thermogram also revealed relatively small transition at a slightly lower temperature (−51.9 °C), which is often referred to as “real” glass transition temperature of the frozen solution. A frozen L-lysine solution (500 mM) had a T_g' transition at −59.1 °C. Some frozen single-solute solutions containing 500 mM H_2SO_4, CH_3COOH, or HCOOH showed eutectic crystal melting endotherms at approximately −72.3, −26.4, and −50.7 °C, respectively. No apparent peaks or transitions were observed prior to the large ice-melting endotherm in other frozen acid solutions.

The effects of various co-solutes on the T_g' of frozen 500 mM L-arginine solution are shown in Fig. 2. Some acids and inorganic salts (e.g., H_2SO_4, H_3PO_4, H_3BO_3,

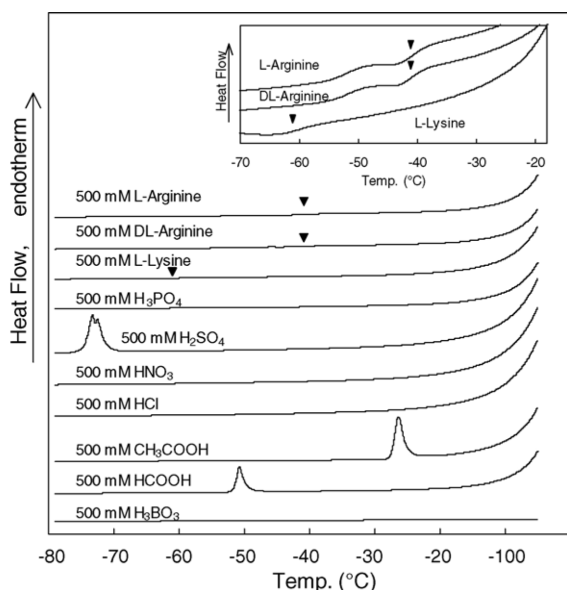

Fig. 1. Thermal profiles of frozen aqueous solutions containing L-arginine, L-lysine, and various acids. Aliquots (10 μl) of frozen solutions in aluminum cells were scanned from −85 °C at 5 °C/min. Glass transition temperatures of maximally freeze-concentrated solutes are indicated by inverted triangles (▼). Transitions of some frozen solutions (500 mM) were magnified in the small figure.

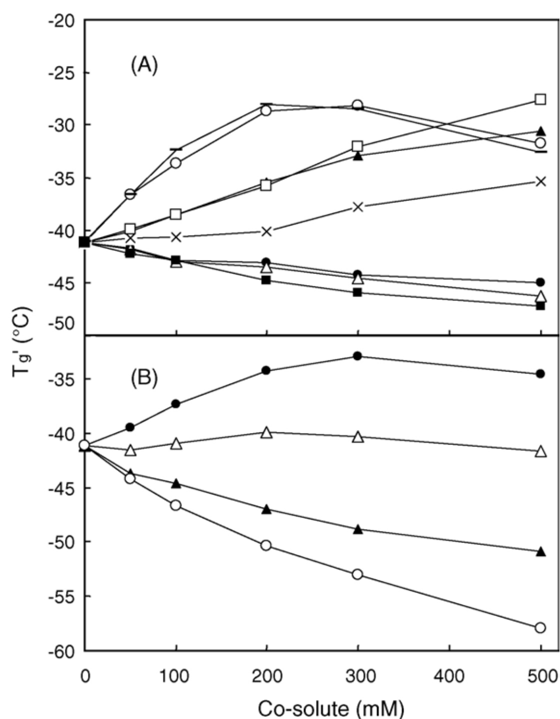

Fig. 2. Effect of co-solutes on the T_g' of frozen 500 mM L-arginine solutions. ($n = 2$). Symbols denote the addition of (○) H_3PO_4, (▲) H_2SO_4, (□) H_3BO_3, (△) CH_3COOH, (■) HCOOH, (●) HCl, (X) HNO_3 (A); (●) NaH_2PO_4, (△) Na_2HPO_4, (▲) $NaNO_3$ and (○) NaCl (B). T_g's (-) of frozen solutions containing 500 mM DL-arginine and H_3PO_4 (A).

NaH_2PO_4, and HNO_3) raised the transition temperature. Phosphoric acid showed the maximum effect, raising the L-arginine T_g' at 200–300 mM, above which the effect was reduced (500 mM). The acid shifted the transition of frozen L- and DL-arginine solutions to a similar extent. Nitric acid showed less of a T_g'-raising effect. In contrast, other acids and salts (HCl, CH_3COOH, HCOOH, Na_2HPO_4, $NaNO_3$, and NaCl) were found to have little effect, or even lowered the T_g' of the frozen L-arginine solution. Sodium hydrogen phosphates altered the L-arginine T_g' to various extents. Monosodium salt (NaH_2PO_4) showed an effect slightly less than H_3PO_4, whereas the disodium salt (Na_2HPO_4) shifted the transition only slightly. A eutectic salt crystallization exotherm was observed in frozen solutions containing L-arginine and higher concentrations of certain inorganic salts (e.g., 100 mM Na_2SO_4, data not shown). The solution pH of the L-arginine and acid combinations were 11.2 (500 mM L-arginine), 9.3 (L-arg and 200 mM HCl), 7.7 (L-arg and 500 mM HCl), 8.9 (L-arg 200 mM H_3PO_4), and 5.6 (L-arg and 500 mM H_3PO_4) at room temperature, suggesting the pH change is not the main reason for the altered physical properties.

The T_g' values of frozen solutions containing L-lysine (500 mM) and various concentrations of acids or inorganic salts are shown in Fig. 3. L-Lysine is also an alkaline amino acid, the hydrochloride salt of which has a cryoprotective effect in the process of the freeze-thawing of certain enzymes (Seguro et al., 1990). The acids that raised the L-arginine T_g' (H_3PO_4, H_2SO_4, H_3BO_3, HNO_3) showed similar effects on the T_g' of L-lysine, which was suggestive of the same mechanism that altered the transition temperature. The addition of HCl did not significantly alter the transition temperature. A disodium hydrogen phosphate (Na_2HPO_4) increased the transition temperature to less of an extent than did either H_3PO_4 or NaH_2PO_4.

Fig. 4 shows the thermograms of freeze-dried solids from solutions containing L-arginine (500 mM) and

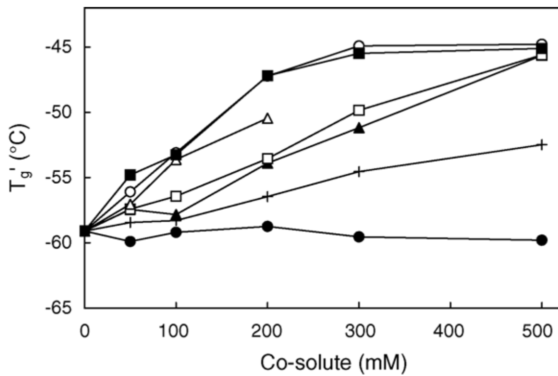

Fig. 3. Effect of co-solutes on the T_g' of frozen 500 mM L-lysine solutions. ($n = 2$). Symbols denote the addition of (○) H_3PO_4, (■) NaH_2PO_4, (△) Na_2HPO_4, (□) H_3BO_3, (▲) H_2SO_4, (+) HNO_3, and (●) HCl.

various concentrations of co-solutes. The freeze-dried L-arginine showed a broad glass transition (T_g), with a midpoint at around 55 °C, and a large crystallization exotherm at 84 °C; these results indicated that the freeze-dried cake was largely amorphous. Co-lyophilization with H_3PO_4 raised the T_g, and simultaneously reduced the L-arginine crystallization peak. The maximum effect on the T_g was observed at

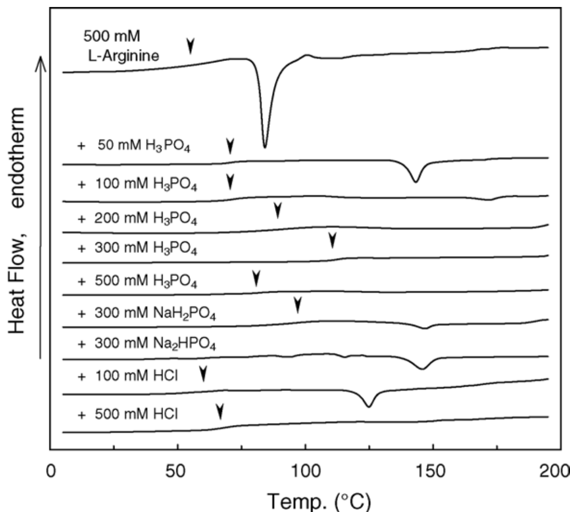

Fig. 4. Thermal profiles of solids freeze-dried from solutions containing L-arginine and co-solutes. Approximately 2 mg of freeze-dried solids in hermetic aluminum cells were scanned from −10 °C at 10 °C/min. Glass transition temperatures are indicated by inverted triangles (▼).

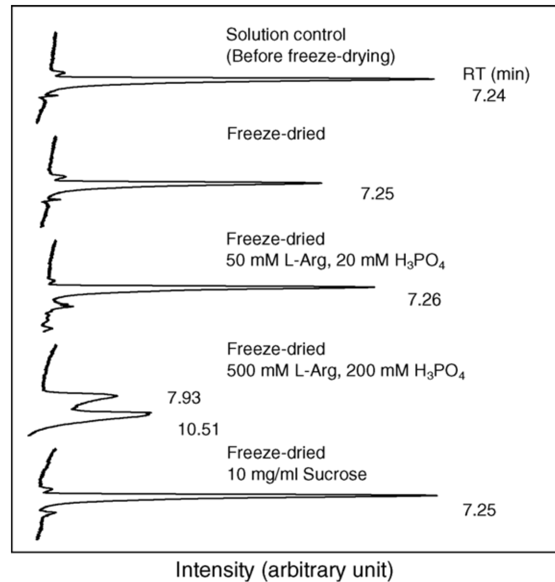

Fig. 5. Size-exclusion HPLC chromatograms of LDH freeze-dried with co-solutes. Re-hydrated solutions of freeze-dried solids from initial solutions containing 0.1 mg/ml LDH and various concentrations of co-solutes were applied for HPLC analysis.

300 mM H_3PO_4. Thermal analysis of the L-arginine phosphate monohydrate crystal showed two endotherm peaks at around 142 and 150 °C (data not shown) (Mazumder et al., 1995). Mono- and disodium hydrogen phosphates showed smaller or complicated effects on the physical properties of the co-lyophilized L-arginine. A small L-arginine crystallization exotherm was observed in the freeze-dried solids with 300 mM NaH_2PO_4 or Na_2HPO_4. The lyophilized sample containing 300 mM Na_2HPO_4 showed several transitions. Co-lyophilization with HCl also reduced the L-arginine crystallization peak, without exerting any apparent effect on the glass transition temperature. The changes in the transition temperature and the reduced tendency toward crystallization were consistent with the report by Mattern et al. (1999).

Fig. 5 shows the effects of L-arginine and acids on the lyophilization-induced aggregation of lactate dehydrogenase (LDH), as studied by size-exclusion chromatography. Rabbit muscle LDH is a typical freeze- or lyophilization-labile oligomer protein composed of four subunits, and it tends to show reduced activity, especially at lower concentrations (Chilson et al., 1965; Seguro et al., 1990; Prestrelski et al., 1993;

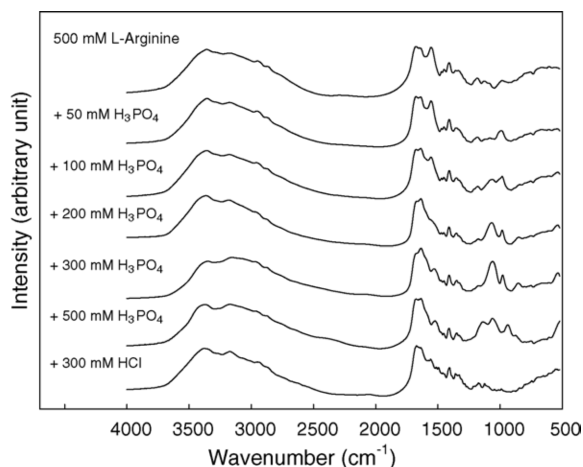

Fig. 6. FT-IR spectra of freeze-dried solids containing L-arginine and acids obtained by a KBr tablet method.

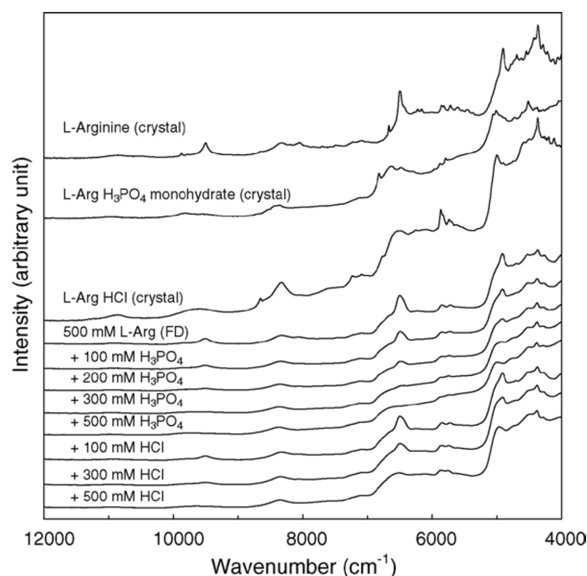

Fig. 7. Near-infrared spectra of crystal powders and freeze-dried solids from solutions containing L-arginine and various acids obtained by a diffuse-reflection method.

Anchordoquy et al., 2001). The freeze-drying of LDH in the absence of stabilizing co-solutes was found to reduce the main peak at 7.2–7.3 min without the appearance of other peaks; this finding is suggestive of the insoluble aggregation of structurally altered enzyme molecules. The enzyme retained most of its original structure under the condition of freeze-drying with 10 mg/ml sucrose. Co-lyophilization with L-arginine and H_3PO_4 exerted concentration-dependent effects on LDH aggregation. The combination of 50 mM L-arginine and 20 mM H_3PO_4 retained more LDH molecules than was observed in the case without the stabilizing co-solutes. Higher concentrations of L-arginine (500 mM) and H_3PO_4 (200 mM) apparently shifted and reduced the main LDH peak, inducing smaller peaks that suggested the separation of subunits. Both of the frozen solutions containing L-arginine (50, 500 mM), LDH, and H_3PO_4 had T_g' at −32 to −33 °C (data not shown). The present results indicate that the effects of L-arginine and H_3PO_4 depend on a delicate balance between the stabilizing and destabilizing factors (Taneja and Ahmad, 1994; Arakawa and Tsumoto, 2003).

The freeze-dried solid containing L-arginine and its salts were analyzed by spectroscopic methods (FT-IR, NIR) in order to elucidate the mechanisms associated with the altered physical properties. Freeze-dried L-arginine showed an IR spectrum typical to that of the amino acids in the measurement of KBr tablets (Fig. 6). Co-lyophilization with H_3PO_4 reduced a NH_3^+ vibration absorption band centered at 1552 cm^{-1}, indicating an altered environment around the amino or guanidyl group of L-arginine. The acid also led to the shifting of broad, superimposed NH_3^+ and OH stretching bands (3200–3400 cm^{-1}) to a lower wavenumber in the freeze-dried solids (Meera et al., 2004). A band at 1065 cm^{-1} appeared at an initial H_3PO_4 concentration higher than 200 mM. The NH_3^+ vibration band was also reduced in the process of freeze-drying L-arginine with 300 mM HCl.

Fig. 7 shows the near-infrared spectra of L-arginine freeze-dried with H_3PO_4 or HCl, as obtained by the reflection probe approach. The spectra of the “as is” L-arginine and L-arginine HCl crystal reagent, and L-arginine phosphate monohydrate (LAP) crystallized from aqueous solution, are also shown. The freeze-dried L-arginine showed several absorption bands, which are slightly broader, but in the same wavenumber as that of the L-arginine crystal. Co-lyophilization with H_3PO_4 reduced the intensity of the bands centered at 9510, 8060, 6500, and 4915 cm^{-1} without inducing any apparent changes in the bands at 8320, 5847, 5716, and 4370 cm^{-1}. The resulting spectra showed broad bands at the wavenumbers similar to that of the

LAP crystal. The significant reduction of the band at 6505 cm^{-1} (first overtone of primary and secondary amine vibration) in the case of freeze-drying L-arginine with H_3PO_4 indicated significant changes in the environment around the functional groups (Ozaki and Kawata, 1996). The band at 6505 cm^{-1} disappeared at H_3PO_4 concentrations (200–300 mM), revealing a maximum effect on the T_g' and T_g. The assignments of the other reduced bands were not immediately available. Co-lyophilization with HCl was associate with similar but smaller changes in the L-arginine spectrum, and these changes were also observed in the L-arginine HCl crystal. The IR and NIR spectra indicated changes in the local environment around the L-arginine amino or guanidyl group due to interactions with counterions.

4. Discussion

The results indicated that certain acids with di-or trivalent anions significantly affect the physical properties of L-arginine in frozen solution and in amorphous freeze-dried solids. Higher T_g' and T_g values are desirable to avoid cake collapse during freeze-drying and to ensure the long-term stability of the amorphous freeze-dried matrix and that of the embedded minor molecules (Nail et al., 2002). A slow evaporation of some L-arginine and acid (e.g., H_3PO_4, HCl, CH_3COOH, HCOOH) solutions results in salt crystals that can be potentially used for non-linear optical materials (Meera et al., 2004). L-Arginine phosphate monohydrate (LAP: $^{+}(H_2N)_2CNH(CH_2)_3CH(NH_3)^{+}COO^{-}\cdot H_2PO_4^{-}\cdot H_2O$) crystal consists of alternate layers of phosphate groups and arginine molecules (Aoki et al., 1971). The phosphate group is linked to zwitterionic L-arginine by via a H···O–H hydrogen bond to carboxyl oxygen and via O···H–N hydrogen bonds to the amino and guanidyl nitrogens of another arginine molecule in the LAP crystal. Chloride ions in L-arginine hydrochloride monohydrate crystal interact mainly with amino and guanidyl nitrogens (Dow and Jensen, 1970). L-Arginine cations in various salt crystals also interact with each other through hydrogen bonds, as they contain negatively charged carboxylate group and positively charged protonated guanidyl and α-amino groups. It is plausible that, despite the lack of long-range spatial ordering of molecules, the ionized L-arginine and acid anions interact similarly in the crystal and the amorphous state. The IR and NIR spectra (Figs. 6 and 7) suggested changes in the environment around the amino or guanidyl groups of L-arginine in the salt crystals and the amorphous solids co-lyophilized with H_3PO_4 or HCl. Acids with divalent or trivalent anions may raise the transition temperatures of amorphous L-arginine because of the intense interaction networks. Phosphate ions also raise the glass transition temperature of amorphous freeze-dried disaccharide solids by increasing the extent of hydrogen bonding between molecules (Kawai et al., 2002; Ohtake et al., 2004; Wolkers et al., 2004).

The diffuse-reflectance NIR method enabled an analysis without opening the top rubber cup. Usage of this non-invasive method has been reported for the measurement of residual water and component crystallinity in freeze-dried solids (Kamat et al., 1989; Lin and Hsu, 2002; Brulls et al., 2003). This method may also be useful for the detection of other changes in the physico-chemical properties of amorphous freeze-dried solids, as it reduces the chances of water absorption and the various subsequent changes that can occur during sample preparation required for other analytical methods (e.g., KBr tableting in FT-IR).

The L-arginine-H_3PO_4 combination was shown to have various effects on the aggregation of freeze-dried LDH, probably due to a number of stabilizing and destabilizing mechanisms. Various stresses during the freeze-drying process (e.g., low temperature, freeze-concentration with unfavorable co-solutes, pH change, contact with ice surface, dehydration) often induce protein subunit separation and conformational changes in the oligomer protein (Zettlmeissl et al., 1979; Carpenter et al., 1997; Anchordoquy et al., 2001). The structurally altered protein molecules aggregate non-covalently if their recovery to the native structure takes place at a slower rate than that of protein–protein binding between exposed hydrophobic surfaces (Rudolph et al., 1979). Here, L-arginine and H_3PO_4 exerted different stabilizing effects, depending on their concentration ratios and the freeze-drying process. Stable, glass-state L-arginine-H_3PO_4 solids may protect the embedded protein molecules by substituting water molecules inevitable to retain the conformation, and by limiting the molecular mobility of the system. The ability of L-arginine to reduce protein–protein interactions in aqueous solution may prevent the irreversible aggre-

gation of lyophilization-induced structurally altered protein molecules in re-hydrated solution, whereas the same effect might also induce the subunit dissociation of the enzyme that leads to conformation changes. L-Arginine may also affect the thermodynamic stability of the protein conformation in aqueous solutions, and these effects may depend largely on the solution pH (Taneja and Ahmad, 1994). The use of L-arginine-acid combinations for parenteral formulations, especially for the freeze-drying of oligomer proteins, will require substantial future research in order to optimize the balance of different effects.

5. Conclusions

The choice of counterions greatly affected the physical properties of frozen L-arginine solutions and the amorphous freeze-dried solids. The higher T_g' and T_g values achieved by the addition of certain acids or salts may reduce the chances of cake collapse during the freeze-drying process, and may also have improved stability for the storage of dried solids. Several other co-solutes (e.g., HCl) showed the opposite effects. Spectroscopic studies (FT-IR and NIR) revealed the presence of interactions between L-arginine amino or guanidyl groups and some acids (H_3PO_4, HCl). It is plausible that the interactions between L-arginine and the multivalent counterions, together with the resulting hydrogen-bonding network, lead to a reduction in the mobility of molecules in the highly concentrated aqueous phase and in the dried solids. The combination of L-arginine and phosphoric acid exerted both stabilizing and destabilizing effects in the process of freeze-drying oligomeric proteins, which requires careful optimization in formulation design.

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