Freeze-Drying of Proteins in Glass Solids Formed by Basic Amino Acids and Dicarboxylic Acids

Ken-ichi IZUTSU,*[,a] Saori KADOYA,[b] Chikako YOMOTA,[a] Toru KAWANISHI,[a] Etsuo YONEMOCHI,[b] and Katsuhide TERADA[b]

[a] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya, Tokyo 158–8501, Japan: and* [b] *Faculty of Pharmaceutical Sciences, Toho University; 2–2–1 Miyama, Funabashi, Chiba 274–8510, Japan.*
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The purpose of this study was to produce and characterize glass-state amorphous solids containing amino acids and organic acids that protect co-lyophilized proteins. Thermal analysis of frozen solutions containing a basic amino acid (*e.g.*, L-arginine, L-lysine, L-histidine) and a hydroxy di- or tricarboxylic acid (*e.g.*, citric acid, L-tartaric acid, DL-malic acid) showed glass transition of maximally freeze-concentrated solute at temperatures (T_g') significantly higher than those of the individual solute solutions. Mixing of the amino acid with some dicarboxylic acids (*e.g.*, oxalic acid) also suggested an upward shift of the transition temperature. Contrarily, combinations of the amino acid with monocarboxylic acids (*e.g.*, acetic acid) had T_g's between those of the individual solute solutions. Co-lyophilization of the basic amino acids and citric acid or L-tartaric acid resulted in amorphous solids that have glass transition temperatures (T_g) higher than the individual components. Mid- and near-infrared analysis indicated altered environment around the functional groups of the consisting molecules. Some of the glass-state excipient combinations protected an enzyme (lactate dehydrogenase, LDH) from inactivation during freeze-drying. The glass-state excipient combinations formed by hydrogen-bonding and electrostatic interaction network would be potent alternative to stabilize therapeutic proteins in freeze-dried formulations.

Key words freeze-drying; protein formulation; amorphous; stabilization; glass

Freeze-drying is a popular method of ensuring the stability of proteins that are not stable enough in aqueous solutions during the period required for storage and distribution.[1,2] Various freeze-dried protein formulations contain excipients (*e.g.*, sugars, polymers, and amino acids) that protect proteins from physical and chemical changes. Disaccharides (*e.g.*, sucrose, trehalose) are the most popular among them because they stabilize proteins both thermodynamically and kinetically in aqueous solutions and freeze-dried solids.[3—5]

The development of freeze-dried protein formulations containing amino acids is often more challenging than the development of formulations with saccharides because of the varied physical and chemical properties (*e.g.*, crystallinity, glass transition temperature) of the freeze-dried amino acids, as well as their tendency to form complexes with other ingredients.[6] Many amino acids are considered to protect proteins basically in similar mechanisms with disaccharides. They thermodynamically stabilize protein conformation in aqueous solutions and probably in frozen solutions by being preferentially excluded from the immediate surface of proteins.[7] Glass-state amorphous solids formed by freeze-drying of the disaccharides or some amino acids protect proteins from structural changes thermodynamically by substituting surrounding water molecules.[8] They also reduce chemical degradation of freeze-dried proteins kinetically by reducing the molecular mobility.[2,8] In addition, some amino acids (*e.g.*, L-arginine) also prevent protein aggregation in aqueous solutions prior to the drying process and after reconstitution.[9] Choosing appropriate counterions that form glass-state solid should be one of the key factors in designing amino acid-based amorphous freeze-dried formulations.[10,11] For example, glass transition temperatures (T_g) of freeze-dried L-histidine salts depend largely on the counterions.[12] Co-lyophilization of L-arginine and multivalent inorganic acids (*e.g.*, H_3PO_4, H_2SO_4) results in glass-state amorphous solids that protect proteins during the process and storage (*e.g.*, tissue plasminogen activator formulation, PDR 2003).[13] Some organic acid and inorganic cation combinations (*e.g.*, sodium citrates) also form high glass transition temperature amorphous solids.[14] Various functional groups (*e.g.*, amino, carboxyl, hydroxyl) in the constituting molecules contributes significantly to form the glass-state amorphous salt solids.[15] Producing glass-state amorphous solids by freeze-drying of amino acid and organic acid combinations, and their application in pharmaceutical formulations are interesting topics to explore.[15]

The purpose of this study was to produce stable amorphous solids that protect proteins by freeze-drying combinations of amino acids and organic acids. The physical properties of frozen solutions and freeze-dried solids containing the popular excipients and model chemicals were studied. The effect of the excipient combinations on the freeze-drying of lactate dehydrogenase (LDH) was also examined.

Experimental

Materials LDH (rabbit muscle) was obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Succinic acid was produced by Kanto Chemical Co. (Tokyo, Japan). L-(+)-Tartaric acid, DL-malic acid, and other chemicals were of analytical grade and were purchased from Wako Pure Chemical (Osaka, Japan). The protein solutions were dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and then centrifuged (1500 g×5 min) and filtered (0.45 μm, polyvinyliden difluoride (PVDF), Millipore) to remove insoluble aggregates before the freeze-drying study.

Freeze-Drying A pH meter (HM-60G, TOA-DKK Co., Tokyo, Japan) was used to determine the pH of the aqueous solutions at 25 °C. A freeze-drier (Freezvac 1C, Tozai-Tsusho, Tokyo, Japan) was used to lyophilize the aqueous solutions. Aliquots of aqueous solutions (250 μl) in flat-bottom glass vials (10 mm diameter) were frozen by immersion in liquid nitrogen. The solutions were freeze-dried without shelf temperature control (20 h), and then at 35 °C (8 h). Solid samples for diffuse-reflection near-infrared analysis were prepared by freeze-drying the aqueous solutions (2 ml) in glass vials (21 mm diameter).

Thermal Analysis Thermal analysis of frozen solutions and dried solids

* To whom correspondence should be addressed. e-mail: Izutsu@nihs.go.jp

was performed using a differential scanning calorimeter (DSC) (Q-10, TA Instruments, New Castle, DE, U.S.A.) and software (Universal Analysis 2000, TA Instruments). Aliquots of aqueous solutions (10 μl) in aluminum cells were cooled from room temperature at 10 °C/min, and then scanned from −70 °C at 5 °C/min. The effect of heat-treatment (annealing) on the thermal properties of the frozen solutions was studied after the initial heating scan paused at −10 °C, then the samples were maintained at this temperature for 10 min. Thermal data were acquired in the subsequent heating from −70 °C at 5 °C/min. Freeze-dried solids (1—2 mg) in hermetic aluminum cells were subjected to the thermal analysis from −20 °C at 5 °C/min under nitrogen gas flow. Melted organic acids (approx. 5 mg, 200 °C) in aluminum cells were rapidly cooled to −50 °C, and then scanned at 5 °C/min to obtain the glass transition temperatures. Glass transition temperatures were determined as the midpoint (maximum inflection) of the discontinuities in the heat flow curves.

Powder-X-Ray Diffraction (XRD) The powder X-ray diffraction patterns were measured at various temperatures by using a Rint-Altima diffractometer (Rigaku, Tokyo, Japan) with Cu$K\alpha$ radiation at 40 kV/40 mA. The samples were scanned in the area of $5° < 2\theta < 35°$ at an angle speed of 15°/min by heating at 2 °C/min from room temperature.

Mid- and Near-Infrared Analysis A Fourier-transform infrared spectrophotometer (MB-104, Bomen, Quebec, Canada) with a gas generator (Balston, Haverhill, MA, U.S.A.) and Grams/32 software were used to obtain mid-infrared spectra of freeze-fried solids. Approximately 0.5 mg of the solid was mixed with dried KBr powder (250 mg) and made into tablets by compression. The KBr tablets were scanned 128 times to obtain the spectra in the 400—4000 cm^{-1} region. Near-infrared spectroscopy was performed by using a Bruker MPA system with a diffuse-reflectance integrating-sphere probe (PbS detector) and OPUS software (Ettlingen, Germany). Near-infrared light was directed upward from the bottom of the glass vials containing freeze-dried solids to obtain the reflected signal over a range of 4000—12000 cm^{-1} with a resolution of 4 cm^{-1} in 128 scans. The freeze-dried solids were measured twice by rotating the sample vials between measurements.

Activity of Lactate Dehydrogenase in Freeze-Dried Solids Aqueous solutions (250 μl) containing LDH (0.05 mg/ml) and excipients were freeze-dried in flat-bottom glass vials (10 mm diameter). One of the enzyme solutions was freeze-dried at a higher sodium phosphate buffer concentration (50 mM, pH 7.0). Other enzyme solutions contained the added excipients and lower concentration buffer components (<1 mM) diluted from the dialyzed protein solutions. Activity of LDH was obtained spectrophotometrically at 25 °C. Each 1.0 ml of assay mixture contained 0.35 mM pyruvic acid and 0.07 mM reduced nicotinamide-adenine dinucleotide (NADH) in 50 mM sodium phosphate buffer (pH 7.5). The enzyme reaction was started by the addition of LDH solution (50 μl), and the decrease in the absorbance at 340 nm was monitored. The enzyme activity (%) relative to that before freezing was shown.

Results

Physical Property of Frozen Solutions The thermal profiles of frozen solutions containing L-histidine and citric acid at various concentration ratios (total 200 mM) are shown in Fig. 1. The single-solute frozen L-histidine solution (200 mM) showed a T'_g (glass transition temperature of maximally freeze-concentrated solute) at −33.5 °C, and an exotherm peak that suggests eutectic crystallization at around −8 °C.[12] Freeze-drying of solutions at above their T'_g often induces physical collapse because of the significantly reduced local viscosity in the freeze-concentrated phase.[1] The second scan of the 200 mM L-histidine solutions after the heat-treatment (−10 °C, 10 min) gave flat thermograms that indicate crystallized solute up to the ice melting temperature (data not shown). The citric acid solution (200 mM) had a T_g' at −55.1 °C, indicating that the solute remained amorphous in the freeze-concentrated phase surrounding ice crystals. The L-histidine crystallization peak disappeared in the presence of citric acid. The two-solute frozen solutions showed transitions (T_g's) at temperatures as high as −22.8 °C at the equal (100 mM) L-histidine and citric acid concentrations.

Figure 2 shows transition temperatures (T'_g) of frozen solutions containing amino acids and organic acids at various concentration ratios. Some single-solute frozen amino acid or organic acid solutions (200 mM) had apparent T'_g transitions at −44.2 °C (L-arginine), −55.1 °C (citric acid), and

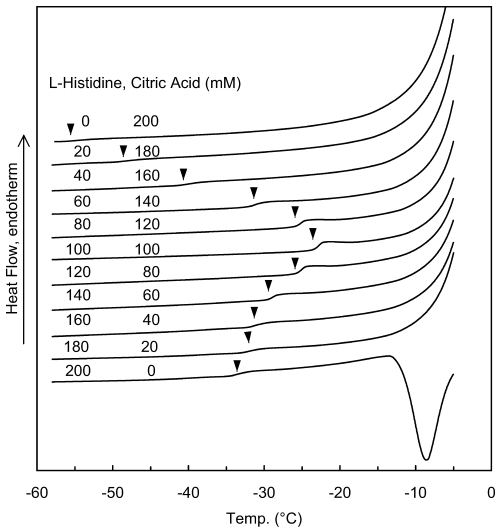

Fig. 1. Thermal Profiles of Frozen Solutions Containing L-Histidine and Citric Acid

Aliquots (10 μl) of solutions in hermetic aluminum cells were scanned from −70 °C at 5 °C/min. Glass transition temperatures of maximally freeze-concentrated solutes (T'_g) are indicated by inverted triangles (▼).

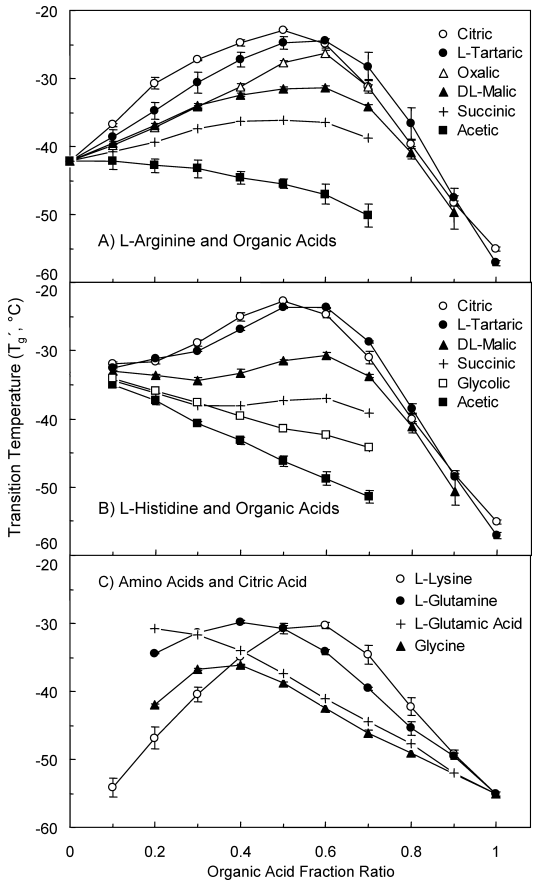

Fig. 2. Glass Transition Temperatures of Maximally Freeze-Concentrated Solute (T'_g) in Frozen Solutions Containing an Amino Acid and an Organic Acid at Varied Concentration Ratios (Total 200 mM, Average±S.D., n=3)

−57.1 °C (L-tartaric acid). The frozen L-glutamine solution showed both T'_g (−42.8 °C) and the subsequent eutectic crystallization peak (approx. −25 °C) in the heating scan (data not shown). Thermograms of the frozen L-lysine and DL-malic acid solutions inclined gradually without apparent transition up to the ice melting endotherm, which suggested T'_gs lower than −60 °C. Exotherm peaks either in the cooling process (glycine, acetic acid) or in the heating scan (oxalic acid) indicated eutectic crystallization in the frozen solution.[16] Potential T'_g transitions of some frozen solutions that also showed eutectic crystallization peaks (*e.g.*, 200 mM L-histidine or L-glutamine) were not included in the figure. The limited solubility of some amino acids and organic acids (*e.g.*, L-glutamic acid, fumaric acid, maleic acid) prevented them from undergoing thermal analysis at 200 mM. A lower concentration glutamic acid solution (100 mM) showed a T'_g at −32.2 °C and an exotherm peak that suggests eutectic crystallization at around −11.0 °C (data not shown).

Mixing of the solutes induced some unique physical properties in the frozen solutions that depend on the number of functional groups in the consisting molecules. The transition temperatures (T'_gs) of frozen solutions containing a basic or neutral amino acid (L-histidine, L-arginine, L-lysine, L-glutamine, glycine) and a hydroxy di- or tricarboxylic acid (citric acid, L-tartaric acid, DL-malic acid) showed bell-shaped profiles. The frozen solutions containing a hydroxy di- or tricarboxylic acid (citric acid, L-tartaric acid) and an acidic amino acid (L-glutamic acid) did not show the mixing-induced upward T'_g shift. Citric acid also effectively prevented the crystallization of glycine in the frozen solutions. Dicarboxylic acids (succinic acid, maleic acid, fumaric acid, oxalic acid) showed a high tendency to crystallize in the single-solute frozen solutions and in some mixture frozen solutions.[15,17] The frozen solutions containing L-arginine and oxalic acid or succinic acid also presented the high transition temperature (T'_g) by mixing. A mono-carboxylic acid (acetic acid), a hydroxy mono-carboxylic acid (glycolic acid), and HCl did not show the upward T'_g shift in the mixture with the basic amino acids.[13]

Physical Property of Freeze-Dried Solids Freeze-drying of the single-solute amino acid solutions resulted in cylindrical cakes that showed varied crystallinity in the powder X-ray diffraction (XRD) and thermal analyses (Figs. 3, 4). Freeze-dried L-arginine showed the typical harrow XRD pattern of amorphous solids. Thermal scan of the solid showed the glass transition (52.6 °C) and subsequent crystallization exotherm (105—110 °C). Freeze-dried L-histidine showed largely amorphous XRD pattern (30 °C) with the broad glass transition (65—100 °C) and crystallization at varied temperatures (120—150 °C). The L-arginine and L-histidine solids showed apparent crystallization peaks in the XRD patterns at the elevated temperature (150 °C). The dried L-glutamine (200 mM) solids showed features of both crystalline (*e.g.*, peaks in the XRD pattern) and amorphous (*e.g.*, glass transitions and heat-induced crystallization exotherm) solids. The solute concentration in the initial solution and thermal history in the freeze-drying process should determine the crystallinity of the freeze-dried L-histidine and L-glutamine.[12] Glycine was freeze-dried as β polymorph crystal.[18] Freeze-drying of citric acid or L-tartaric acid solutions (200 mM) resulted in unstructured or particulate solids that

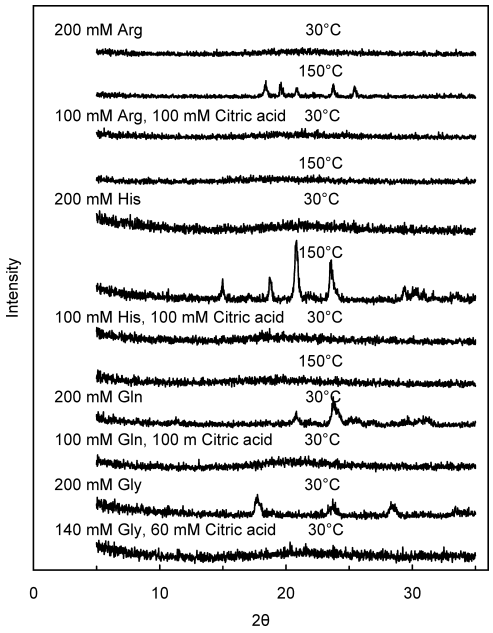

Fig. 3. Powder X-Ray Diffraction Patterns of Freeze-Dried Solids Containing Amino Acids and Citric Acid

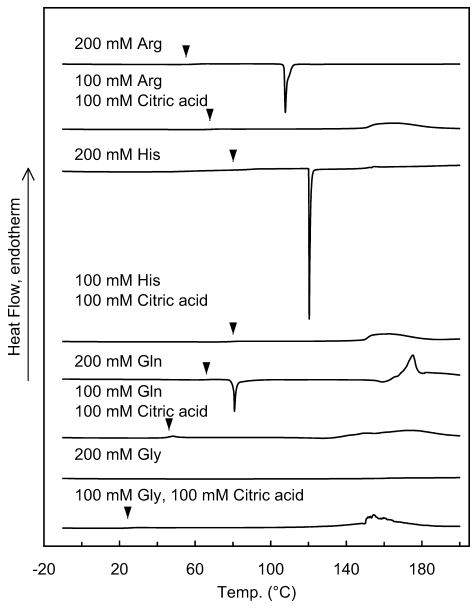

Fig. 4. DSC Thermograms of Freeze-Dried Solids Containing Amino Acids and Citric Acid

Freeze-dried solids (1—2 mg) in hermetic aluminum cells were scanned from −20 °C at 5 °C/min.

indicate physical collapse in the primary during process. Amorphous solids of the organic acids prepared by rapid-cooling of the melt liquid showed glass transition at 9.2 °C (citric acid) and 68.1 °C (L-tartaric acid) in the thermal scan (n=3).[19]

Co-lyophilizing the basic or neutral amino acids (L-arginine, L-histidine, L-glutamine, glycine) and the organic acid (citric acid, L-tartaric acid) produced cylindrical non-crystalline cake solids at wide initial concentration ratios (Figs. 3—5). The solids obtained by freeze-drying the basic amino acids (L-arginine, L-histidine) with citric or L-tartaric acid showed glass transition at temperatures (T_gs) much higher

than those of the individual components. The transitions were observed at temperatures as high as 89.5 °C (140 mM L-arginine, 60 mM citric acid) or 98.5 °C (160 mM L-histidine, 40 mM citric acid). Shrinking of some solids containing higher ratio of organic acid during the freeze-drying process suggested their low glass transition temperatures. The XRD and thermal analysis also indicated that the co-lyophilized solids remained amorphous up to 150 °C. Some binary freeze-dried solids showed a broad endotherm that suggests component decomposition at the elevated temperatures. The mixing of L-arginine with citric acid and with L-tartaric acid showed similar T_g profiles, in spite of the large difference in their transition temperatures of the cooled-melt solids. The bell-shaped profiles of the transition temperatures were significantly different from the reported transitions of binary nonionic molecule systems that follow Gordon–Taylor equation.[20] Glass transition temperatures of amorphous solids containing ideally mixed nonionic molecules without particular attractive or repulsive interactions shift between those of the individual components. Contrarily, the glass transition temperatures of co-lyophilized L-glutamine and citric acid combination solids shifted linearly between those of the individual components, which suggested absence of the particular attractive interactions between the heterogeneous molecules in the solids. Co-lyophilization of glycine and citric acid resulted in amorphous cake solids only at limited molar ratios.

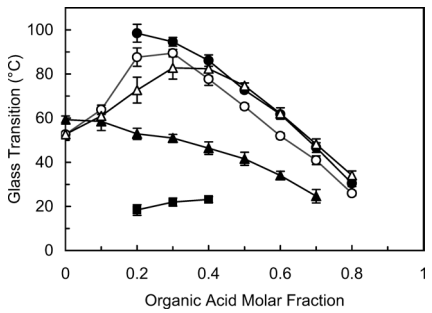

Fig. 5. Glass Transition Temperatures of Freeze-Dried Binary Solids

Each symbol denotes transition of solids containing L-arginine and citric acid (○), L-arginine and tartaric acid (△), L-histidine and citric acid (●), L-glutamine and citric acid (▲), or glycine and citric acid (■) (total: 200 mM, average±S.D., $n=3$).

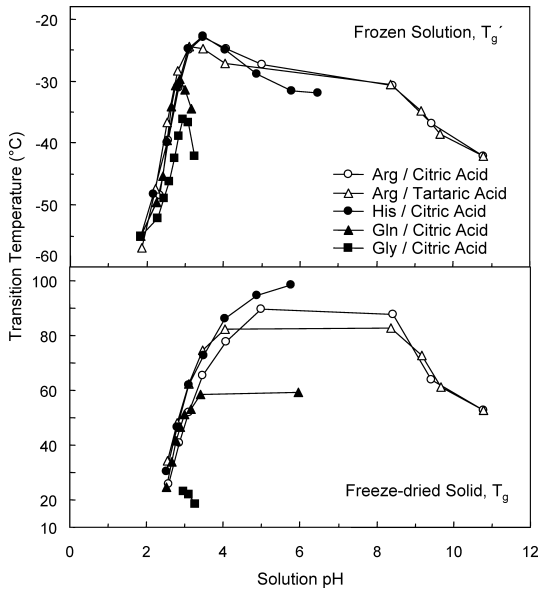

Fig. 6. Effect of Initial Solution pH (25 °C) on the Transition Temperatures of Frozen Solutions (T'_g) and Freeze-Dried Solids (T_g) Containing an Amino Acid and an Organic Acid at a Fixed (0.1) Molar Concentration Ratio Intervals (200 mM Total, $n=3$)

Transition temperatures (T'_g, T_g) of the excipient combinations obtained at a fixed (0.1) molar ratio interval were plotted against the pH of the initial solutions (25 °C, Fig. 6). Some mixtures (*e.g.*, L-arginine and citric acid, L-histidine and citric acid) yielded high T'_g frozen solutions and high T_g freeze-dried solids from weakly acidic initial solutions ($-35\,°C < T'_g$, $80\,°C < T_g$, pH 4—6), which are preferable in parenteral protein formulations. Small changes in the L-arginine and organic acid compositions (0.1 molar fraction) significantly shifted pH at the neutral region.

The mid- and near-infrared spectra of the freeze-dried L-arginine and citric acid combinations showed broad absorption bands that are typical of amorphous solids (Figs. 7, 8).[21] Co-lyophilization with citric acid reduced an amino group absorption band of L-arginine at 1550 cm^{-1} in the mid-IR spectra (KBr method), indicating altered environment of the functional group. Similar reduction of the amino group band has been reported in L-arginine–HCl salt crystal and L-argi-

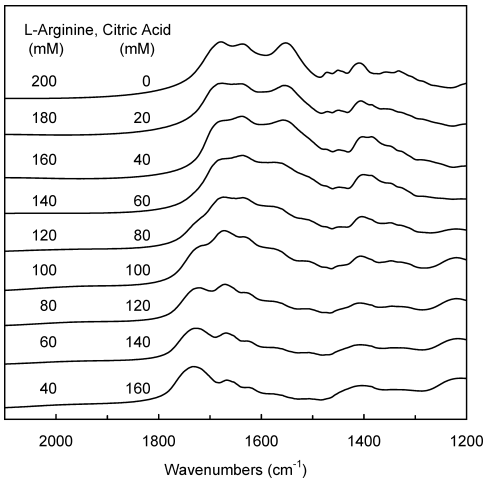

Fig. 7. Mid-Infrared Spectra of Freeze-Dried L-Arginine and Citric Acid Combinations Obtained by a KBr Tablet Method (128 Scans)

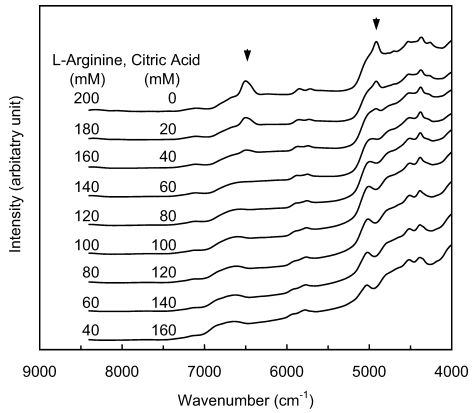

Fig. 8. Diffuse-Reflection Near-Infrared Spectra of Freeze-Dried L-Arginine and Citric Acid Combinations Obtained at the Bottom of the Glass Vials (128 Scans)

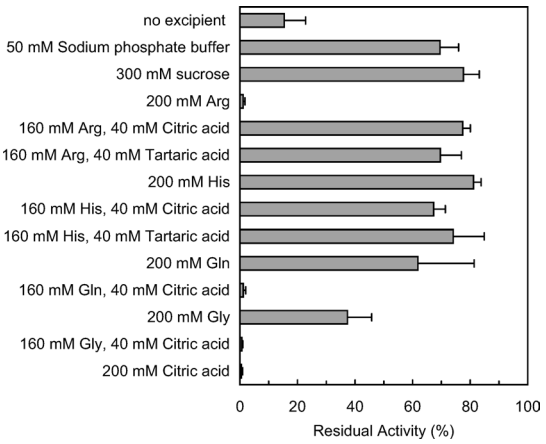

Fig. 9. Effect of Amino Acid and Organic Acid Combinations on the Activity of Freeze-Dried Lactate Dehydrogenase (50 μg/ml, Average±S.D., n=3)

nine freeze-dried with inorganic acids (*e.g.*, HCl, H_3PO_4).[13] A carboxyl group band at 1725 cm^{-1} appeared when the citric acid ratio was increased. Diffuse-reflection near-infrared spectra obtained non-destructively at the bottom of the glass vials also indicated the altered local environment of the functional groups. A large amino band of L-arginine (6505 cm^{-1}, N–H stretching 1st overtone) disappeared in the presence of lower molar concentration ratio of citric acid in the initial solution (140 mM L-arginine, 60 mM citric acid). Increasing the citric acid ratio also reduced the large absorption band at 4920 cm^{-1}, and concomitantly induced band at 5030 cm^{-1} in the co-lyophilized solids. Assignment of these bands remains to be elucidated. The results strongly suggested hydrogen-bonding and/or electrostatic interactions between L-arginine and citric acid in the lyophilized solids.

Effect of Excipients on Inactivation of Freeze-Dried LDH Freeze-drying of LDH in the absence of the stabilizing excipients resulted in significant reduction of the activity (approximately 15% of the initial solution) (Fig. 9). Higher enzyme activity was retained in freeze-drying at a higher phosphate buffer concentration (50 mM). Some amino acid and organic acid combinations that provide neutral to weakly acidic initial solution (pH 5—8) and amorphous dried solids also retained the enzyme activity. The enzyme lost most of the activity in freeze-drying from extreme pH solutions (*e.g.*, 200 mM L-arginine, pH 10.6). Addition of citric acid or L-tartaric acid slightly reduced the effect of L-histidine to retain the activity of LDH during freeze-drying. Crystallization of glycine in the single-solute frozen solution, and concomitant loss of the protecting effect, should explain the lower remaining enzyme activity.[1,2,22]

Discussion

The freeze-drying of aqueous solutions containing some basic or neutral amino acid (*e.g.*, L-arginine, L-histidine) and hydroxy di- or tricarboxylic acid (*e.g.*, citric acid, L-tartaric acid) combinations resulted in the glass-state amorphous solid cakes that protect proteins from dehydration stresses. Some of the solids showed glass transition temperatures comparable to those of disaccharides (*e.g.*, sucrose, trehalose).[4] The data and recent literature on the properties of related substances in different physical states (*e.g.*, complex crystals, ionic liquids) strongly suggested contribution of the multiple functional groups of the consisting molecules to form the interaction (*e.g.*, electrostatic, hydrogen-bonding) networks required for the glass-state amorphous solids.[23—25] Multiple amino, carboxyl, and hydroxyl groups in the solute molecules raise transition temperatures of the mixture frozen solutions (T'_g) and the freeze-dried solids (T_g).[15] The ammonium carbohydrate ion pairs form multiple hydrogen-bondings in some non-polar solvents.[23,24] Differently protonated carboxyl and carboxylate groups also form an intermolecular hydrogen-bonding network.[25]

The amino acids and organic acids containing plural amino or carboxyl groups should have large chance to form the interactions with multiple counterpart molecules. The contribution of the multiple functional groups should explain the high transition temperatures (T'_g, T_g) of the L-arginine and citric acid combination. L-Arginine also forms stable amorphous freeze-dried solids with multivalent inorganic acids (*e.g.*, H_3PO_4).[11,13] Frozen sodium citrate and tartrate buffer solutions exhibit the highest T'_g at certain sodium concentration ratios.[17] Supramolecular interactions (*e.g.*, peptide-like periodic interactions) reported in some complex crystals of amino acid and dicarboxylic acid (*e.g.*, L-arginine and adipic acid, X-ray analysis)[26] should support the possible multi-molecular interaction network in the less-ordered amorphous phase.

Hydroxyl groups in the citric acid, L-tartaric acid, and DL-malic acid should introduce additional hydrogen bonding to the amorphous phase. The number of hydroxyl groups in the component, and the accompanying change in the molecular interactions are major factors in determining the glass transition temperature of some ionic liquids composed of an amino acid and a 1-allylimidazolium cation.[27] The intense interactions and resulting reduction of the molecular mobility may prevent the crystallization of amino acids (*e.g.*, glycine, glutamine) at concentration ratios much lower than those of "inert" nonionic solutes (*e.g.*, sucrose) or inorganic salts (*e.g.*, NaCl).[17,30—32]

The high glass transition temperature amorphous solids formed by combinations of popular excipients would be a practical alternative to disaccharides in the design of freeze-dried protein formulations. The excipient combinations would satisfy the two major protein-stabilizing mechanisms postulated on saccharides, namely substitution of the surrounding water molecules by hydrogen-bonding and reduction of the chemical reaction by embedding in the glass-state solids.[6—8] Additional effects of some amino acids (*e.g.*, reduced aggregation in aqueous solution by L-arginine) preferable in protein formulations are also anticipated.[9] The limited crystallinity and low volatility of the amino acid and organic acid should reduce the risk of pH change and the resulting protein inactivation in the freeze-drying process reported in some buffer systems.[28]

Various proteins degrade during the freeze-drying process and subsequent storage through several chemical and physical mechanisms.[3,29] The low concentration LDH solution is often used as a model system for studying the effect of co-solutes in the freeze-thawing and freeze-drying processes because of its apparent tendency to lose its activity due to irreversible subunit dissociation and conformation change.[30] The ability of excipient combinations to retain the enzyme

activity in the freeze-drying process should indicate the stabilization of the quarterly structure against freeze-concentration and dehydration stress. Different molecular mobility, local pH, water content, and crystallinity of the excipients may affect the chemical degradation rate of the freeze-dried enzyme in the subsequent storage. The freeze-dried basic amino acid and organic acid combination solids should provide the embedded proteins with unique local environments that are significantly different from those of the nonionic excipients (*e.g.*, saccharides). The structural and chemical stability of proteins in these solids during the freeze-drying process and storage is an intriguing topic that needs further study through various model protein and stress systems.

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