Surface Denaturation at Solid-Void Interface—A Possible Pathway by Which Opalescent Particulates Form During the Storage of Lyophilized Tissue-Type Plasminogen Activator at High Temperatures

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During protein lyophilization, it is common practice to complete the freezing step as fast as possible in order to avoid protein denaturation, as well as to obtain a final product of uniform quality. We report a contradictory observation made during lyophilization of recombinant tissue-type plasminogen activator (t-PA) formulated in arginine. Fast cooling during lyophilization resulted in a lyophilized product that yielded more opalescent particulates upon long term storage at 50 °C, under a 150 mTorr nitrogen seal gas environment. Fast cooling also resulted in a lyophilized cake with a large internal surface area. Studies on lyophilized products containing 1% (w/w) residual moisture and varying cake surface areas (0.22 - 1.78 m^2/gm) revealed that all lyophilized cakes were in an amorphous state with similar glass transition temperatures (103 - 105 °C). However, during storage the rate of opalescent particulate formation in the lyophilized product (as determined by UV optical density measurement in the 360 to 340 nm range for the reconstituted solution) was proportional to the cake surface area. We suggest that this is a surface-related phenomenon in which the protein at the solid-void interface of the lyophilized cake denatures during storage at elevated temperatures. Irreversible denaturation at the ice-liquid interface during freezing in lyophilization is unlikely to occur, since repeated freezing/thawing did not show any adverse effect on the protein. Infrared spectroscopic analysis could not determine whether protein, upon lyophilization, at the solid-void interface would still be in a native form.

KEY WORDS: recombinant tissue-type plasminogen activator (t-PA); freezing; lyophilization; cake surface area; opalescent particulates.

INTRODUCTION

Recombinant tissue-type plasminogen activator (t-PA) is an approved drug used for the management of acute myocardial infarction in adults. The efficacy of t-PA is derived from the molecule's ability to convert plasminogen to plasmin, the protease which is able to rapidly dissolve the thrombi obstructing coronary arteries (1). The drug is biosynthesized in the fermentation of Chinese hamster ovary (CHO) cells that have been transfected with DNA encoding the gene for human t-PA. Subsequent manufacturing procedures are used to recover, purify and formulate the protein into a drug product. Finally, the drug product is lyophilized and stored as a solid to preserve protein activity. There are three dosage vial configurations for the lyophilized drug, namely 20, 50, and 100 mg in 20, 50, and 100 mL glass vials, respectively. Reconstitution of the drug with water for injection (WFI) is required prior to intravenous administration to patients. One quality specification for this drug product is that the reconstituted solution must be optically clear. The presence of opalescent protein particulates will disqualify the product for release.

Lyophilization of t-PA, as with other proteins, requires a freezing step as the first stage of the lyophilization process. In freezing a protein solution, the following series of physical events generally occur: supercooling, ice nuclei formation, ice crystal growth, and glass formation (2). During the phase transition of water into ice crystals, protein and excipient molecules become progressively concentrated in the uncrystallized, liquid portion of the system. As the temperature of the system is further reduced, some excipients may also crystallize as pure substances due to their lowered solubility, or as eutectic mixtures with low solubility at that temperature. Accordingly, significant changes in local ionic strength, pH, and protein solubility are highly probable (3). If the protein is susceptible to denaturation caused by these changes, slow cooling, which exposes the protein to the concentrated liquid phase for longer periods and at a higher temperature, should be avoided. In addition, changes in the concentrated liquid phase may not be uniform throughout the system during freezing by slow cooling. As a result, with slow cooling, some parts of the lyophilized cake may contain more excipients and buffer components. If cake uniformity is critical for long term stability, fast cooling is certainly the procedure of choice. Thus, it is understandable why fast cooling with liquid nitrogen has been preferred, and has also become a common practice during lyophilization of some protein solutions (4-8).

Interestingly, in a recent freeze/thaw study, Eckhardt *et al.* (9) observed that fast cooling has a more damaging effect on recombinant human growth hormone in a mannitol/phosphate formulation, especially with regards to insoluble aggregate formation. This phenomenon cannot be explained simply by "cold denaturation," a protein degradation process in which the low temperature alone induces protein unfolding or dissociation of subunits (10-12). The authors hypothesize that the protein may undergo surface denaturation at the ice-liquid interface during the freeze/thaw process. Fast cooling produces small ice crystals which, in turn, provide a relatively large ice-liquid interface, thereby promoting surface denaturation. This study did not explore whether fast cooling might also have additional adverse consequences on the stability of lyophilized growth hormone during storage.

Historically, reports concerning lyophilized protein products emphasize a dependence between protein stability and product residual moisture content (13-17). Hageman (15)

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proposed that with a high residual moisture content, the possibility of chemical reactions occurring (e.g. cleavage, oxidation, deamidation, denaturation and aggregation) is increased. Greiff (16) suggested that a minimum moisture content is necessary to protect the protein. More recently, a study by Hsu *et al.* (17) noted that an optimum residual water content, approximately 6 to 7% (w/w) is required to balance the physical stability (both insoluble aggregate and soluble aggregate formation) and the biological stability (*in vitro* activity) of lyophilized, excipient-free t-PA.

Recently, the physical state of the lyophilized cake was proposed to be a crucial factor in protein stability as well. Franks *et al.* (18,19) hypothesized that degradation of lyophilized protein during storage depends upon the ability of protein molecules to diffuse and react with each other or with reactants (impurities, salts, etc.). An amorphous lyophilized cake with a high glass transition temperature is believed to constrain the protein more tightly, thereby minimizing the protein's reactivity. Roy *et al.* (20) successfully applied the theory to explain the stability results of a lyophilized monoclonal antibody conjugate.

More recently, hydrogen bonding interactions between protein and excipient were demonstrated to be another factor in stabilizing a lyophilized protein. With the use of infrared spectroscopy. Carpenter *et al.* (21-23) concluded that trehalose and lactose act as water substitutes by satisfying the hydrogen-bonding requirements for maintaining proteins in a native state.

While literature exists on the effects of residual moisture, physical state, and protein-excipient interaction, we did not find reports describing the effect of internal surface area of the lyophilized cake on protein stability. In the present study of the lyophilization of t-PA, we found that fast cooling during lyophilization resulted in a less stable product. During storage at elevated temperature, opalescent particulates formed faster in a product prepared by fast cooling than in one prepared by slow cooling. We also observed that fast cooling resulted in a lyophilized cake with a large internal surface area, and that the increase in the rate of opalescence change appeared strongly dependent on this cake surface area. All lyophilized cakes used in this study contained similar amounts of residual moisture and similar glass transition temperatures. We hypothesize that the formation of opalescent particulates in lyophilized t-PA during storage at elevated temperatures is a surface-related phenomenon in which protein denatures at the internal surface of the cake. This paper describes our observations and interpretations of the phenomenon. We hope this case study could add more information to aid in the understanding of protein denaturation in the lyophilized state.

MATERIALS AND METHODS

Protein Source

The t-PA protein was produced at Genentech Inc. It is a glycoprotein of 527 amino acid residues. The molecule exists as two differently glycosylated variants, Type I and Type II. Type I is glycosylated at asparagine residues 117, 184, and 448. Type II is glycosylated at residues 117 and 448. CHO cells produce approximately equal levels of Type I and Type II. Details of the molecular structure are described in the literature (24, 25). Aqueous protein bulk (2.5 mg/mL) was formulated with 0.5 *M* L-arginine (Ajinomoto, USP grade) and 0.004% (w/v) polysorbate 80, adjusted with phosphoric acid to a pH of 7.3 ± 0.1.

Lyophilized Samples

Samples were prepared by lyophilizing the formulation in 20, 50, and 100 mL glass vials (Wheaton) using a 20 ft^2 freeze-dryer (Leybold Heraeus, Model GT20). Fill volumes were 8.2, 20.6, and 41.2 mL and fill heights were 1.2, 1.7, and 2.2 cm for the 20, 50, and 100 mL vials, respectively. Total content of the t-PA protein was 20.5 mg in each 20 mL vial, 51.5 mg in each 50 mL vial, and 103 mg in each 100 mL vial. In the normal cooling operation, filled vials were loaded into the dryer and equilibrated at 5 °C for 1 hour, then cooled down by the silicone oil circulated in the dryer's shelves. Silicone oil temperature was ramped down from 5 to −55 °C at a constant rate of 0.5 °C/min. In the fast cooling operation, vials were loaded into the dryer prechilled with −55 °C silicone oil coolant. In the very fast cooling operation, vials were cooled by immersion in a dry ice/isopropanol bath. When vial contents reached a temperature of −55 °C, the frozen vials were removed from the cooling bath and loaded into the dryer prechilled with −55 °C silicone oil coolant.

Temperature of the vial contents was measured by a thermocouple placed vertically through the center of the vial opening. The tip of the thermocouple was positioned at 5 mm below the meniscus of the vial contents. Using the approach proposed by Hartmann *et al.* (26), cooling rate was calculated by dividing the temperature difference between the temperature at which ice formation occurred (see explanation in RESULTS AND DISCUSSION) and −40 °C by the number of minutes that it took to go between these two temperatures. In a separate experiment, cooling rate was also measured at various locations in the vial. Using the method proposed by Hartmann, it was concluded that cooling rates measured at 5 mm below the meniscus of the vial contents represented at least 80% of the contents in each vial. This representative value met the criteria described by Hartmann for a uniform cooling operation, indicating that the contents in each vial were cooled evenly.

After freezing, all vials were held in the dryer at temperatures between −50 and −55 °C for 6 to 7 hours before proceeding with lyophilization. Lyophilization was conducted at a lyophilizer chamber pressure of 150 mTorr nitrogen (Linde, ultra-pure grade) and a product temperature of −35 °C for 48 hours, followed by secondary drying at 40 °C for 12 hours. The duration of secondary drying was 12, 14, and 24 hours for the 20, 50, and 100 mL vials, respectively.

After lyophilization, all product vials were sealed under 150 mTorr nitrogen. The residual moisture content of the lyophilized cakes was determined by the Karl-Fischer method (Metrohm, Model 658) and expressed as a weight percentage, % (w/w), based on the total weight of the lyophilized cake in the vial.

To determine whether impurities present in the gas used during sealing of the vials would have an adverse effect on the lyophilized product, a set of the above sealed vials were again placed in a lyophilizer, equilibrated with nitrogen at 750 Torr and restoppered under that pressure. Vials before and after this nitrogen reequilibration had the same residual moisture content.

Cake Surface Area

The Quantasorb System (Quantachrome, Model QS-15) was used to determine the specific surface area of the lyophilized cakes. Sample cores were cut from a lyophilized cake by inserting a rubber stopper borer into the lyophilized vial in the vertical direction. Each core measured approximately 0.8 cm in diameter and 2 cm in height. Three of these cores were weighed and placed in the sample cell of the system. The loaded cell was outgassed at 37-40 °C with helium gas (Linde, ultra-pure grade) at a rate of 30 to 40 mL/min overnight. After outgassing, a gas mixture containing a known concentration of krypton in helium (0.015 to 0.101% v/v) was passed through the cell at 20 mL/min to allow the krypton atoms to adsorb onto the cake in a liquid nitrogen cooled environment. The adsorption process was repeated five times for each helium/krypton gas mixture. The value of the cake internal surface area was determined by calculation of the area covered by a monolayer of adsorbed krypton from the Brunauer-Emmett-Teller (B-E-T) equation (27).

Cake Structure

The lyophilized sample was placed on a 1 cm^2 glass slide and scanned by a Cu X-ray powder diffractometer from a 10° to 100° diffraction angle at intervals of 0.1° every 4 seconds. The diffraction data were compared with the X-ray diffraction patterns of raw L-arginine powder and lyophilized arginine cake. Lyophilized arginine was prepared in the 20 mL vial using the same fill volume, normal cooling condition and lyophilization cycle as those for the preparation of lyophilized t-PA product in the 20 mL vial.

Cake T_g

The glass transition temperature (T_g) measurements for lyophilized samples were carried out in a Perkin-Elmer Model 7 differential scanning calorimeter (DSC). An aluminum sample pan was filled with approximately 11 mg of a lyophilized sample and then sealed with a pan cover. An empty pan was sealed with the same type of pan cover as the reference. The sealed pans were placed in the appropriate cells within the DSC unit. Helium was directed through the cells to provide an inert environment. After thermal equilibration at 30°C, the DSC unit was heated at a constant rate of 20 °C/min. During heating, the thermogram (heat flux through the sample cell versus temperature) was recorded. T_g was taken as the mid-point in the thermogram as measured from the extensions of the pre- and post-transition baselines.

Infrared Spectroscopy

Infrared spectroscopic analysis was performed to determine whether different cooling conditions in lyophilization would result in any change in the lyophilized t-PA protein. The analysis was conducted using a Nicolet Fourier-Transform Infrared Spectrometer (Model 5DXB). Approximately 3 mg of each lyophilized sample was mixed with 300 mg of dry infrared grade KBr, and ground to a fine powder with an agate mortar and pestle. Approximately 200 mg of the resulting powder was placed in a casting die and pressed with a 20,000 psi pressure into a disk. The disk was scanned by the spectrometer. Spectra of lyophilized protein samples were subtracted from the spectrum of lyophilized arginine excipient for data analysis.

Product Stability

Lyophilized product vials were stored at 50 °C for sixteen months. At selected time points, two vials were reconstituted with WFI to 1 mg/mL protein concentration for all the assays described below.

Opalescense of the reconstituted protein solution was determined according to the UV spectroscopy method of Eckhardt *et al.* (9, 28). Briefly, each reconstituted solution was placed in a Kontron Uvikon 860 double beam spectrophotometer and scanned from 360 to 340 nm. The mean UV optical density over this wavelength range was recorded in OD/cm. In comparison with the optical density of opalescence reference suspensions described in 1980 European Pharmacopeia V.6.1, all solutions exhibiting an optical density less than 0.014 OD/cm were classified as clear. Solutions with an optical density from 0.014 to 0.029 OD/cm were classified as slightly opalescent, from 0.029 to 0.087 OD/cm as opalescent, from 0.087 to 0.144 OD/cm as a very opalescent, from 0.144 to 0.201 OD/cm as slightly cloudy, from 0.201 to 0.568 OD/cm as cloudy, and greater than 0.568 OD/cm as very cloudy.

The mass of opalescent particulates in each reconstituted solution was determined by measuring the difference in protein concentration (Kontron Uvikon 860, UV detection at 277 nm) of the reconstituted sample before and after centrifugation (Beckman AccuSpin FR, Rotor AA-24 at 3000 rpm for 20 minutes) and filtration (Millipore, Millex-GV 0.22 μm). Particulates of size less than 0.22 μm were not necessarily removed by the filtration.

The percentage of soluble protein aggregates in each reconstituted solution was determined by size-exclusion HPLC (UV detection at 214 nm) using a silica-based Tosoh TSKG3000SWXL column (7.8 mm ID × 30 cm L, particle size 5 μm) (17). Typically, 5 μL of each filtered, reconstituted sample was loaded onto the column and eluted with mobile phase consisting of 0.2 *M* sodium phosphate monobasic and 0.1% (w/v) sodium dodecyl sulfate (SDS), pH 6.8, at a flow rate of 0.5 mL/min. SDS was added to the mobile phase in order to prevent strong hydrophobic binding of the protein to the column. This analysis therefore only determines the percentage of soluble, nondissociable aggregates. The absolute error in this assay is usually less than 1%.

The *in vitro* clot lysis activity of the t-PA samples was determined by dissolution of a fibrinogen and plasminogen clot monitored by UV absorbance at 340 nm. The dissolution time of the samples was compared to the dissolution time of a t-PA standard (29). The relative errors in this assay are typically ±10%.

Freeze/Thaw Study

In the study to determine whether freezing would induce the denaturation of t-PA, two formulated t-PA solutions were separately frozen, one to −55 °C in a temperature chamber (Planer Biomed Kryo 10) at a constant cooling rate of 5 °C/min, while the other was quick frozen in a dry ice/isopropanol bath. Both were then thawed at a constant heating rate of 5 °C/min in the temperature chamber. This freeze/thaw cycle was repeated three times. After the third cycle, optical density of the solutions, soluble aggregate content, as well as the *in vitro* bioactivity of the protein were determined.

DSC for Frozen Solution

DSC measurements were carried out to ensure that the concentrated phase in all t-PA solutions subjected to different cooling conditions was in a glassy state at temperatures below −40 °C. The measurements were conducted in the same DSC instrument as that used for the determination of T_g of the lyophilized cake. Approximately 10 μL of the formulated t-PA solution was sealed in a sample pan. The pan was equilibrated at 10 °C in the DSC unit and then cooled at a constant rate of 0.2, 1, 5, or 50 °C/min to −55 °C. After the sample reached −55 °C, it was held at that temperature for 15 minutes, then warmed at 20 °C/min to 20 °C. From the thermogram obtained during heating, glass transition temperature of the freeze-concentrated phase in frozen sample, referred to as $T_{g'}$ in literature, was determined.

For the case of very fast cooling, a t-PA solution was sealed in a sample pan and cooled by touching the pan bottom to a dry ice/isopropanol bath (−75 °C) for one minute. Cooling rate was measured in a separate experiment using a thermocouple (0.025 mm OD) imbedded in the sample pan. After cooling, the pan was quickly transferred to the DSC unit that had been precooled to −55 °C. After sample transfer, the DSC was held at −55 °C for 15 minutes and then operated by the same procedure described above.

RESULTS AND DISCUSSION

Figure 1 shows the temperature changes for formulated t-PA solutions in 20, 50, and 100 mL vials under different cooling conditions. When cooled at 0.5 °C/min (the lyophilizer shelf temperature ramping rate), the contents of all vial sizes underwent supercooling in the early phase of cooling. Supercooling ended at approximately −10 °C with a sudden change in the appearance of the vial contents from a clear liquid to an opaque/translucent ice slush. This instantaneous ice formation released a significant amount of latent heat that could not be immediately dissipated by cooling. As a result, the temperature of the vial contents increased sharply. After the ice formation was complete, the temperature decreased continuously. This decrease appeared to follow an S-shaped profile with the inflection point occurring between −25 and −35 °C. The 20 mL vial had the fastest temperature response during the entire course of cooling because this vial had the thinnest wall for heat transfer and the least volume for cooling. Figure 1 also indicates that supercooling could not be determined by temperature measurements when a t-PA solution was cooled in dry ice/isopropanol or by the prechilled lyophilizer. The temperature of the vial contents remained between −1 and −2 °C for a period of 3 to 15 minutes, then decreased sharply toward the temperature of cooling medium. In this case, ice formation was visually observed to start during this constant temperature period.

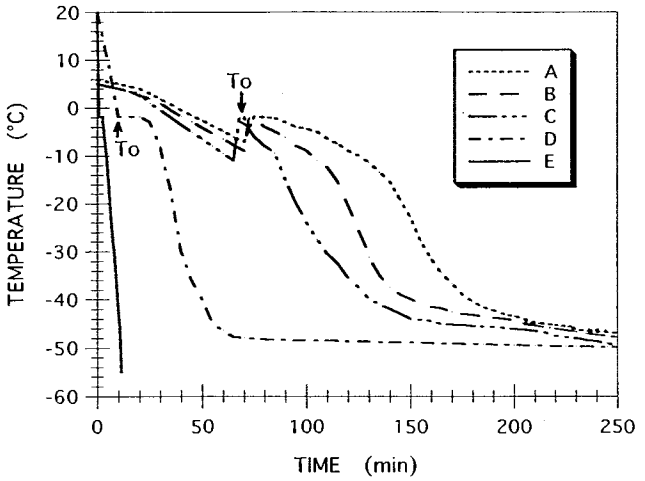

Figure 1. Typical cooling curves of L-arginine formulated t-PA solution. A: 100 mL vial in lyophilizer at a 0.5 °C/min shelf temperature ramping rate. B: 50 mL vial in lyophilizer at a 0.5 °C/min ramping down rate in silicone oil coolant. C: 20 mL vial in lyophilizer at a 0.5 °C/min ramping down rate in silicone oil coolant. D: 20 mL vial by prechilled lyophilizer (−55 °C silicone oil coolant temperature), E: 100 mL vial in dry ice/isopropanol. Fill volumes were 8.2, 20.6, and 41.2 mL for the 20, 50, and 100 mL vials, respectively. Temperature of the vial contents was measured by a thermocouple positioned at 5 mm below the meniscus in each vial. T_0 is defined in text.

As seen in Figure 1, the temperature of the system did not decrease at a constant rate during cooling. For purposes of comparison, a cooling rate was calculated by dividing the temperature difference between T_o and −40 °C by the number of minutes that it took to go between these two temperatures. In the case of supercooling, T_o was defined as the maximum temperature reached after supercooling. In the case of nonsupercooling, T_o was defined as the temperature at which the constant temperature period started. The −40 °C temperature was selected based on the following two reasons. First, the results of the DSC analysis indicate that the freeze-concentrated phase in a t-PA solution is transformed from fluid into glass at −28.9 ± 0.4 °C (Figure 2). Thus, the selected temperature is far below the glass transition point of

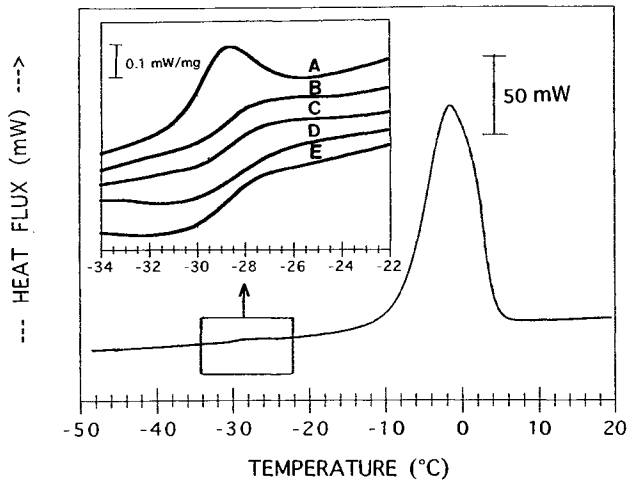

Figure 2. DSC thermogram of frozen t-PA solution upon warming at 20 °C/min, illustrating the magnitude of the glass transition in freeze-concentrated phase relative to the melting endotherm of ice. The inset shows the effect of cooling rate on the glass transition. Sample A was cooled in DSC at 0.2 °C/min, $T_g' = -29.5$ °C. Sample B was cooled in DSC at 1 °C/min, $T_g' = -29.1$ °C. Sample C was cooled in DSC at 5 °C/min, $T_g' = -28.6$ °C. Sample D was cooled in DSC at 50 °C/min, $T_g' = -28.6$ °C. Sample E was cooled in dry ice/isopropanol bath at 274 °C/min, $T_g' = -28.6$ °C. Sample A exhibits an endothermic enthalpic relaxation during the T_g' transition, indicating that slowing cooling increases the extent of structural relaxation in the glassy state.

the system. The second reason is for improved reproducibility; during cooling, the change in temperature with time (shown in Figure 1) at temperatures below −40 °C is relatively small, resulting in a large variability in the determination of the final time point.

The DSC results shown in Figure 2 also revealed that the freeze-concentrated phase in the frozen sample prepared with the slowest cooling rate (0.2 °C/min) underwent an endothermic enthalpic relaxation during glass transition. This observation is consistent with the results reported for the frozen aqueous solutions of various sugars (30). At slow cooling rates, there is more time for molecules in the freeze-concentrated phase to relax to lower-energy conformations and minimize the "free volume" in the phase. Consequently enthalpy of the system is reduced. When the system is heated up from glass to fluid, it must suddenly absorb energy from the environment to compensate for the energy of relaxation at the glass transition. This explains why an endothermic enthalpic relaxation was seen in the frozen solution prepared by slow cooling (30). Whether this energy relaxation could affect the t-PA protein is yet to be determined.

Nine sets of lyophilized t-PA samples (four in 20 mL vials, two in 50 mL vials, and three in 100 mL vials) were prepared for a product stability study. Cooling conditions used in the preparation of these samples are summarized in Table I. All samples were dried to a 1% (w/w) residual moisture. Figure 3 presents the stability data of samples prepared by a cooling rate of 0.61, 11.28, and 21.78 °C/min. As expected for a lyophilized protein product exposed to high temperatures, t-PA formed soluble dimer aggregates and the bioactivity of the protein decreased in all samples after storage

TABLE I. Summary of cooling conditions used in preparing lyophilized t-PA samples for the stability study

Vial size mL	Protein content mg /vial	Cooling rate (°C/min) Normal cooling[a]	Fast cooling[b]	Very fast cooling[c]
20	20.5	0.61	0.96	21.78 8.12[d]
50	51.5	0.49		11.28
100	103.0	0.37	0.56	4.36

[a] Conducted by ramping down the temperature of silicone oil coolant in lyophilizer at 0.5 °C/min.
[b] Conducted by loading vials in a lyophilizer prechilled by -55 °C silicone oil coolant.
[c] Conducted by immersing vials in a dry ice/isopropanol bath. Unless specified, the bath contained 1300 mL of isopropanol and 1516 grams of dry ice. Bath temperature was -75 °C.
[d] Temperature bath contained 1300 mL of isopropanol and 520 grams of dry ice. Bath temperature was -62 °C.

for sixteen months at 50 °C. As seen in Figure 3, the time courses of soluble aggregate formation and loss in clot lysis activity for the samples prepared by fast cooling rate were essentially identical to those for the sample prepared by slow cooling rate, suggesting that faster cooling does not influence these routes of degradation during product storage.

The most interesting observation in the stability study was the effect of cooling rate during lyophilization on the formation of opalescent particulates during storage. Protein particulates can arise from denaturation of the protein, leading to insoluble species. Upon product reconstitution, these particulates often form a hazy solution due to scattered light. The degree of turbidity caused by opalescent particulates may be quantitatively assessed as an increase in optical density over a wavelength region known not to contain chro-

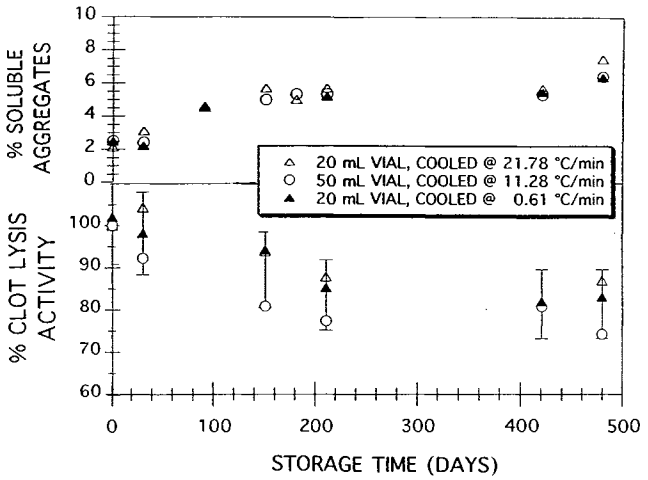

Figure 3. Protein soluble aggregate (dimer) content and *in vitro* clot lysis activity of t-PA lyophilized products upon storage at 50 °C. Vial sizes and cooling rates for preparing the samples are shown in legend. Each point represents a mean of at least two determinations. Relative errors in clot lysis assay were ±10%. (-I-) denotes the mean of all measurements from the samples with a 10% relative error.

mophoric absorption (28). For most protein solutions the average absorption in the spectral range from 360 to 340 nm fulfills these requirements (28). It should be noted that, although the amount of optical density may be quantified, these values are not necessarily linearly dependent upon the mass of the insoluble particulates. Figure 4 shows the relationship between the optical density and the amount of t-PA particulates in solution. The particulates were isolated by centrifugation at 3000 rpm and removed by a 0.22 μm filter. As seen in Figure 4, an amount of only 0.01 mg/mL of protein particulates was sufficient to cause the reconstituted t-PA solution to become "very opalescent" or "slightly cloudy". The figure also demonstrates that mass determination by centrifugation and filtration is extremely variable for solutions exhibiting an optical density value less than 0.15 OD/cm. Thus, particulate levels are reported using optical density values only. Figure 5 shows optical density measurements for reconstituted samples that were prepared using 0.61, 11.28, and 21.78 °C/min cooling rates during lyophilization. In all samples, optical density increased with increasing storage time. Data were analyzed by linear regression in order to quantify the rate of increase in optical density. In all cases, the correlation coefficient was greater than 0.93. Thus, the rate of increase in optical density was determined by the slope of the fitted curve. Results are plotted against cooling rate in Figure 6. This figure indicates that, for each vial size, increasing the cooling rate during lyophilization operation results in increased opalescent particulate formation during storage at elevated temperatures.

An important question to address then, was whether the freezing step alone was responsible for protein degradation which would lead to the protein particulate formation. To investigate this possibility, a freeze/thaw study of the formulated t-PA bulk was conducted. One protein solution was

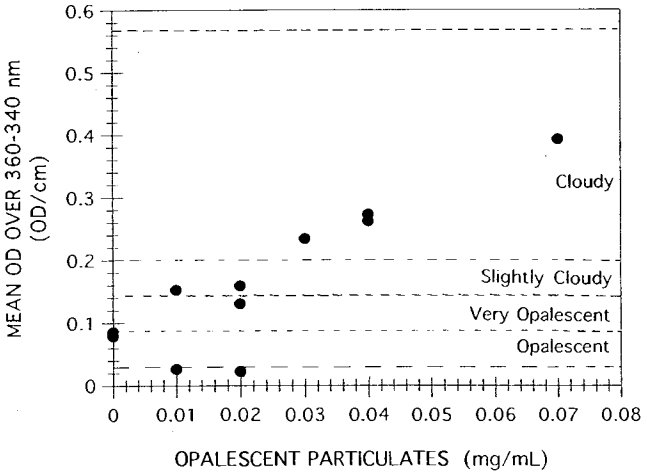

Figure 4. Optical density (mean over 360 to 340 nm range) of the reconstituted solution of lyophilized t-PA sample versus the amount of insoluble particulates isolated from each solution by centrifugation at 3000 rpm followed by filtration using a 0.22 μm filter. OD less than 0.014 OD/cm was classified as clear, 0.014 to 0.029 OD/cm as slightly opalescent, 0.029 to 0.087 OD/cm as opalescent, 0.087 to 0.144 OD/cm as very opalescent, 0.144 to 0.201 OD/cm as slightly cloudy, 0.201 to 0.568 OD/cm as cloudy, and greater than 0.568 OD/cm as very cloudy.

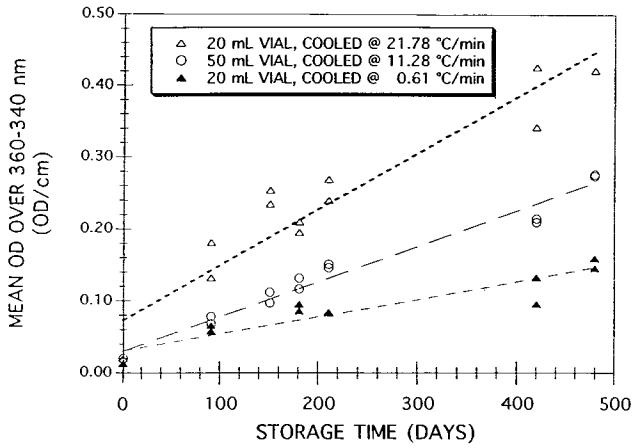

Figure 5. Optical density (mean over 340 to 360 nm range) for reconstituted solutions of lyophilized t-PA products upon storage at 50 °C. Vial sizes and cooling rates for preparing the samples are shown in legend. Straight lines represent least square linear regression of data.

frozen to −55 °C in a temperature chamber at a constant cooling rate of 5 °C/min. Another protein solution was quickly frozen in the dry ice/isopropanol bath. Both were then thawed at a constant heating rate of 5 °C/min in the temperature chamber. After three freeze/thaw cycles, all liquid samples appeared clear, with a mean optical density (360 to 340 nm) of 0.0031 ± 0.0006 and 0.0032 ± 0.0002 OD/cm (n = 5), respectively. This OD value is not significantly different from that of the samples before the freeze/thaw runs. Dimer content and *in vitro* bioactivity of the protein also remained unchanged through these freeze/thaw cycles. These results imply that t-PA was not denaturated simply by freezing and that fast cooling alone was not the factor causing the increase in opalescent particulate formation.

Another hypothesis was whether lyophilization by different cooling rates could yield product cakes with different physical states (crystalline or amorphous) which, in turn, could affect the stability of the lyophilized protein. An X-ray

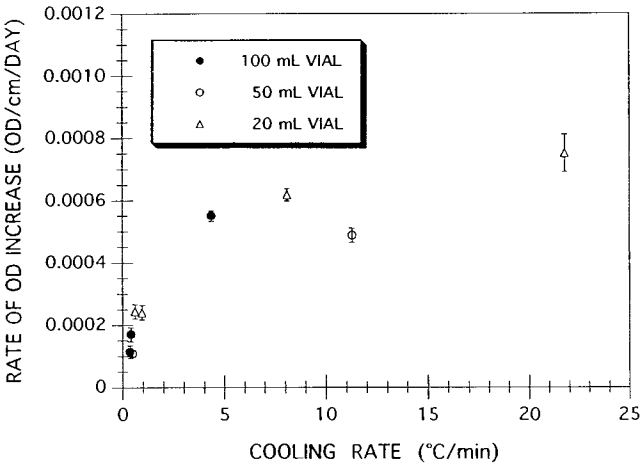

Figure 6. Dependence of rate of increase in optical density (mean over 360 to 340 nm) on cooling rate used in preparation of the lyophilized t-PA product.

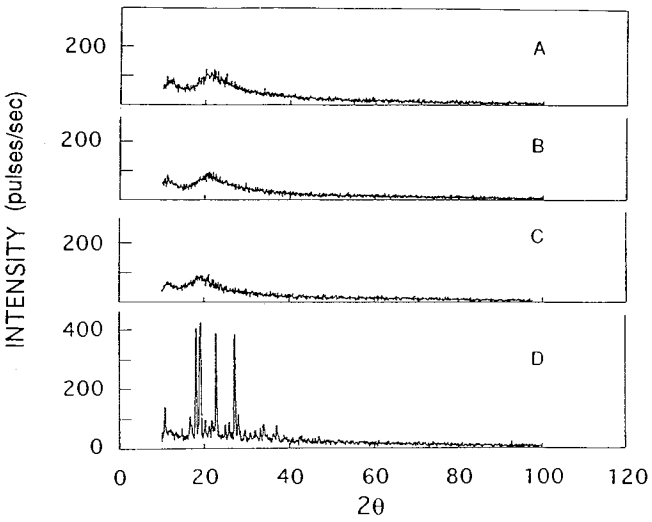

Figure 7. X-ray powder diffraction patterns of lyophilized arginine excipient and t-PA product, as well as raw arginine powder. Sample A was lyophilized t-PA product, prepared in a 20 mL vial by dry ice/isopropanol cooling at 21.78 °C/min. Sample B was lyophilized t-PA product, prepared in a 20 mL vial by normal cooling at 0.61 °C/min. Sample C was lyophilized arginine excipient, prepared in a 20 mL vial by normal cooling at 0.61 °C/min. Sample D was raw arginine powder.

powder diffraction study on the lyophilized cakes was conducted and is shown in Figure 7. Results suggest that, despite being prepared by different cooling rates, all lyophilized cakes were in an essentially amorphous state. The DSC study revealed that all amorphous cakes exhibit a glass transition at temperatures between 103 and 105 °C, and during the phase transition a slight endothermic enthalpic relaxation occurs in the lyophilized sample prepared by slow cooling during lyophilization (Figure 8). As discussed in the DSC results of frozen t-PA solutions, at slow cooling rates, there is more time for molecules in the freeze-concentrated phase to relax to lower-energy conformations. These molecules remain in the lower-energy conformations after lyophilization, and upon heating to the glass transition temperature they absorb energy from the environment and thus show an endothermic enthalpic relaxation in DSC. This is the only difference found in the physical state of lyophilized t-PA product cakes prepared by various cooling conditions during lyophilization.

A Fourier-transform infrared (FTIR) spectroscopic study was used to determine whether different cooling rates during lyophilization would result in any difference in protein structure in the lyophilized state. Applying the solid sample preparation method used in most FTIR spectroscopic studies, each lyophilized t-PA cake was ground in an agate mortar, mixed with KBr and compressed into a disk for analysis. The method is considered to be nondestructive to a lyophilized protein, for the protein still remains in the solid state. Results (spectra not shown) revealed that all lyophilized t-PA products and the arginine excipient exhibited identical spectra, suggesting that the arginine component (approximately 98 % w/w) in the t-PA product dominated the IR absorption over the whole spectral range. Thus, determination of the FTIR spectrum of t-PA could not be measured in the formulation matrix.

In studying various physical properties of the lyophilized t-PA product, it was found that a faster cooling rate during lyophilization resulted in a larger internal surface area of the lyophilized cake (Figure 9). Lyophilization is an ice sublimation process. The vast internal surface area in a lyophilized product results from the porous cake structure left by the ice sublimation. Ice crystals are formed in the protein solution during cooling. Different cooling conditions can create different ice crystal sizes and populations. Small ice crystals may grow to larger sizes at the expense of smaller crys-

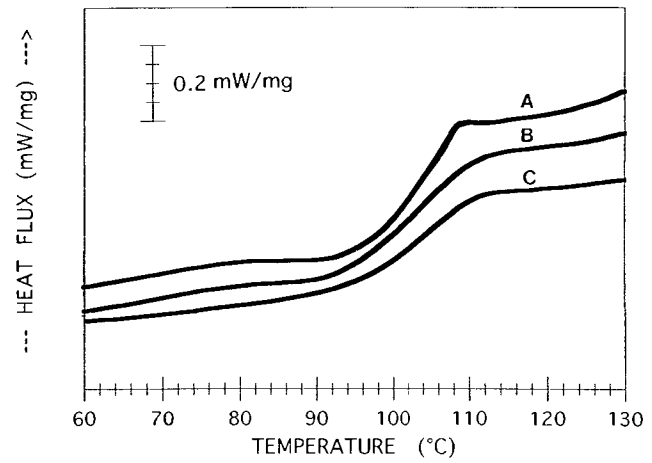

Figure 8. The effect of cooling rate during lyophilization on the DSC thermogram of lyophilized t-PA product. During DSC analysis, all samples were warmed at a constant rate of 20 °C/min. All thermograms were normalized based on 1 mg sample weight. Sample A was prepared in a 50 mL vial by normal cooling at 0.49 °C/min, T_g = 103.8 °C. Sample B was prepared in a 20 mL vial by prechill cooling at 0.96 °C/min, T_g = 102.9 °C. Sample C was prepared in a 20 mL vial by dry ice/isopropanol cooling at 21.78 °C/min, T_g = 104.9 °C. Sample A exhibits a slight endothermic enthalpic relaxation during the glass transition.

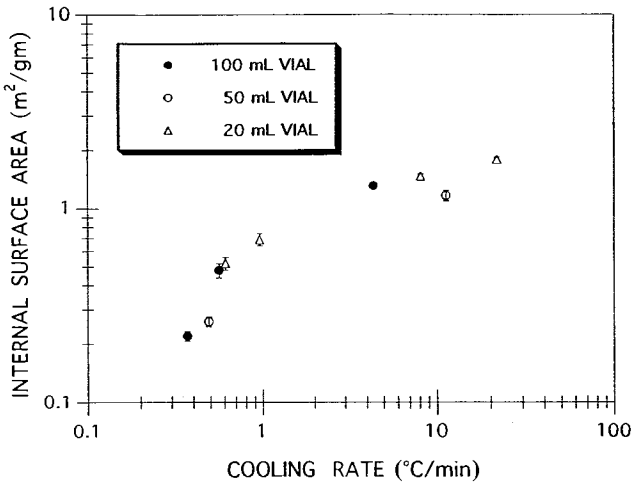

Figure 9. Lyophilization cooling effect on the cake internal surface area of t-PA products. Each point represents the determination from 15 gas adsorption measurements (3 gas mixtures and 5 replicates for each gas mixture).

tals. To understand the phenomenon of ice crystal growth in the t-PA solution would require detailed kinetic studies on solution super cooling and ice nuclei formation and is beyond the scope of this paper. Despite the possibility for migratory recrystallization of ice, which we recognize, it is evident that faster cooling leads to a larger internal surface area of the lyophilized cake for the t-PA solution frozen in each vial size.

Interestingly, replotting the data shown in Figure 6 versus the internal surface area of lyophilized cakes yields a linear correlation between the rate of opalescent particulate formation and the cake surface area (Figure 10). This correlation strongly suggests that the rate of increase in optical density is dependent on the surface area, and that opalescent particulate formation will be minimized if a lyophilized cake contains a very small surface area.

One concern regarding this surface-related phenomenon was whether trace impurities (e.g. oxygen) in the nitrogen gas used for pressure regulation during lyophilization could induce protein degradation and lead to protein particulate formation. The same nitrogen was also used as a vial seal gas. If such a gas-solid type of reaction did occur, it might help to explain why the rate of protein particulate formation was proportional to the internal surface area of the lyophilized cake. To investigate this possibility, a set of lyophilized samples were divided into two groups. The long term stability of a group sealed under nitrogen at 150 mTorr was compared with that of the other group sealed under nitrogen at 750 Torr. Both groups contained a cake surface area of 1.46 ± 0.04 m^2/gm and a residual moisture content of 1%. If gas impurities could react with the protein, the 750 Torr sample, which contained more nitrogen and therefore more impurities, should show a faster rate of opalescent particulate formation. Such an adverse effect, however, was not found in this study, suggesting that the opalescent particulate formation is not related to gas impurities.

The precise mechanism of insoluble aggregate formation, including that of opalescent particulates in lyophilized protein products is still unknown. Aggregate formation in protein solutions, however, has been examined in detail (31). Aggregates may form through either covalent and/or noncovalent interactions between protein molecules. Covalent bonds can be formed by esterification of the carboxyl groups in aspartic acid and glutamic acid side chains with the hydroxyl groups of serine or threonine residues. Interaction can also occur by amidation of these same carboxyl groups with the free amino groups of lysine or with the N-terminus. Linkage between free SH groups, or intermolecular disulfide bond interchange can also result in extensive covalent aggregation. In the case of glycoproteins, the carbohydrate groups in one molecule may cross-link with the amino groups of lysine on another molecule. Noncovalent interactions may also occur between hydrophobic sites of adjacent protein molecules, which can lead to the formation of noncovalent aggregates. In some cases, aggregation may become quite extensive, resulting in the production of insoluble oligomers. The protein can also unfold at the gas-liquid, liquid-solid, or even ice-liquid interfaces. These conformational changes may facilitate aggregate formation.

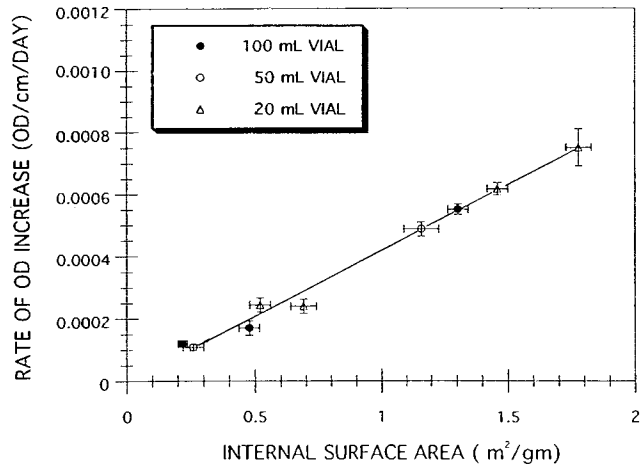

Figure 10. Dependence of rate of increase in optical density (mean over 360 to 340 nm) on cake internal surface area of a lyophilized t-PA product. Each point is presented by the mean ± standard error of cake surface area and the mean ± standard error of rate of optical density increase. Straight line ($Y = -2.26 \times 10^{-6} + 4.20 \times 10^{-4} X$, $R = 0.995$) is the least square linear regression of all mean values.

The t-PA molecule contains 527 amino acid residues of which 207 contain hydrophilic side groups (hydroxyl, carboxyl, amino and quanidino). However, not all of these hydrophilic groups are available for intermolecular interaction at the surface of the folded t-PA molecule. The lyophilized t-PA product used in this study contains approximately 98% (w/w) arginine phosphate as an excipient and 2% (w/w) protein. This is equivalent to one mole of protein to 1.2×10^4 moles of arginine. In lyophilized form, the protein is immobilized in the excipient cake and is incapable of significant diffusion. While still in solution prior to freezing, the protein will usually become concentrated during ice crystal growth. It is plausible that some t-PA molecules may diffuse to the ice-liquid interface during the concentrating step. These protein molecules remain at the interface between the solid cake and the voids after lyophilization, and are thereby less protected by the arginine excipient. During storage at elevated temperatures, these "interfacial" protein molecules slowly denature irreversibly and, when reconstituted, immediately aggregate to form opalescent particulates. Faster cooling yields a lyophilized product with a larger internal surface area where more protein molecules are exposed to this progressive surface denaturation. This mechanism may explain why opalescent particulate formation on storage is proportional to the total surface area of the solid-void interface in the lyophilized cake.

CONCLUSIONS

Freezing, the first step in the lyophilization process, plays an important role in determining the cake structure of a lyophilized protein product. A faster cooling operation results in a larger dry cake surface area for an arginine-formulated t-PA product. Stability studies using lyophilized t-PA samples containing 1% (w/w) residual moisture indicate that soluble aggregates increased and product bioactivity decreased during storage at 50 °C. These two degradation events were not enhanced in t-PA products that contain a

larger cake surface area. However, in sharp contrast, opalescent particulate formation (as detected by the rate of increase in optical density of the reconstituted solution) appeared to be proportional to the internal surface area of the lyophilized cake. This suggests that soluble aggregate (t-PA dimer) and insoluble aggregate (t-PA opalescent particulates) formation are separate events with different mechanisms. This surface area-related phenomenon was shown not to be caused by an interaction between the lyophilized protein and seal gas impurities, including oxygen. The fact that all lyophilized samples under study were in an amorphous state and exhibited similar glass transition temperatures leads us to believe that this surface area-related phenomenon is not due to differences in the physical state of the lyophilized cake. Irreversible denaturation during freezing was not found to occur, since repeated freezing/thawing did not show any adverse effect on the protein. Infrared spectroscopic analysis could not determine whether protein, upon lyophilization, at the solid-void interface would still be in a native form. We suggest that t-PA molecules present at the interface between the solid cake and the voids within the cake are less protected by the arginine excipient, and thus are more prone to irreversible denaturation. These denatured molecules then interact to form insoluble particulates during reconstitution. This is a possible mechanism by which opalescent particulates form during the storage of the t-PA product at elevated temperatures.

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