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*Research Article*

# Feasibility Study on Spray-Drying Protein Pharmaceuticals: Recombinant Human Growth Hormone and Tissue-Type Plasminogen Activator[1]

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The feasibility of spray-drying solutions of recombinant methionyl human growth hormone (hGH) and tissue-type plasminogen activator (t-PA) was investigated. hGH was formulated in a mannitol phosphate buffer and t-PA was used in an arginine phosphate formulation containing 0.004% (w/v) polysorbate 80. Using filtered air (90–150°C) as the drying medium, hGH could be dried to a residual moisture content of ≤4%. However, approximately 25% of the protein was degraded during the processing. Results of atomization studies suggest that surface denaturation at the air–liquid interface of the droplets in the spray plays a major role in the degradation of the protein. The addition of 0.1% (w/v) polysorbate 20 into the hGH formulation reduced the formation of soluble and insoluble aggregates by approximately 90% during atomization. During spray-drying the addition of 0.1% (w/v) polysorbate 20 reduced the formation of soluble and insoluble aggregates by approximately 70 and 85%, respectively. In contrast, t-PA remained intact upon atomization. Depending on the spray-drying conditions, product powders with a residual moisture content between 5 and 8% were obtained. No oxidation, aggregation, or denaturation occurred in the protein under several operation conditions. Overall, this study demonstrates that it is feasible to spray-dry t-PA in the current marketed formulation.

KEY WORDS: spray-drying; recombinant methionyl human growth hormone; tissue-type plasminogen activator.

## INTRODUCTION

The stability of proteins in aqueous solution varies widely. Some proteins such as albumin remain stable at room temperature for days, while others such as lipoproteins are quite labile and lose their activity very rapidly. Several factors (pH, ionic strength, temperature, surfactants, interfaces, and agitation) and a variety of degradation pathways (deamidation, hydrolysis, oxidation, aggregation, and denaturation) are known to affect protein stability in aqueous media (1,2).

In pharmaceutical formulation research, when a protein is found to be unstable in solution, an attempt is made to preserve the product in a dry form. Today, the most common method for preparing dry protein pharmaceuticals is lyophilization. Lyophilization, as operated on the principle of ice sublimation, is usually considered to have less of a thermal effect on the protein than other drying methods. The process, however, has several disadvantages. For instance, during the freezing step, proteins and buffer components tend to be concentrated in the phase between ice crystals. Such a concentration effect can result in a dramatic change in the pH and ionic strength of the protein environment and, thus, lead to protein denaturation, aggregation, and precipitation (3–9). The fact that lyophilization is an energy-intensive and time-consuming process also raises the concern of high production costs.

Alternative techniques for preparing dry forms of proteins have been reported in the literature in recent years. These include coprecipitation of enzymes with water-soluble starch in an organic solvent, followed by dehydration of the precipitate in a vacuum (10), as well as "air-drying" of enzymes in the presence of carbohydrates as glass-forming stabilizers (11–13).

Among the conventional drying processes that rely on evaporation, spray-drying is currently a well-established method for processing liquids into powders. Unlike lyophilization, spray-drying utilizes heat from a hot gas stream to evaporate microdispersed droplets created by atomization of a continuous liquid feed and is therefore a very fast and cost-effective dehydration method. For example, spray-drying has already been successfully applied to many heat-sensitive materials of biological (enzymes), alimentary (milk), and pharmaceutical (antibiotics) interest (14,15). Its application to therapeutic proteins is, however, rather unexplored, undoubtedly because of the concern that proteins may be thermally degraded during the operation (16–19).

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In the present study we investigated spray-drying of recombinant methionyl human growth hormone (hGH) and tissue-type plasminogen activator (t-PA). Recombinant hGH is an approved drug used in the long-term treatment of children with growth failure due to lack of adequate endogenous growth hormone secretion (20). Recombinant t-PA is an approved drug used in the management of acute myocardial infarction in adults (21). Both drugs are currently available in lyophilized dosage forms. Our study was therefore centered on preparing spray-dried protein powders, which upon reconstitution, possessed a quality comparable to the liquid bulk. Overall, the objective of this study was to use these two approved drugs in their current formulations as model compounds to explore the feasibility of applying the spray-drying technique to the production of dry protein pharmaceuticals.

## MATERIALS AND METHODS

### Materials

Recombinant methionyl human growth hormone (hGH), molecular mass 22.25 kDa, was produced at Genentech Inc. from bacterial fermentation of a strain of *Escherichia coli*. The protein contains the same 191 amino acid residues as the natural, pituitary-derived hGH, with the addition of an N-terminal methionine residue. Details of the molecular structure are described in the literature (22). For this study, the protein was formulated into an aqueous solution containing 2 mg hGH/mL, 88 m*M* mannitol, and 5 m*M* sodium phosphate at pH 7.8. The solution was filtered through a 0.22-μm sterile filter before use.

Recombinant tissue-type plasminogen activator (t-PA), molecular mass approximately 60 kDa, was produced at Genentech Inc. from the fermentation of Chinese hamster ovary (CHO) cells that had been transfected with DNA encoding the gene for human t-PA. It is a glycoprotein of 527 amino acid residues and exists as equally active one-chain and two-chain forms. The two-chain form arises due to proteolytic cleavage between amino acid residues 275 (arginine) and 276 (isoleucine). Details of the molecular structure are described in the literature (23,24). The bulk was formulated to contain 2.5 mg t-PA/ml, 0.5 *M* L-arginine, and 0.004% (w/v) polysorbate 80 and adjusted with phosphoric acid to a pH of 7.3 ± 0.1. Polysorbate 80 was added during bulk manufacturing in order to prevent protein binding to the purification columns. No manufacturing method was used to remove this trace amount of surfactant. Thus no polysorbate-free t-PA bulk could be produced for this study. Solutions of higher protein concentration were obtained by reconstituting a lyophilized product of the initial bulk with deionized water. Solutions of lower protein concentration were obtained by diluting the initial bulk with deionized water. Both starting materials have been shown to be of identical quality, particularly with regard to protein aggregation and bioactivity. The solution was filtered through a 0.22-μm sterile filter before processing.

Arginine was obtained from Ajinomoto Co. (Tokyo), and mannitol from Aldrich Chemical Co. (Milwaukee). All other chemicals were of analytical grade.

### Procedure

The spray-drying runs were performed using a laboratory scale spray-dryer (Büchi, Model 190). During operation, protein solution was fed at a constant rate with a peristaltic pump to a nozzle (0.5-mm I.D.), where atomization occurred by means of a pressurized air stream. Drying air entered the drying chamber (105-mm I.D. × 450-mm L) in the same direction as the descending spray droplets (co-current operation). The flow rate of drying air was measured at the inlet of the dryer using a Side-Trak gas flow meter (Sierra Instruments). In order to avoid the aspiration of dust particles from the laboratory atmosphere, drying air and atomization air were prefiltered with 70- and 0.22-μm filters, respectively. In all spray-drying runs, the relative humidity of drying air at room temperature was determined by the dry–wet bulb method (25) to be approximately 50%. The product powders were clarified from the drying-air stream by means of a cyclone and collected in a water-cooled receiving vessel. After spray-drying, the collected powders were transferred to glass vials, stoppered under atmospheric pressure, and kept at −40°C until assayed.

Atomization experiments were conducted using the same spray nozzle as described above. Protein bulk was sprayed into a beaker at room temperature with no drying-air stream. Liquid samples were collected for assays.

Thermal effects on the protein were determined by exposing the formulated bulk to high temperatures for increasing time periods. Approximately 1-mL samples of solution were added to 5-mL glass vials which had been equilibrated for 20 min in a water bath at 50 or 80°C. After 5, 10, 20, and 40 sec of heat exposure the reaction was quenched by rapidly moving the vials to an ice bath. The samples were analyzed immediately after reaching room temperature. The experiment was run in duplicate.

### Product Characterization

#### *Physical*

Residual moisture was assayed by the Karl Fischer titration method; each reported value (%; w/w) represents the mean of at least three determinations. Particle size was determined by scanning electron microscopy using the built-in calibration scale. Clarity of reconstituted protein solution was determined by UV spectroscopy using a Kontron Uvikon 860 double-beam spectrophotometer. The optical absorbance in the 340- to 360-nm range was compared to the mean absorbance of reference suspensions recommended by the European Pharmacopoeia V.6.1 (26).

#### *Chemical*

hGH and t-PA product powders were reconstituted with sterile water for injection (SWFI) to a 1 mg/mL protein concentration for all the assays described below.

The amount of soluble protein aggregates was determined by size-exclusion HPLC (UV detection at 214 nm). A silica-based Tosoh TSKG2000SWXL column (7.8-mm I.D. × 30-cm L; particle size, 5 μm) was used for hGH. A silica-based Tosoh TSKG3000SWXL column (7.8-mm I.D. × 30-cm L; particle size, 5 μm) was used for t-PA. Typically, 50 μl

of each filtered reconstituted protein sample was loaded onto the column and eluted with mobile phase at a flow rate of 0.5 mL/min. The mobile phase used for the determination of the total amount of soluble covalently and noncovalently bound hGH aggregates was an aqueous solution of 50 m*M* sodium phosphate monobasic and 150 m*M* sodium chloride, pH 7.2. The mobile phase used for the determination of soluble covalently bound hGH aggregates only was an aqueous solution of 200 m*M* sodium phosphate monobasic and 0.1% (w/v) sodium dodecyl sulfate (SDS), pH 6.8. This same mobile phase was used for the determination of soluble t-PA aggregates. SDS had to be added to the mobile phase for the t-PA aggregate assay to prevent strong hydrophobic binding of the protein to the column. Thus only covalently bound soluble aggregates were determined in this study.

For both hGH and t-PA, the amount of insoluble protein aggregates 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 min) and filtration (Millipore, Millex-GV, 0.22 μm). Insoluble aggregates less than 0.22 μm in size were not necessarily removed by the filtration.

For both hGH and t-PA, protein fragmentation was determined by silver-stained SDS–polyacrylamide gel electrophoresis (PAGE) under both DL-dithiothreitol (DTT)-reduced and nonreduced conditions. This assay was performed to confirm the presence of covalently bound soluble protein aggregates as well.

Optical circular dichroism (CD) was performed using an Aviv CD spectrometer (Model 62DS) to determine whether the secondary and tertiary structure of the t-PA molecule would be affected by spray-drying. CD spectra were obtained in triplicate in the "far-UV" (200- to 250-nm) and "near-UV" (250- to 360-nm) region with a bandwith of 1.0 nm and a sampling interval of 0.5 nm.

Hydrophobic interaction chromatography (HIC) was performed to determine whether the t-PA molecule would be oxidized by the drying air during spray-drying. A Tosoh TSKPHENYL5PW column (4.6-mm I.D. × 15-cm L; particle size, 20 μm) was used at a 60°C column temperature with a flow rate of 0.8 mL/min. Mobile phase A was an aqueous solution of 0.6 *M* ammonium sulfate, 0.1 *M* monopotassium phosphate, 20% glycerol, and 0.04% C12E8, pH 7.0. Mobile phase B was an aqueous solution of 0.1 *M* monopotassium phosphate, 20% glycerol, and 0.04% C12E8, pH 7.0. A linear gradient from 100% mobile phase A to 100% mobile phase B in 140 min was used for elution. Typically, 5 μL of each reconstituted protein sample was loaded onto the column and UV detection was at 214 nm.

The *in vitro* clot lysis activity of 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 (27).

### Water Vapor Pressure Depression of the Protein Solution

Approximately 10 μL of the protein bulk solution was pipetted onto a solute-free paper disk in the sample chamber of the dew-point osmometer (Wescor, Model 5100C). Osmolality of the sample was determined based on the dew-point temperature depression in the chamber. From the osmolality data, the vapor pressure depression of the protein solution was calculated according to Raoult's law.

### Calorimetry of the Protein Solution

Approximately 1.5 mL of the protein bulk solution was added to the sample chamber of the differential scanning calorimeter (DSC, Microcal MC-2). An identical volume of sample buffer was placed in the reference cell. The chambers were temperature equilibrated at 20°C and the runs were performed in the temperature range of 30 to 95°C at a scan rate of 48°C/hr. The reported thermogram is the average of three determinations. Reversibility of the endothermic transition was examined after reequilibration of the cells at 20°C after the first run, by rescanning the same sample over the same temperature range.

## RESULTS AND DISCUSSION

### hGH

With a 600 L/hr atomization air rate for the spray nozzle, a 5 mL/min protein solution feeding rate, and a drying air rate of 36,000 L/hr at 25°C, 1 atmosphere (STP condition), spray-dried products containing residual moisture of 3.5, 3.4, and 2.3% were obtained for respective inlet air temperatures of 90, 120, and 150°C. These moisture values are comparable with those of a lyophilized hGH product. Unlike lyophilization, which normally leads to a cake-shaped product, spray-drying produces white, free-flowing powders of particle size ranging from 1.3 to 3.5 μm. Reconstituted solutions were visibly opalescent.

A major concern in the spray-drying of proteins is the risk of heat-induced degradation caused by exposure of the molecule to high temperatures. Table I presents the effect of different inlet air temperatures on the formation of insoluble and soluble aggregates in mannitol-formulated hGH. Compared to the bulk, the spray-dried products contained more insoluble aggregates which scatter UV light in the 340- to 360-nm range. In fact, compared to reference suspensions of the European Pharmacopoeia, the degree of opalescence for the spray-dried and reconstituted 1 mg/mL solution was found to be at the transition between slightly opalescent and opalescent, whereas the bulk could be classified as clear. After removing the particulates by centrifugation and filtration, the UV light-scattering analysis of reconstituted hGH solutions (1 mg/mL) revealed that approximately 40, 46, and 44 μg/mL of insoluble aggregates were present in the reconstituted solution of products prepared by spray-drying at 90, 120, and 150°C, respectively. The spray-dried products also contained approximately 21% soluble aggregates (dimer and higher molecular weight species) as compared to the bulk sample, which contained only about 1%. Table I also shows that approximately 97% of these soluble aggregates are noncovalently linked, as they almost completely dissociate to monomers in the presence of SDS. The presence of trace amounts of covalently bound soluble aggregates was further confirmed by silver-stained SDS-PAGE (Fig. 1). All the spray-dried samples present the same gel patterns: a 21-kDa (monomer) band and a 45-kDa (dimer) band for the nonre-

Table I. Effect of Inlet Air Temperature ($T_{ia}$) on the Formation of Insoluble and Soluble Aggregates in hGH During Spray-Drying

| Inlet air temperature (°C) | Clarity[a] | % aggregates | | |
|---|---|---|---|---|
| | | | Soluble | |
| | | Insoluble | Covalent and noncovalent | Covalent |
| Bulk | Clear | 0.2 | 0.6 ± 0.1 | 0.2 ± 0.1 |
| 90 | Opalescent | 4.0 | 20.2 ± 0.8 | 0.6 ± 0.0 |
| 120 | Opalescent | 4.7 | 20.3 ± 1.0 | 0.6 ± 0.0 |
| 150 | Slightly opalescent | 4.2 | 20.2 ± 1.5 | 0.7 ± 0.0 |

[a] According to European Pharmacopoeia V.6.1. reference suspensions (26).

duced samples. No higher molecular weight species can be detected on the SDS-PAGE gel, thus indicating their noncovalent nature. Furthermore, no low molecular weight bands can be detected on the SDS-PAGE gel of the reduced sample, thus indicating that no cleavage of the hGH molecule was taking place during spray-drying.

Table I also demonstrates that increasing drying air temperature from 90 to 150°C does not significantly affect the extent of soluble and insoluble aggregate formation. Drying air provides heat to evaporate moisture from the droplets in the spray. As a result, the drying air cools down as it approaches the outlet of the drying chamber. In practice, immediately after atomization the droplet surface temperature ($T_{ps}$) approximates the wet-bulb temperature of the inlet air and can be determined by the enthalpy–humidity chart of the pure water–air system, if the presence of solutes in the hGH solution has a negligible effect on the saturation water vapor pressure (28). The saturation water vapor pressure of the mannitol-formulated hGH solution at room temperature was determined to be only 0.03 mm Hg lower than the 23.8 mm Hg saturation vapor pressure of pure water at the same temperature, indicating that it is appropriate to use the enthalpy–humidity chart of the pure water–air system for this study. Thus the $T_{ps}$ values were determined to be 33, 37, and 41°C when inlet air temperatures of 90, 120, and 150°C were used, respectively.

While moving with the hot air stream through the drying chamber, the drying droplets absorb more heat and thereby increase their surface temperature. To determine the surface temperature of a drying particle at any time and location during the spray-drying operation requires a complex mathematical modeling for heat and mass transfer and is beyond the scope of this study. However, when spray-dried in a hot air stream that flows in the same direction as the descending spray droplets, the product can reach a theoretical maximum temperature no greater than the temperature of the air

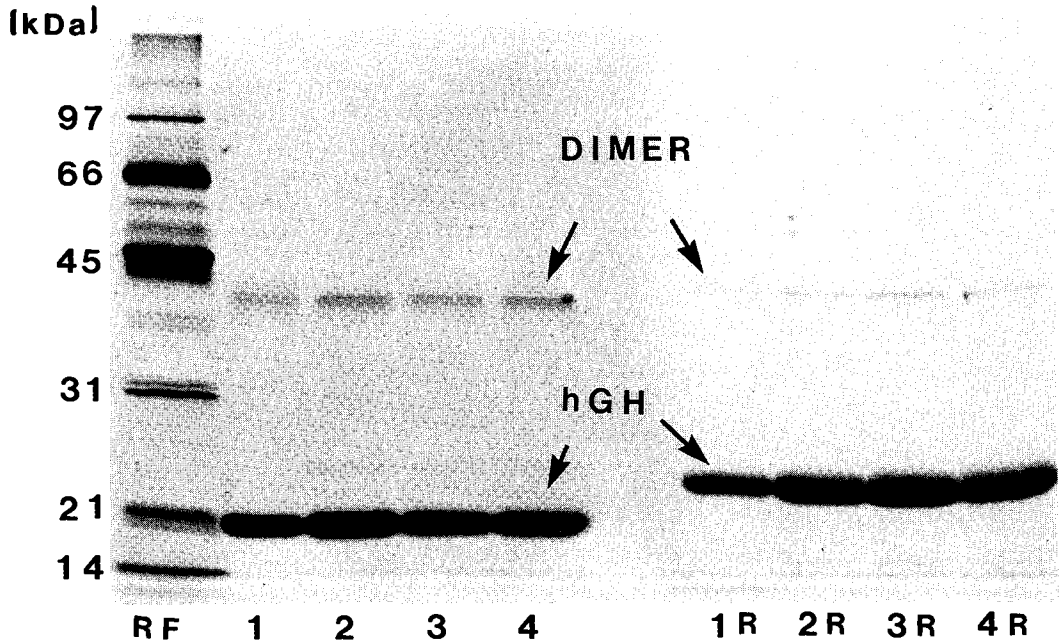


Fig. 1. Silver-stained SDS-PAGE patterns of hGH samples in DTT-reduced and nonreduced form (14% resolving gel). RF, molecular mass reference; 1, bulk not spray-dried, nonreduced; 2, bulk spray-dried at $T_{ia}$ 90°C, nonreduced; 3, bulk spray-dried at $T_{ia}$ 120°C, nonreduced; 4, bulk spray-dried at $T_{ia}$ 150°C, nonreduced; 1R, bulk not spray-dried, reduced; 2R, bulk spray-dried at $T_{ia}$ 90°C, reduced; 3R, bulk spray-dried at $T_{ia}$ 120°C, reduced; 4R, bulk spray-dried at $T_{ia}$ 150°C, reduced. $T_{ia}$ is the inlet air temperature in spray-drying.

stream at the dryer's outlet ($T_{oa}$). In practice, it can generally be assumed that the actual maximum temperature is approximately 15 to 25°C below the outlet air temperature (29). In this study, $T_{oa}$ was measured to be 50, 70, and 85°C when an inlet air temperature of 90, 120, and 150°C was used, respectively. The DSC thermogram depicted in Fig. 2 shows that the hGH molecule starts an endothermic transition at approximately 70°C and that the endothermic heat capacity peak maximum is at 76°C. The second DSC scan of the same sample does not show any transition, indicating the irreversibility of the shown endothermic transition. If, at an inlet air temperature of 150°C, the product temperature had indeed reached the $T_{oa}$ value of 85°C, spray-drying of hGH would have resulted in an extensive endothermic transition and would therefore have caused significantly higher damage to the protein than the lower inlet air temperatures. However, according to the results shown in Table I no significantly higher damage to the protein was found for the spray-drying experiment conducted at 150°C inlet air temperature. This indicates that the actual temperature the protein is exposed to is lower than the measured outlet air temperature.

Results from a separate experiment shown in Fig. 3 reveal that exposure of the hGH solution to high temperature is not the sole cause of protein degradation. In this experiment, 1-mL samples of protein solution were pipetted into preheated glass vials set in a water bath of 80°C. After a given amount of time, the heat exposure was quenched by rapidly moving the vials to an ice bath. The samples were then analyzed for soluble aggregate formation. Typically, during spray-drying the time period of exposure of the drying droplets to elevated temperature is approximately 5 to 30 sec (15). Figure 3, however, shows that even 40 sec of exposure of the protein solution to 80°C (4°C higher than the temperature of the endothermic heat capacity peak maximum) fails to degrade the protein to the same extent as spray-drying (Table I). Consequently, it appears that additional process parameters play a role in hGH degradation during the spray-drying operation.

In studying process parameters, we found that hGH is very susceptible to aggregation during atomization. Figure 4

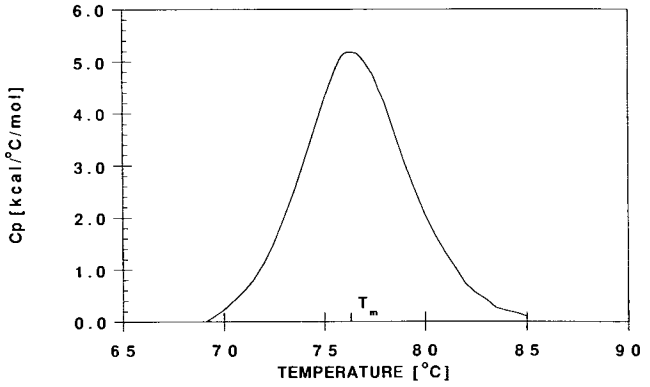


Fig. 2. DSC thermogram of mannitol-formulated, 2 mg/mL hGH at pH 7.8. Each data point represents the mean of three determinations. The second scan of the same sample does not show any transition, indicating the irreversibility of the shown endothermic transition at these operation conditions. $T_m$ denotes the temperature at which the heat capacity peak maximum occurs.

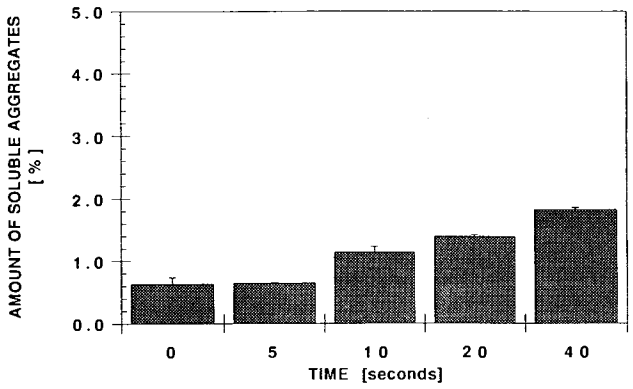


Fig. 3. Effect of time exposure to 80°C on the formation of soluble aggregates in hGH bulk. Each value represents the mean ± standard error of four determinations. In the experiment, 1-mL samples of the protein solution were pipetted into preheated glass vials set in a water bath of 80°C. After a given amount of time, the samples were quenched by rapidly moving the vials to an ice bath. The experiment was run in duplicate. Each sample was analyzed twice.

shows that the formation of insoluble and soluble aggregates of hGH increases with increasing atomization air rate. This result is corroborated by the observation that an air–liquid interface can facilitate denaturation of hGH (30). Higher atomization air rates generate smaller droplets in the sprays and thus increase the air-liquid interface for surface denaturation. Figure 4 also shows that the formation of aggregates will be minimized if spray-drying is conducted at atomization rates between 200 and 300 L/hr. Unfortunately, even at a lower protein solution feeding rate of 2.5 mL/min, an inlet air temperature of up to 180°C still could not provide enough heat to dry the large droplets resulting from such low atomization rates. No powder product could therefore be obtained.

Of interest in the atomization study (Fig. 4) is the finding that the liquid sample obtained at an atomization rate of 600 L/hr contains the same amount of insoluble aggregates (4.7%) as the spray-dried samples discussed in Table I (4.0–4.7%). This result suggests that during spray-drying the formation of insoluble aggregates is induced mainly by surface denaturation. Figure 4 also shows that atomization of the

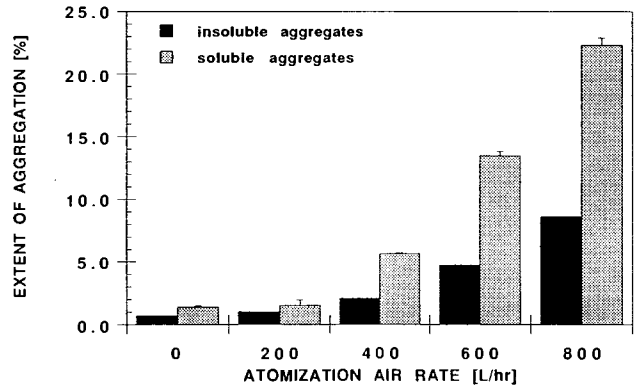


Fig. 4. Effect of atomization air rate on the formation of insoluble and soluble aggregates in hGH during atomization of 2 mg/mL mannitol-formulated bulk at room temperature. Each value represents the mean ± standard error of three determinations.

Table II. Effect of 0.1% Polysorbate 20 (T20) on the Formation of Insoluble and Soluble Aggregates in hGH During Atomization and Spray-Drying

| Sample preparation conditions | | | | % aggregates | | |
|---|---|---|---|---|---|---|
| | | | | | Soluble | |
| T20 | Atomized | Spray-dried[a] | Clarity[b] | Insoluble | Covalent and noncovalent | Covalent |
| No | No | No | Clear | 0.2 | 0.6 ± 0.1 | 0.2 ± 0.1 |
| No | 600 L/hr | No | Opalescent | 4.7 | 13.5 ± 0.4 | 0.1 ± 0.1 |
| 0.1% | No | No | Clear | 0.2 | 0.7 ± 0.1 | 0.0 ± 0.0 |
| 0.1% | 600 L/hr | No | Clear | 0.3 | 1.3 ± 0.3 | 0.0 ± 0.0 |
| 0.1% | 600 L/hr | 90°C | Clear | 0.6 | 5.4 ± 0.2 | 0.0 ± 0.0 |
| 0.1% | 600 L/hr | 150°C | Clear | 0.5 | 7.9 ± 0.5 | 0.0 ± 0.0 |

[a] Corresponding data for samples spray-dried in the absence of polysorbate 20 are given in Table I.
[b] According to European Pharmacopoeia V.6.1. reference suspensions (26).

protein solution at room temperature at an air rate of 600 L/hr results in degradation of 13% of the protein molecules to soluble aggregates. This amount of degradation is two-thirds of the soluble aggregate found in the spray-dried samples (Table I), suggesting that during spray-drying surface denaturation plays a role in the formation of soluble aggregates as well. In addition, results shown in Table II demonstrate that the formation of insoluble and soluble aggregates during atomization and spray-drying can be significantly reduced by adding 0.1% (w/v) polysorbate 20 into the formulation. Polysorbate 20 is a surfactant. We hypothesize that the surfactant replaces hGH molecules at the air–liquid interface of droplets in the spray and thus reduces exposure of the protein to the surface denaturation.

### t-PA

Contrary to what was found for hGH, atomization did not have a significant effect on the t-PA molecule. The amounts of insoluble and soluble aggregates measured in the samples at different atomization rates (0–800 L/hr) were equivalent to the corresponding values determined for the t-PA bulk. These results suggest that t-PA is less susceptible to the air–liquid interface than hGH. Differences in molecular structure (e.g., number of disulfide bonds, degree of glycosylation, etc.) as well as in bulk formulation (e.g., surfactant) may explain the protein-specific sensitivity to the phenomenon of surface-induced denaturation. As described under Materials and Methods, no polysorbate-free t-PA bulk could be manufactured. Thus no study was conducted to determine whether the presence of 0.004% (w/v) polysorbate 80 in the formulation was the factor preventing t-PA from surface denaturation during atomization.

Spray-drying operated at an atomization rate of 600 L/hr, a solution feeding rate of 5 mL/min, and a drying air rate of approximately 36,000 L/hr at STP condition resulted in products containing residual moisture of 5.0, 5.4, 6.2, and 7.4%, at inlet air temperatures of 180, 165, 120, and 90°C, respectively. The product powders had good free-flowing properties with a particle size ranging from 3 to 8 μm. All the reconstituted samples, except the one obtained at 180°C air temperature, were clear.

Table III shows the effect of increasing inlet air temperature ($T_{ia}$) on the degradation of t-PA during spray-drying. After reconstitution to a 1 mg/mL solution, significantly higher amounts of insoluble and soluble aggregates were found in the samples spray-dried at 180°C, whereas at $T_{ia} \leq$ 165°C aggregation was minimal and comparable to corresponding data obtained for the t-PA bulk. UV light-scattering analysis of t-PA solutions after removing the particulates by centrifugation and filtration revealed that approximately 6, 4, 9, and 30 μg/mL of insoluble aggregates were present in the reconstituted solution of products prepared by spray-drying at 90, 120, 165, and 180°C, respectively. In all spray-dried products, the soluble aggregates were determined to be protein dimers by the SDS size-exclusion HPLC assay (chromatograms not shown). Because of nonspecific hydrophobic binding of the protein to the column, the size-exclusion HPLC assay cannot be conducted under truly native conditions. The method used for the determination of soluble aggregates therefore only measures aggregates that do not dissociate in the presence of SDS (covalent aggregates). Consequently, the total amount of soluble aggregates present in each sample may be greater than that shown in Table III. The products obtained at $T_{ia} \leq$ 165°C also maintained full *in vitro* clot lysis activity, whereas products obtained at an 180°C inlet air temperature had a decrease in bioactivity by approximately 10%. It was also found that spray-dried samples showed SDS-PAGE patterns identical to those of the t-PA bulk, under reducing as well as nonreducing conditions (Fig. 5): a 60-kDa (monomer, one-chain) band and two bands of approximately 30 kDa (monomer, two-chain) resulting from proteolytic cleavage of the molecule.

Table III. Effect of Inlet Air Temperature ($T_{ia}$) on the Formation of Insoluble and Soluble Aggregates in t-PA During Spray-Drying

| Inlet air temperature (°C) | Clarity[a] | % aggregates | |
|---|---|---|---|
| | | Insoluble | Soluble |
| Bulk | Clear | 0.5 | 1.1 ± 0.2 |
| 90 | Clear | 0.6 | 1.8 ± 0.2 |
| 120 | Clear | 0.5 | 1.8 ± 0.2 |
| 165 | Clear | 0.9 | 1.8 ± 0.2 |
| 180 | Opalescent | 3.0 | 2.9 ± 0.6 |

[a] According to European Pharmacopoeia V.6.1. reference suspensions (26).

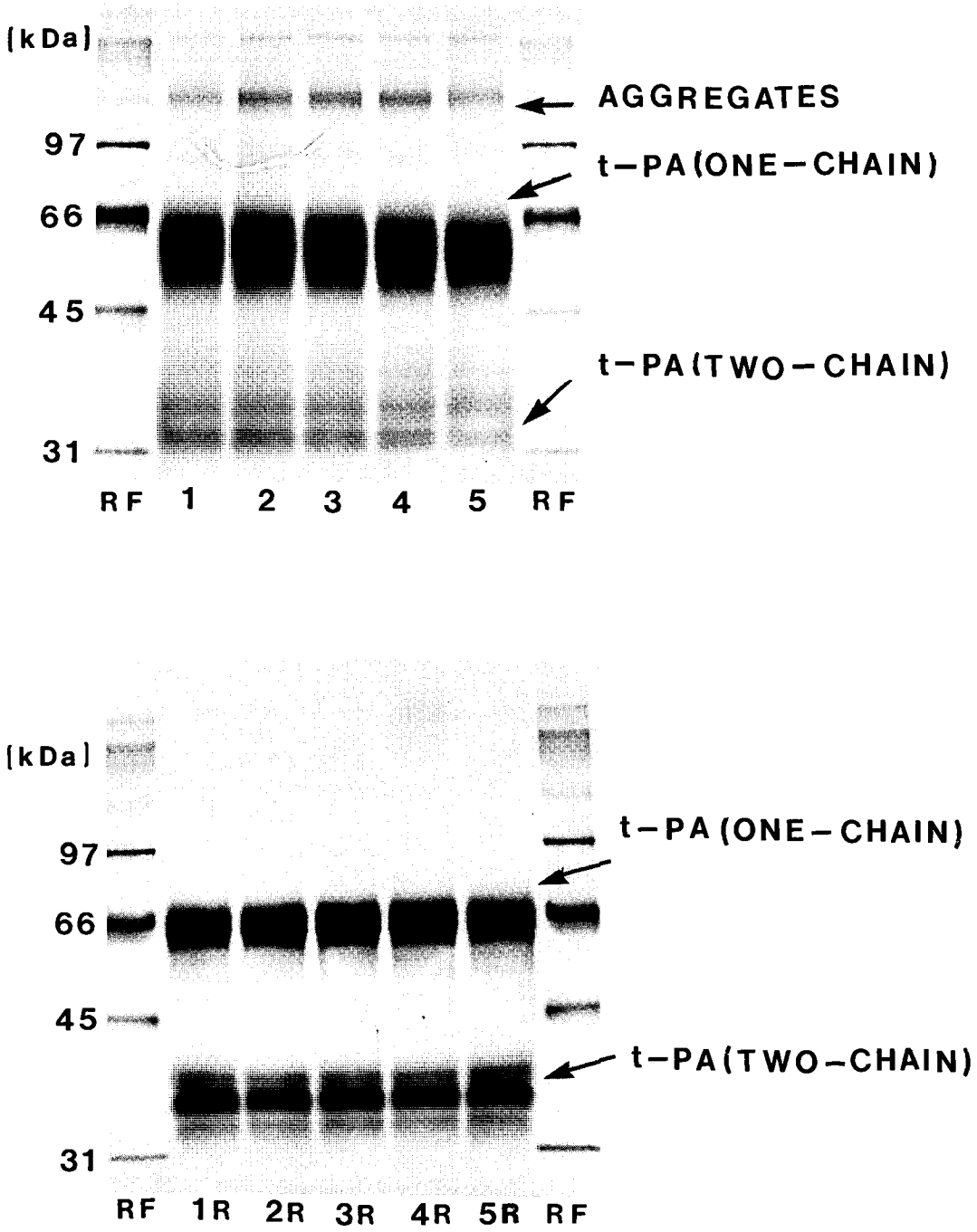


Fig. 5. Silver-stained SDS-PAGE patterns of t-PA samples in DTT-reduced and nonreduced form (12% resolving gel). RF, molecular mass reference; 1, bulk not spray-dried, nonreduced; 2, bulk spray-dried at $T_{ia}$ 90°C, nonreduced; 3, bulk spray-dried at $T_{ia}$ 120°C, nonreduced; 4, bulk spray-dried at $T_{ia}$ 165°C, nonreduced; 5, bulk spray-dried at $T_{ia}$ 180°C, nonreduced; 1R, bulk not spray-dried, reduced; 2R, bulk spray-dried at $T_{ia}$ 90°C, reduced; 3R, bulk spray-dried at $T_{ia}$ 120°C, reduced; 4R, bulk spray-dried at $T_{ia}$ 165°C, reduced; 5R, bulk spray-dried at $T_{ia}$ 180°C, reduced. $T_{ia}$ is the inlet air temperature in spray-drying.

The temperature effect mentioned above can be explained by the DSC thermogram shown in Fig. 6. When formulated in arginine, t-PA starts an endothermic transition at approximately 55°C, and the endothermic heat capacity peak maximum is at 70°C. The second DSC scan of the same sample does not show any transition, indicating the irreversibility of the shown endothermic transition. The water vapor depression of the arginine formulated t-PA solution at room temperature was determined to be less than 1% of the saturation water vapor pressure of pure water at the same temperature. Thus the enthalpy–humidity chart of a pure water–air system was applied for the estimation of the droplet surface temperature. At 180°C inlet air temperature, the surface temperature of droplets immediately after spray-air con-

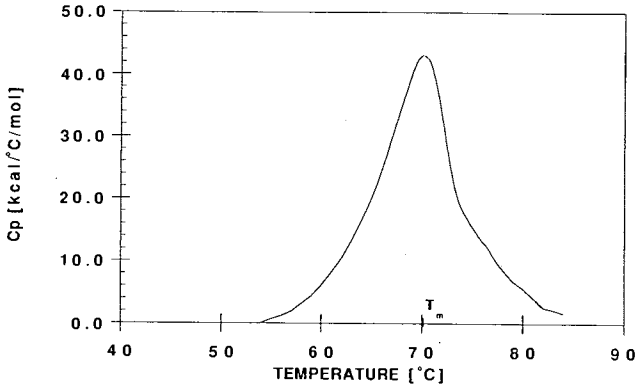


Fig. 6. DSC thermogram of arginine-formulated, 2.5 mg/mL t-PA at pH 7.3. Each data point represents the mean of three determinations. The second scan of the same sample does not show any transition, indicating the irreversibility of the shown endothermic transition at these operation conditions. $T_m$ denotes the temperature at which the heat capacity peak maximum occurs.

tact was estimated to be approximately 45°C and the outlet temperature of the drying air stream was measured to be 124°C. Assuming that the drying particles reach a maximum temperature which is 25°C below the air's outlet temperature, the protein is exposed to an environment of approximately 100°C, i.e., to a temperature which is approximately 30°C higher than the temperature of endothermic heat capacity peak maximum for t-PA (Fig. 6). Therefore, it can be expected that samples spray-dried at 180°C inlet air temperature contain significantly higher amounts of degraded protein than those spray-dried using lower air temperatures.

A separate experiment was conducted to understand the time course of t-PA degradation in solution at elevated temperatures (Fig. 7). The experiment was performed in preheated glass vials using the same procedure as described previously for hGH. Figure 7 shows that even 40-sec exposure of the solution to a 50°C environment fails to degrade

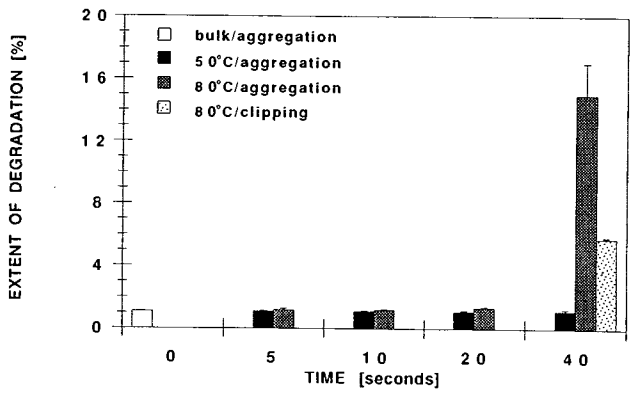


Fig. 7. Effect of time exposure to 50 and 80°C on the extent of degradation, expressed as the formation of soluble aggregates and the fragmentation of the protein molecule, in t-PA bulk. Each value represents the mean ± standard error of four determinations. In the experiment, 1-mL samples of protein solution were pipetted into preheated glass vials set in water baths of 50 and 80°C. After a given amount of time, the samples were quenched by rapidly moving the vials to an ice bath. The experiment was run in duplicate. Each sample was analyzed twice.

the protein. The results also indicate that the extent of protein degradation is negligible even after exposure of the solution to 80°C for up to 20 sec. However, a 40-sec exposure of the solution to 80°C results in clipping of 6% of the protein to low molecular weight fragments as well as in degradation of 16% of the protein to soluble aggregates. An 80°C temperature is the estimated droplet surface temperature in spray-drying using 165°C inlet air. The facts that the extent of protein degradation in the 165°C spray-dried samples is almost negligible and there is no protein clipping in the spray-dried samples (Table III) demonstrate that the droplet–hot air contact time in the spray-drying chamber must be quite limited.

Optical circular dichroism (CD) analysis was further conducted to determine whether the structure of the t-PA protein would be affected by spray-drying. CD is a common technique used to study changes in the three-dimensional structure of a protein in solution (31). The "near-UV" CD spectrum serves as a fingerprint of the protein molecule, providing information about the conformation and local environments of the aromatic and disulfide chromophores (tertiary structure) of the molecule. The "far-UV" CD spectrum is dominated by the amide bond absorption and is generally interpreted in terms of the sum of the contribution of each of the secondary structural elements (α-helix, β-sheet, and random coil). Both the "near-UV" CD (Fig. 8) and the "far-UV" CD spectra (spectra not shown) obtained for the protein samples spray-dried at inlet air temperatures ≤165°C did not show any significant deviation from the spectrum obtained for the t-PA control. Consequently the temperature–time conditions encountered by the protein during spray-drying did not provide enough thermal energy to induce any irreversible conformational changes in either the secondary or the tertiary structure of t-PA.

A further concern in spray-drying of protein pharmaceuticals is the risk of protein oxidation. Polypeptides containing free cysteine (Cys) and methionine (Met) residues are especially prone to oxidation. Even atmospheric oxygen can oxidize the thioether group of Met residues to their corresponding sulfoxides (2). t-PA contains Met at positions 13, 207, 455, 490, and 525 of the amino acid sequence. Consequently, oxidation of the protein can potentially occur at those sites during spray-drying. Oxidized t-PA can be sepa-

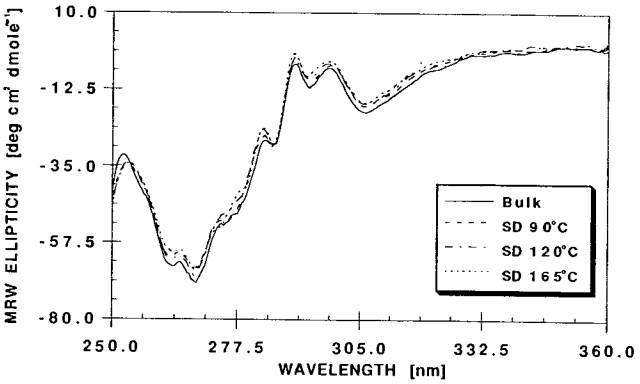


Fig. 8. Optical circular dichroism spectra in the "near-UV" region for t-PA bulk and t-PA spray-dried (SD) at 165, 120, and 90°C, respectively.

rated from native t-PA using hydrophobic interaction chromatography (HIC) as described under Materials and Methods. Using this analytical method, we found the HIC chromatograms of all spray-dried samples to be identical to that of a t-PA standard reference (chromatograms not shown). Thus, no oxidation was occurring during the spray-drying operation.

## CONCLUSIONS

Spray-drying of mannitol formulated human growth hormone results in extensive protein aggregation. Insoluble aggregates are formed primarily by surface-induced denaturation of the protein at the air–liquid interface during atomization, whereas the mainly noncovalently linked soluble aggregates result from surface-induced denaturation as well as from thermal degradation. Arginine and 0.004% (w/v) polysorbate 80-formulated tissue-type plasminogen activator is not degraded by atomization. Spray-drying conducted under conditions that expose the protein to the temperature range of endothermic transition (≥55°C) for minimal periods of time (<20 sec) can yield a free-flowing, powdered product free from oxidized protein or fragments. Insoluble and soluble aggregates are comparable to the starting bulk.

It must be emphasized that the performance of a spray-dryer is strongly dependent upon the design of the individual dryer. The operating conditions used in a laboratory scale spray-dryer have to be evaluated for a scale-up operation. Nevertheless, this study demonstrates that spray-drying may be a potential alternative to lyophilization for the drying of pharmaceutical proteins.

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