Spray-Freeze-Drying for Protein Powder Preparation: Particle Characterization and a Case Study with Trypsinogen Stability

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Received 21 December 2001; revised 7 March 2002; accepted 3 April 2002

ABSTRACT: This work investigates the use of spray freeze-drying (SFD) to produce protein loaded particles suitable for epidermal delivery. In the first part of the study, the effects of formulation and process conditions on particle properties are examined. Aqueous solutions of trehalose produce SFD particles in the size range 20–80 μm, with a smooth, textured surface, but having high internal porosity. The latter was visualized using SEM and a novel particle embedding and sectioning technique. Use of an annealing step during the freeze-drying cycle caused the particles to shrink, reducing hereby porosity and also the measured rate of moisture uptake into these amorphous particles. SFD pure mannitol was approximately 40% amorphous, but not hygroscopic. Incorporation of dextran 37,500 into a combined amorphous trehalose/mannitol formulation led to increased particle shrinkage and lower particle porosity on annealing. The model protein trypsinogen lost approximately 15% activity during SFD of solutions containing 50 mg/mL protein, but was only marginally aggregated (1.4%). It is suggested that trypsinogen forms an irreversible partially unfolded state or molten globule on SFD/rehydration. The pure protein was also partially inactivated without aggregation during atomization into air. Surprisingly, neither activity loss nor aggregation were detected on atomization of the protein solution into liquid nitrogen. Quench-freezing of small droplets may reverse the partial unfolding of trypsinogen occurring on atomization into air. The origin of the trypsinogen inactivation during SFD must therefore be the subsequent freeze-drying step of this multistep process. Isolated freeze drying of trypsinogen produces strong aggregation and equivalent inactivation. This result suggests that trypsinogen behaves differently during freeze drying from frozen droplets and from bulk solution in a vial. In the former case the protein forms an irreversible partially unfolded state, whereas in the latter case aggregates are formed. Trypsinogen inactivation during SFD could be completely prevented by the presence of trehalose in the formulation. Electron Spectroscopy for Chemical Analysis (ESCA) showed a high surface excess of the protein in the SFD particles, which was reduced on inclusion of Polysorbate 80, but not trehalose. Taken together, these results help to elucidate the complex destabilization behavior of trypsinogen during SFD.


Keywords: spray drying; freeze drying; protein; atomization; particle

INTRODUCTION

The increasing demand for protein particles suitable for pulmonary or parental application is currently met using a number of methods of preparation. These can conveniently be divided into destructive and constructive particle formation...
methods. The destructive methods involve milling of a protein/carrier solid solution prepared, for example, by freeze drying an aqueous solution thereof. Milling of a proteinaceous formulation give rise, however, to concerns about possible protein degradation and to problems associated with obtaining a sterile product. In addition, the control of particle size and particle size distribution is difficult. Constructive techniques include spray-drying, supercritical fluid technology, and more recently spray-freeze drying. Spray drying (SD) is now an established production process for producing protein/carrier particles that are both process and storage stable. Super critical fluid technology has been widely propounded, and its utility in practice already demonstrated.

Compared with these two technologies, there is little known about tailoring the properties of protein particles using spray freeze drying (SFD). This method involves atomization of a solution of a protein plus a suitable carrier substance into liquid nitrogen. The resulting coarse dispersion is then transferred to a precooled freeze dryer and dried using a standard freeze-drying program. Three studies of SFD pharmaceutical protein powders have been published recently. In the first, spray-freeze drying aqueous solutions of pure proteins or protein/sugar combinations produced larger, more porous particles than those prepared by SD. Additionally, the SFD protein particles had superior aerosol performance than the SD particles, indicating their suitability for pulmonary delivery. In the second, the friability of SFD protein particles was examined and found to be dependent on their size. It was proposed that because small particles freeze in a shorter time than do large ones, a finer microstructure, and hence higher friability and also greater protein aggregation, is the result. In the third study, it was found that the degree of aggregation of bovine serum albumin was directly proportional to the specific surface area of SFD trehalose or mannitol particles. There has, however, been no systematic examination of the effects of SFD conditions and formulation variables on protein/sugar particle properties and protein process stability.

In this article we present our first work to prepare SFD protein/sugar particles suitable for intradermal delivery. This application method requires particles of larger size (diameter approximately 20–70 μm) than those suitable for pulmonary application. Additionally, a high particle density is advantageous. Our aim was to determine how particle morphology is influenced by SFD process conditions and formulation, and also to investigate protein stability during SFD in a systematic fashion. Trehalose was used as a glass former and mannitol as a bulking agent for particle production. In some experiments a polymer (dextran 37500) was added as an auxiliary adjuvant. We selected the enzyme trypsinogen as a model protein, because it is known to be partially inactivated during SD, has a simple activity assay, and its aggregation status can readily be determined by size-exclusion chromatography. Particle morphology was investigated using scanning electron microscopy, wide-angle X-ray diffraction, and mercury porosimetry. Additionally, the internal particle structure was visualized using a novel embedding and sectioning technique. Moisture uptake kinetics were measured to determine how change in process conditions effect particle porosity and hence hygroscopicity. Comparison of the extents of protein aggregation and inactivation during SFD with those occurring during separate atomization and freeze-drying produced some unexpected results. We discuss the possible causes of protein inactivation during SFD, which are complex in this multi-step process of atomization, rapid freezing and freeze drying.

MATERIALS AND METHODS

Materials

Mannitol (Merck, D-Darmstadt), trehalose, dextran 37500, and polysorbate 80 (all Sigma Chemicals, D-Munich) were used as received. Bovine pancreas trypsinogen (molecular weight = 24 kD, dialyzed and lyophilized) was purchased from Sigma Chemicals (D-Munich). Nα-benzoyl arginine ethylester and other substances required for the trypsinogen activity assay, size-exclusion chromatography, and gel chromatography (SDS-PAGE) were obtained from Sigma. The cold-curing resin Technovit 7100 was supplied by Heraeus Kulzer (D-Wehrheim). Water was double distilled from an all-glass apparatus.

Methods

**Spray-Freeze Drying (SFD)**

Aqueous solutions of the excipient(s) under consideration were prepared either with or without trypsinogen and immediately spray freeze dried. A laboratory scale SFD rig was constructed, comprising an ultrasonic nozzle (Sonotek, 120 kHz)
held at a height of 10 cm above a circular metal bowl of diameter 16 cm and height 6 cm standing on a magnetic stirrer. A conical cover was designed to fit over the bowl during operation to minimize loss of liquid nitrogen (LN2). The bowl was filled to within 1 cm of its top with LN2, and after a short pause to allow stabilization of the LN2 surface, 2–10 mL of the solution under consideration was sprayed into the LN2. The solution feed rate was 3 mL/min controlled by an accurate, low-pulse peristaltic pump (Pharmacia Biotech, S-Uppsala). The dispersion of frozen droplets in LN2 thus produced was stirred continuously, and on completion of spraying was topped up with LN2 and transferred to a shelf of a precooled Christ freeze-dryer (Model D1–24 kD with total shelf area of 0.24 m²) at a shelf temperature, $T_{shelf} = -45^\circ C$. On completion of a hold period of 1/2 h at $T_{shelf} = -45^\circ C$ the frozen droplets were freeze dried using the program given in Figure 1, as required with an annealing step outlined in the Discussion. At the end of the cycle the dried powders were removed from the bowls and immediately transferred to a glove box filled with nitrogen at a maximum relative humidity (RH) of 20% at 25°C. The powders were then filled into glass containers and hermetically sealed.

Characterization of SFD Powder Morphology

**Scanning Electron Microscopy (SEM)**

The external morphology of the SFD particles was examined using an Amray 1810 T scanning electron microscope. The SFD powders were first Au sputtered using a Hummer JR Technics unit.

**X-Ray Powder Diffraction (XRPD)**

The crystalline/amorphous state of the powders was examined at 25°C ± 0.5°C using wide-angle X-ray powder diffraction on a Philips model Expert MPD at 40 kV, 40 mA.

**Pore Size Distribution**

Powder porosity was examined using a Hg porosimeter (Porosimeter 2000, Carlo Erba, I-Milan).

**Moisture Content**

Karl-Fischer titration was used to measure the moisture contents of the SFD powders (T 82/50 Titrator, Schott, D-Mainz). Approximately 50–100 mg of powder was dissolved in methanol/formamide (2:1) before titration.

**Internal Morphology**

The internal structure of the SFD particles was assessed by embedding 2 mg powder in Technovit 7100 cold curing resin contained in a hard gelatine capsule (size 00). After hardening was complete the capsule was microtome sectioned into slices of 10–16 μm thickness. These were then Au sputtering to enable SEM examination.

**Moisture Uptake**

The moisture uptake kinetics of the SFD powders was determined after their removal from the freeze-drying chamber. A 100–200-mg sample of the powder was transferred to a balance (Sartorius MC 1, D-Göttingen) at 33% RH and 25°C, and the increase in powder weight measured over 2 h.

**Electron Spectroscopy for Chemical Analysis (ESCA)**

This technique was used to measure the atomic composition of the surface layer (outer 10 nm) of the SFD particles, as fully described before.14

**Measurement of Trypsinogen Activity**

An equivalent mass of a preparation (powder or liquid) containing 2.5 mg trypsinogen was dissolved/diluted in 10 mL of Tris buffer (46.7 mmol/L TRIS + 19 mmol/L CaCl₂, pH 7.6). Autocatalytic reduction of the trypsinogen to trypsin was initiated by adding 100 μg of trypsin to the solution.
After 4 h at 25°C the reaction was stopped by addition of 50 µL 2 N HCl. Of this solution, 0.2 mL was placed in a quartz cuvette and mixed with 3 mL of a solution of 0.9 mmol N₂-benzoyl arginine ethylester in the same Tris buffer. The increase in absorption of the solution, ΔA(1/min), was measured at λ = 253 nm over 6 min. The activity of the trypsin, U/L [units/L], was then calculated from ΔA using:

\[
U/L = \frac{\Delta A \cdot \frac{1}{\text{min}} - 3.2 \times 10^{-3} \cdot 1000}{\varepsilon \cdot d \cdot 0.2 \times 10^{-3}}
\]

where \( \varepsilon = 0.811 \, \text{cm}^{-1} \cdot \text{mmol}^{-1} \) and \( d = 1 \, \text{cm} \).

The activity in U/L was converted to U/mg powder from the weighed-out amount of SFD powder initially dissolved in the TRIS buffer. Pure trypsinogen was always run as a standard in the assay, and the value measured for U/mg fixed as 100% activity.

**Examination of Aggregation Status of Trypsinogen**

**Size-Exclusion Chromatography (SEC)**

Size-exclusion was performed using a BioSep-SEC-S3000 column (Phenomenex) in phosphate-buffered saline pH 6.8 as an isocratic mobile phase. Duplicate samples (10 µL) were analyzed at a concentration of 2–5 mg/mL and a flow rate of 0.5 mL/min with detection at λ = 280 nm.

**Gel Electrophoresis**

Electrophoresis was performed on a Mini Protean II unit (Biorad) controlled by a Power Pack 300 power supply (ISCO). The samples were separated according to molecular weight in nonreducing conditions on a 12% polyacrylamide gel with a Tris buffer system. A molecular weight calibration kit was used in the range of 14.4 kDa (bovine serum albumin) up to 97 kDa (phosphorylase). After fixing, the gels were silver stained and scanned.

**RESULTS AND DISCUSSION**

**SFD Trehalose Powder**

SFD pure trehalose particles obtained from a 20% w/w solution (Figure 2a) show diameters between approximately 20 and 90 µm. The ultrasonic nozzle used in this work (120 kHz; 3 mL/min solution feed rate) gives a wide droplet size distribution between 15 and 100 µm, as measured by laser diffraction (data not shown). It is therefore evident that no substantial shrinkage of the frozen droplets occurs during SFD, in contrast to that occurring with evaporative water loss during SD. Figure 2a also shows a microporous surface morphology of the particles. The result from Hg porosimetry shown in Figure 2b illustrates large interparticulate spaces in the powder at around 20 µm, and smaller intraparticulate pores in the submicron range. The latter are the “ice ghosts” remaining after sublimation of the ice crystals formed on freezing in the LN2. Their cumulative pore volume equals approximately 1000 mm³/g, as shown by the dotted line in Figure 2b. Freezing of small, aqueous droplets is very rapid (3.2 ms calculated for a droplet diameter of 30 µm) and is expected to cause a high degree of supercooling, and hence formation of very small ice crystals within the frozen droplets. High internal particle porosity is confirmed from the microtome section of the cold curing resin containing the embedded SFD trehalose particles (Figure 2c). The particles are not hollow as frequently produced by SD, but rather solid and contain numerous channel-like pores within the spherical shape of a particle. The microporous appearance of the particles’ surface under SEM (cf. Figure 2a) reflects therefore a highly porous inner structure. The Hg porosimetry result in Figure 2b is thus a measure of those intraparticulate pores that open to the outside of the particle and can be penetrated by the Hg. As with SD and freeze drying (FD), the SFD trehalose particles are fully amorphous under XRPD (result not shown, but identical to previously published diffractogram).

When stored at 33% RH/25°C the SFD trehalose powder takes up some 4.5% moisture after 1 h (Figure 3). It is therefore much more hygroscopic than SD trehalose powders of particle diameter <5 µm, which took up only 1.5% moisture after 2 h storage in a 33% RH atmosphere. The SFD trehalose particles are some 10 times larger than these SD particles, yet much more hygroscopic because of their higher internal porosity and the resulting larger specific surface area for moisture uptake. As a comparison, SFD rhDNase particles had a 40 times greater specific surface area than the spray-dried protein, as measured by Hg porosimetry. The use of an annealing step at the beginning of the primary drying phase (initiated on reduction of chamber pressure) can reduce this hygroscopicity. The total moisture uptake after 2 h annealing at \( T_{shelf} = 0°C \) (Figure 3) is reduced from approximately 4.5 to 3.5% after 1 h exposure to 33% RH. Annealing up to 6 h does not further
improve this result. The reason for this reduction in hygroscopicity can be found by examining the SEMs. SFD trehalose after 30 min annealing at $T_{shel} = 0^\circ C$ has the identical appearance to that shown in Figure 2a without annealing. After 6 h annealing, however, the SEM in Figure 4a illustrates that the small and also, to some extent, the medium-sized particles now have a rugged surface and a shrunken appearance. A partial collapse and shrinkage of the trehalose has evidently occurred, which could reduce specific surface area, and hence, moisture uptake. The internal morphology of the annealed SFD trehalose particles also shows an altered appearance (Figure 4b), with loss of much of their pore structure in the particle center. The Hg porosimetry result (not shown) is, however, only marginally changed from that of the nonannealed powder in Figure 2b. This is a further indication that the Hg only penetrates the outer pores in a particle, which are not effected by annealing (cf. Figure 4a—annealed—with Figure 2a—nonannealed). It cannot penetrate into the center region of a particle where annealing causes structural change (Figure 4b), presumably because the pores are not continuous structures. Figure 4a also shows that the larger and also some of the middle-sized particles have not been affected by annealing and still show the typical smooth surface seen without annealing (cf. Figure 2a). Maa and Prestrelski\(^7\) calculated freezing times in LN2 of 0.84 and 3.2 ms for aqueous droplets of diameter 16 $\mu m$ and 32 $\mu m$,

**Figure 2.** Structure of SFD trehalose prepared from a 20% w/w aqueous solution. (a) SEM of particles; (b) Hg porosimetry result presented as pore volume (mm$^3$/g) versus pore diameter ($\mu m$) on logarithmic scale. Solid line is frequency, and the dotted line is cumulative frequency of pores; (c) Section of Technovit-embedded particles viewed via SEM and showing internal structure.
respectively. Since our droplets and SFD trehalose particles show wide distributions of diameters, we expect a substantial difference in freezing time between the smallest (approximately 20 μm) and largest (approximately 90 μm) frozen particles formed. The larger particles freeze more slowly and contain therefore fewer, larger ice crystals, and have therefore a coarser frozen trehalose structure than that in the smaller particles, as suggested by Costantino for SFD polylactate. During annealing this coarser trehalose phase may be less prone to collapse and shrinkage than is the finer structure present in the smaller particles. In this case, the larger SFD particles would be less likely to show effects of annealing than the small particles, as observed in Figure 4a. This lack of effect on large particles can be countered, however, by increasing the annealing temperature to $T_{shelf} = +5^\circ C$, which produced

![Graph](image1.png)

**Figure 3.** Moisture uptake kinetics of SFD trehalose particles (33% RH/25°C) annealed for various times at $T_{shelf} = 0^\circ C$ during primary drying. This figure also includes the negative moisture uptake curve of pure SFD mannitol.

![Image](image2.png)

![Image](image3.png)

**Figure 4.** Effects of annealing on properties of pure SFD trehalose prepared from a 20% w/w aqueous solution. (a) SEM of powders obtained after annealing at $T_{shelf} = 0^\circ C$ for 6 h. (b) Section of Technovit-embedded particles viewed by SEM showing internal structure; (c) SEM of pure SFD trehalose annealed at $T_{shelf} = +5^\circ C$ for 1 h. This should be compared with Figure 2a, which shows the nonannealed particles of pure SFD trehalose.
shrinkage of all particles after just 1 h annealing (Figure 4c). Again, no substantial change in the Hg porosimetry result (not shown) occurs. We could not measure the product (the frozen powder) temperature, \( T_{\text{prod}} \), during these annealing experiments at high \( T_{\text{shelf}} \). Evaporative cooling will, however, certainly make \( T_{\text{prod}} \) lower than \( T_{\text{shelf}} \). Clearly, great care must be taken to avoid excessive collapse or ice melting in the frozen product and consequent failure of primary drying.

**SFD Trehalose/Mannitol Powders**

Mixtures of trehalose + mannitol (consistently at 20% w/w total solids’ content) containing a mannitol/trehalose weight ratio, \( w_{\text{m:t}} \), of up to 0.4:0.6 give fully amorphous SFD powders (Figure 5a). Amorphous mannitol in a formulation containing other excipients has been identified in both FD\(^{17}\) and SD\(^{18}\) systems, where it plasticizes disaccharides because its glass transition temperature, \( T_g \), (fully dry) is 13°C.\(^{17}\) Higher values of \( w_{\text{m:t}} \) lead to crystallinity, which increases linearly in degree up to pure mannitol, as illustrated in Figure 5a. Pure SFD mannitol from a 20% w/w solution has a crystallinity index of approximately 0.6 (e.g., 40% amorphicity) compared with the fully crystalline starting material. Its XRPD (Figure 5b) corresponds mainly to the \( \beta \)-form,\(^{17}\) with small amounts of the \( \alpha \)- or \( \delta \)-polymorphs. The rapid loss of 0.5% w/w moisture on storage of the pure SFD mannitol powder at 33% RH/25°C (see Figure 3) may be a consequence of crystallization of the amorphous part, although this was not confirmed by XRPD. The SFD pure mannitol particles viewed under SEM are evidently porous (Figure 5c), and the particle surface shows a “platelet” structure reported before with SD mannitol and considered to be indicative of crystallinity.\(^{19}\) The section through the embedded particles shows wide channel-like pores (Figure 5d). The Hg porosimetry result (Figure 5e) also shows, apart from the interparticulate pores at approximately 20 \( \mu \)m, a wide intraparticulate porosity between 0.1 and approximately 3 \( \mu \)m. The cumulative pore volume is approximately 1700 mm\(^3\)/g, much larger than the 1000 mm\(^3\)/g found with trehalose. The greater porosity of SFD mannitol than SFD trehalose must reflect different ice growth patterns in these two carbohydrate solutions, despite the extremely rapid freezing process in LN2 (a few ms\(^{-1}\)). Clearly, care must be taken in interpreting the Hg porosimetry results, and Figure 5e only reflects those pores penetrable by the Hg. It is possible, however, that ice crystal growth in the mannitol/water eutectic is easier than in the trehalose/water partially freeze-concentrated system on plunging into LN2.

The residual moisture contents of the trehalose/mannitol powders measured immediately after there preparation decrease linearly with increasing \( w_{\text{m:t}} \) (Figure 5f). It follows that addition of mannitol causes improved freeze drying of the amorphous trehalose in that range of \( w_{\text{m:t}} \) where the mannitol remains noncrystalline. For example, the mixture \( w_{\text{m:t}} = 0.4:0.6 \) is still fully amorphous, but contains only approximately 2.3% w/w moisture compared with 3% w/w moisture in pure amorphous SFD trehalose, \( w_{\text{m:t}} = 0:1.0 \). The improved drying of the solid solution of trehalose/mannitol is attributable to the plasticising action of the mannitol, which—by reducing \( (T - T_g) \) or \( (T - T_k) \)—would increase the diffusivity of the water molecules within the freeze concentrate during secondary (diffusional/evaporational water loss) drying. \( T \) is here the observation temperature, and \( T_k \) is the Kautzman temperature. Recall that although pure SFD mannitol contains approximately 1.2% w/w moisture, 0.5% w/w is evidently held within the amorphous part and is lost on crystallization (cf. Figure 3).

**SFD Trehalose/Mannitol/Dextran Powders**

The addition of dextran 37500 to an amorphous trehalose/mannitol mixture (consistently at 20% w/w total solids’ content) gave improved particle properties. SFD particles of trehalose/mannitol/dextran (0.4:0.4:0.2) show the “platelet” surface structure (Figure 6a) also observed with pure mannitol. Because these particles were purely amorphous (XRPD not shown), the platelets can have nothing to do with mannitol crystallinity.\(^{19}\) We cannot, however, rule out some sort of phase separation of different amorphous phases. There is less visible surface porosity than with either pure trehalose or mannitol. The internal structure shows very fine pores homogeneously distributed within the particle matrix (Figure 6b). Annealing at \( T_{\text{shelf}} = +5°C \) for 1 h produced a notably homogeneous shrinkage of all particles in the viewing field (Figure 6c). Dextran 37500 is a low melting point, polymeric excipient which, we suggest, promotes softening and flow of the solids’ phase during secondary drying at \( T_{\text{shelf}} = 20°C \) (cf. Figure 1). On annealing, the surface can now shrink and collapse readily. The
Figure 5. Properties of SFD mannitol/trehalose and pure mannitol powders. (a) Crystallinity index (CI) determined by wide-angle X-ray diffraction of SFD trehalose containing increasing weight fraction of mannitol.

\[
CI = \frac{AUC_{\text{crys peaks}}^\text{sample}}{AUC_{\text{total}}^\text{starting material}} \times 100;
\]

(b) Wide-angle X-ray diffraction pattern of pure SFD mannitol. The positions of the reflections correspond mainly to the \(\beta\)-form of mannitol. (c) SEM of SFD pure mannitol. Note “platelet” structure of particle surface. (d) Section of Technovit-embedded particles of pure SFD mannitol viewed by SEM showing internal structure. (e) Hg porosimetry result for SFD pure mannitol. The solid line is frequency, and the dotted line is cumulative frequency of pores. (f) Moisture content of SFD mannitol/trehalose powders containing increasing weight fraction of mannitol, \(w_{\text{m:t}}\). The measurements were performed by Karl-Fischer immediately after preparation of the powders by SFD.
Hg porosimetry result for the annealed powder (not shown) has the same cumulative pore volume as with nonannealed trehalose, but, again, is just measuring those pores penetrable by the Hg. These observations again show the strong influence of annealing and formulation on morphology of the SFD particles.

SFD Trypsinogen Formulations

SFD pure trypsinogen from a 20% w/w aqueous solution shows high porosity of the particles viewed from the surface (Figure 7a). If SFD trypsinogen powder is rehydrated (i.e., redissolved in water) immediately after production, substantial loss in enzymatic activity is found compared with the untreated protein. On rehydrating SFD trypsinogen powders prepared from 50 mg/mL and 100 mg/mL aqueous spray solutions, we measured mean losses in protein activity ± standard deviation (n = 4 determinations from two independent experiments performed on different days) of 13.5 ± 5.2% and 15.1 ± 2.1%, respectively (Table 1). If % mean activity loss is equated with mass of trypsinogen inactivated, then these values are equivalent to 6.8 mg/mL and 15.1 mg/mL of protein, respectively. The higher the solution concentration of the protein in the range examined here, the greater the mass of protein inactivated. An obvious, possible cause of such inactivation is molecular aggregation, which is frequently encountered during the solid state processing of proteins.20 The inactivation of trypsinogen

Figure 6. Properties of a trehalose/mannitol/dextran 37,500 (0.4:0.4:0.2) powder prepared by SFD. (a) SEM of powder. Again, note “platelet” structure of surface. (b) Section of Technovit-embedded particles viewed by SEM to illustrate internal structure. (c) SEM of SFD powder obtained after annealing at $T_{shelf} = +5$°C for 1 h. Note highly wrinkled, homogeneously shrunk particle surface structure.
measured after SFD can, however, only marginally be attributed to aggregation of the protein. Consider the size exclusion chromatogram (SEC) in Figure 8a obtained after rehydrating the SFD trypsinogen powder produced from the 50 mg/mL spray solution. There is a strong monomer peak at 24,000 and some clips at lower molecular weight, but only a small dimer peak at 48,000, which was not present in the untreated trypsinogen (see inset to Figure 8a). Integration of the chromatogram (for \( n = 3 \) injections) yields 95.7 ± 0.15% relative monomer, 2.9 ± 0.2% relative clips, and just 1.4 ± 0.09% relative dimer. The rehydrated untreated enzyme shows 97.6% relative monomer, 2.4% relative clips, and 0% relative dimer. The amount of dimer generated during SFD/rehydration is therefore numerically equal to the loss of monomer. The presence of 1.4% aggregates does not, however, compare with the 13.5% inactivation of this SFD trypsinogen powder.

Figure 7. (a) SEM of pure SFD trypsinogen prepared from a 20% w/w solution. (b) Inactivation of trypsinogen on atomization of aqueous solutions into a glass beaker. % Activity loss and inactivated mass of protein in dependence of protein solution concentration. Mean values plus/minus standard deviation of two independent determinations performed on the same day \((n = 2)\). The two points (■) at 5 mg/mL and 50 mg/mL were determined 1 year later in a repeat experiment conducted on a single day in response to the comments of one of the reviewers \((n = 2)\). (c) Effect of trypsinogen/trehalose mass ratio in SFD powders on residual activity of trypsinogen measured immediately after preparation. Mean value plus/minus standard deviation of two independent experiments with one sample being determined from each experiment. (d) Activity loss of a 50 mg/mL aqueous solution of trypsinogen on atomization into a glass beaker in dependence of added amount of polysorbate 80. Although these are single determinations \((n = 1)\), the constant trend to less inactivation is unambiguous.
The aggregation behaviour is confirmed by the result of SDS-PAGE. Lanes 8 and 9 of the gel shown in Figure 9 were obtained from the dissolved SFD trypsinogen powder prepared from the 50 mg/mL spray solution. Apart from the strong monomer band, a dimer band is seen and also a shadow of some higher aggregates lying between 48 kD and 97 kD.

Tzannis and Prestrelski\textsuperscript{13} found similar behaviour on SD of trypsinogen. Pure trypsinogen powder prepared from a 20 mg/mL trypsinogen spray solution showed a 14.9 ± 3.3% activity loss after rehydration, but the presence of only 8.4 ± 0.3% aggregates. These authors proposed three pathways that operate during SD of dissolved trypsinogen to the solid state. The first pathway (denoted S3) retains native structure and hence also activity. The second (S2) leads to protein aggregation, and the third (S1) results in partial protein unfolding without aggregation, both producing partial loss of activity. On rehydration, some of the partially unfolded molecules fail to readopt their native structure and therefore remain unfolded in solution, but not aggregated. In the case of SD, these partially unfolded, inactivated protein molecules account for some 2/5ths of total protein inactivation, i.e., the difference between 14.9% inactivation and 8.4% aggregation from a 20 mg/mL protein spray solution.\textsuperscript{13} On SFD the difference between 13.5% inactivation and 1.4% aggregation for the 50 mg/mL trypsinogen spray solution equals >9/10ths of total protein inactivation. Although this value is much higher than that with SD of the same protein, we take it also to represent partially unfolded trypsinogen present in the SFD powder (pathway S1). This does not refold on rehydration and hence remains inactive, but not aggregated. Circular dichroism and SEC have indeed shown that urea or guanidine-HCl induced unfolding of bovine trypsinogen in water is not a simple transition, but rather involves a molten globule intermediate.\textsuperscript{21} Our results show, therefore, that aggregation of trypsinogen is minimal during SFD/rehydration, but that a substantial fraction of the protein is partially and irreversibly unfolded to an inactive state, i.e., a molten globule.

SFD is a multistep process comprising sequential atomization, rapid freezing in LN2, and freeze drying. The question now arises, in which of these steps a partial, irreversible unfolding of the trypsinogen could occur? We consider first atomi-

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<tr>
<td>50</td>
<td>—</td>
<td>25</td>
<td>FD</td>
<td>0.70 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>95</td>
<td>SFD</td>
<td>+1.55 ± 3.1</td>
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\textsuperscript{a}Mean value plus/minus standard deviation of four determinations from two independent experiments performed on different days.

\textsuperscript{b}Mean value plus/minus standard deviation of two determinations from 1 experiment.
zation of trypsinogen spray solution in the relevant concentration range of 0–100 mg/mL protein. Figure 7b shows protein activity loss measured immediately after atomization with the ultrasonic nozzle from a height of 10 cm into a glass beaker (the same construction as with SFD). Increase in trypsinogen concentration starting at 5 mg/mL leads to higher % activity loss, which reaches a plateau of approximately 15% at protein concentrations ≥50 mg/mL in the spray solution. Again, if we equate % activity loss with mass of trypsinogen inactivated, then Figure 7b shows that the latter increases linearly with protein solution concentration up to 100 mg/mL. At 50 and 100 mg/mL, approximately 7 and 15 mg/mL trypsinogen are inactivated. The values for both % activity loss and inactivated mass are, therefore, the same as those obtained after rehydration of the SFD powders prepared from these same two spray solutions (cf. Table 1). Additionally, the SEC shown in Figure 8b for the atomized 50 mg/mL trypsinogen solution shows no indication of aggregation. Integration of the chromatogram (for n = 3 injections) yields 97.3 ± 0.17% relative monomer, 2.7 ± 0.17% relative clips, and 0% dimer, which is unaltered from the untreated trypsinogen starting material. Lanes 2 and 3 of the SDS-PAGE gel of the atomized 50 mg/mL trypsinogen solution (Figure 9) confirm that no dimers or higher aggregates (<97 kD) are present in the atomized solution. Atomization of the trypsinogen solution therefore produces no measurable aggregation. As with SFD, the measured inactivation of the trypsinogen is attributed to irreversible partial unfolding, i.e., formation of a molten globule state via pathway S1.

Maa et al.20 found that rhGH aggregated on atomization through a two-fluid nozzle (droplet...
It is, therefore, quite reasonable that an increase in trypsinogen solution concentration would produce a greater \( \Gamma \) of protein at times \((\leq 1 \text{s})\) before equilibrium surface adsorption is reached, provided the surface is not saturated with protein.

The second step of SFD is rapid freezing of the spray droplets into LN2. A surprising result is obtained on atomising a 100 mg/mL trypsinogen solution into LN2 followed by boiling off of the LN2, melting of the frozen droplets at room temperature and recovery of the solution without freeze drying. This solution shows no measurable loss in activity \((0.70 \pm 1.5\% \text{ for } n = 4 \text{ determinations}; \text{ see Table 1})\) and also no signs of protein aggregation. Lanes 4 and 5 of the SDS-PAGE gel (Figure 9) have no dimer or higher aggregate \((\leq 97 \text{ kD})\) bands, and are identical to that of the untreated enzyme in Lane 10. The full retention of enzymatic activity and absence of any measurable aggregation means that full native structure of the trypsinogen exists in the recovered solution.

Atomization of the protein solution into LN2 involves the sequential steps of atomization into air followed by freezing of the spray droplets in LN2. We recall, however, that the first of these two steps—atomization into air—results in 14.5 \(\pm 2.0\%\) inactivation of the 100 mg/mL trypsinogen solution (Figure 7b), attributed to irreversible partial unfolding. This paradoxical result can be resolved in one of two ways. The first way postulates that a partial unfolding of trypsinogen does not occur on atomization into the air 10 cm above the LN2 surface. We consider this to be very unlikely. The second way postulates that partial unfolding occurs during atomization as observed in Figure 7b, but that this is reversed during droplet quench freezing in LN2 with subsequent thawing.

The third step of SFD is freeze drying of the frozen spray droplets (the LN2 boils off at \(T_{\text{shelf}} = -45^\circ \text{C}\) before commencement of primary drying). On rehydration of a freeze-dried, non-atomized 100 mg/mL trypsinogen solution (freezing program given in Figure 1), a mean activity loss of 16.0 \(\pm 2.4\%\) \((n = 2 \text{ determinations}; \text{ see Table 1})\) is found. This is accompanied by substantial aggregation of the protein. The SEC in Figure 8c shows two peaks corresponding to 85.9 \(\pm 0.19\%\) relative monomer and 14.1 \(\pm 0.07\%\) relative dimer \((n = 3 \text{ injections})\). Within the experimental error, the % activity loss can therefore be accounted for by the measured % aggregation.

FIGURE 9. SDS-PAGE gel of various trypsinogen formulations prepared in different ways. Lanes: (1) standards; (2+3) trypsinogen solution (50 mg/mL) atomized into glass beaker and recovered; (4+5) trypsinogen solution (100 mg/mL) atomized into LN2, thawed, and recovered; (6+7) trypsinogen solution (100 mg/mL) freeze/thawed; (8+9) rehydrated SFD pure trypsinogen (prepared from 50 mg/mL trypsinogen solution); (10) nontreated trypsinogen starting material.

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The authors suggested that aggregation was a result of protein adsorption and unfolding at the large liquid/air interface of the spray droplets. Although the trypsinogen does not aggregate on atomization, its measured inactivation means some loss in native structure, i.e., irreversible partial unfolding. In our experiments, droplet lifetime was of the order of 1 s before the droplets recombined to form bulk liquid in the glass beaker. Measurements of the dynamic surface tension of trypsinogen at the water/air interface show that a solution concentration of 50 mg/mL produces a surface excess concentration of the protein, \(\Gamma\), of approximately 6 mg/m\(^2\) after 1-ms interface lifetime, increasing to approximately 20 mg/m\(^2\) after 1000-ms interface lifetime. The trypsinogen will therefore have a positive \(\Gamma\) at the large liquid/air interface of the spray droplets during their 1-s lifetime. Could this adsorbed protein be the source of the measured inactivation and, hence, also of irreversible partial unfolding of the trypsinogen during atomization? The observed increase in mass of trypsinogen inactivated with greater protein solution concentration (Figure 7b) suggests this may be the case. The early stages of protein adsorption to a liquid/air interface are rate limited by diffusion of the protein molecules from the adjacent region to the interface. The short-time approximation of Ward and Tordai’s equation predicts that the surface pressure, \(\Pi\) [mN m\(^{-1}\)], is directly proportional to the protein solution concentration, \(c_0\), at any fixed time. It is, therefore, quite reasonable that an increase in trypsinogen solution concentration would produce a greater \(\Gamma\) of protein at times \((\leq 1 \text{s})\) before equilibrium surface adsorption is reached, provided the surface is not saturated with protein.

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of the trypsinogen occurring via pathway S2. On FD/rehydration it is not necessary to consider the formation of an inactive, partially unfolded form via the S1 pathway.

There is, therefore, a fundamental difference between trypsinogen inactivation during SFD/rehydration and that during FD/rehydration. On SFD/rehydration the protein is inactivated by irreversible partial unfolding via pathway S1, whereas on FD/rehydration it is inactivated by irreversible aggregation via pathway S2. It follows that FD, when performed as part of SFD, produces a different effect on trypsinogen (minimal aggregation, partial unfolding) than when it is performed as an isolated process (strong aggregation). The difference between FD and SFD is, of course, the use of an atomization step in the latter process. With FD, the bulk solution contained in a vial is frozen by reducing $T_{\text{shelf}}$ from room temperature to $-20^\circ\text{C}$ at $\Delta T_{\text{shelf}} = 2^\circ\text{C}$/min. With SFD, droplets of diameter $\leq 100\ \mu\text{m}$ are quench frozen in the LN2 before being transferred to the precooled shelf ($T_{\text{shelf}} = -45^\circ\text{C}$). Because atomizing a 100 mg/mL trypsinogen solution into LN2 (followed by remelting) results in neither inactivation (Table 1) nor aggregation (Figure 9), protein damage during FD must follow different pathways for either frozen droplets or frozen bulk solution.

Trypsinogen contained in frozen droplets is not aggregated, but rather suffers irreversible partial unfolding and loss of activity on rehydration. Trypsinogen in frozen bulk solution is irreversibly aggregated and looses thus activity on rehydration. Quench freezing of droplets $\leq 100\ \mu\text{m}$ diameter will not only be exceedingly rapid; it will also result in less freeze concentration of the protein than occurs on slow freezing of the bulk solution during FD. The formation of a partially unfolded state rather than complete aggregation of the protein during the FD step of SFD could, therefore, be a result of higher nonfrozen water content and lower protein concentration within the amorphous phase of the frozen droplets, compared with the maximally freeze-concentrated bulk solution during isolated FD.

The cause of trypsinogen aggregation and inactivation during isolated FD of the protein bulk solution can be deduced from the freeze/thawing result of a 100 mg/mL trypsinogen solution (without drying). This produces no measurable activity loss ($1.3 \pm 2.0, n = 4$ determinations) of the trypsinogen (Table 1). Lanes 6 and 7 of the SDS-PAGE gel (Figure 9) also show no aggregation of the protein. The aggregation and inactivation of trypsinogen observed after isolated FD/rehydration therefore appears to be a result of water removal from the trypsinogen molecule during primary/secondary drying of the maximally freeze-concentrated system. It is not a consequence of freezing stresses such as freeze concentration or ice surface adsorption, because both of these effects would also be expected during freeze/thawing of the solution.

The addition of trehalose to the spray solution stabilizes the trypsinogen during SFD. Figure 7c shows that a trypsinogen/trehalose mass ratio of 2:1 already completely ameliorates the original 15% loss in activity of the pure protein (50 mg/mL) measured on SFD/rehydration. The SEC obtained on rehydrating this powder (Figure 8d) shows no dimer peak, and is identical to that of the untreated trypsinogen shown in the inset. As a result, the activity of the pure protein (Table 1). Isolated FD/rehydration of the bulk trypsinogen/trehalose (2:1) solution produces, however, no loss in protein activity (Table 1), attributable to the effectiveness of trehalose as a lyoprotectant by reason of glassy immobilization/water replacement. This result confirms that the activity loss of trypsinogen during SFD occurs during the FD step. Both SFD and FD yield full protein protection for the trypsinogen/trehalose (2:1) formulation. As elucidated above, there is no activity loss during atomization and droplet freezing in LN2 as part of the SFD.

Further light can be thrown on this matter by considering the effects of added surfactant, which can stabilize proteins during SD or FD. One milligram per milliliter polysorbate 80 in the 100 mg/mL trypsinogen spray solution reduces protein inactivation during SFD from $15.1 \pm 2.1\%$ to $6.9 \pm 1.9\%$ ($n = 2$ determinations, Table 1). To explain this, we again consider the individual, sequential steps of the SFD process. First, atomization into air, where increasing concentration of polysorbate 80 progressively reduces the % activity loss of a 50-mg/mL trypsinogen solution (Figure 7d). Maa et al. reported that the addition of polysorbate 20 reduced aggregation of rhGH on atomization, and claimed this confirmed the likelihood of liquid/air interfacial adsorption as the cause of protein damage. Greater adsorption of surfactant than of protein at the liquid/air interface can occur under equilibrium adsorption.
conditions. At the short times involved in atomization (≤1 s), however, other effects such as binding of surfactant to protein in bulk solution, and/or change in the free energy of protein unfolding by the surfactant may be more likely. At a polysorbate 80 concentration of 1 mg/mL there is no stabilizing effect on the trypsinogen during atomization into air (Figure 7d), although this surfactant concentration more than halved the trypsinogen inactivation during SFD (Table 1). This result confirms the idea voiced above that trypsinogen inactivation on SFD/rehydration occurs during the FD step. The effect of surfactant during isolated FD provides further evidence for this idea. The addition of 1 mg/mL polysorbate 80 to the 100 mg/mL trypsinogen solution reduces % protein inactivation during FD from 16.0 ± 2.4% to 6.6 ± 4.4% (n = 2 determinations, see Table 1). This action of surfactant during FD can therefore account for the measured reduction in inactivation occurring during SFD. Taken together, these results strongly suggest that trypsinogen inactivation on SFD/rehydration occurs during the FD step and not during atomization into the LN2. Indeed, they complement the finding that no inactivation of trypsinogen occurred on atomization into LN2 (Table 1). If the cause of trypsinogen inactivation on FD is water removal, then the protecting effect of the surfactant can most readily be attributed to change in free energy of protein unfolding or reduced dissolution rate of protein on solid rehydration.

The use of electron spectroscopy for chemical analysis (ESCA) provides the surface composition of SFD particles. SFD pure trehalose yields the relative atomic concentrations in the surface layer shown in Table 2, which are reasonably close to the theoretical values for O11C12 shown in brackets. The ESCA spectrum of untreated pure trypsinogen is shown in Figure 10a. As expected for a protein, a strong N1s-peak is evident, equivalent to approximately 15 relative atomic % N (Table 2; note that H is not detected by ESCA). SFD trypsinogen/trehalose (15:85) from a 20% w/w total solids’ solution gives the ESCA spectrum shown in Figure 10b, having 6.6 relative atomic % N in the particle surface (Table 2). This N surface concentration is some three times higher than that expected from a homogeneous distribution of the protein throughout the particle (equal to 2.25 relative atomic % N at Γ = 0). A positive Γ of the protein in the particles therefore exists. A simple calculation shows that 6.6% relative atomic N is equivalent to a particle surface composition of 44 parts trypsinogen and 56 parts trehalose. This relative composition must also have existed at the water/air interface of the atomized droplets on their freezing in the LN2, because molecular motion of the protein and sugar is stopped at this point during the SFD process. The trypsinogen will, therefore, have had a substantial positive Γ at the liquid/air interface of the spray droplets.

Addition of polysorbate 80 to the spray solution causes a progressive, linear reduction in N1s, showing decreasing presence of the protein in the surface of the SFD particles (Figure 10c). Note that in this experiment, the trypsinogen/trehalose mass ratio was 5:95 (from a spray solution containing 50 mg/mL protein), which gives a relative atomic % N of 3% in the absence of surfactant (Table 2). This N surface concentration is some four times higher than a homogeneous protein distribution (0.75% relative atomic % N at Γ = 0), and the surface is composed of 20 parts trypsinogen to 80 parts trehalose. Incidentally, this finding with lower protein loading supports the suggestion made above that increase in trypsinogen solution concentration will produce a greater Γ of protein at the liquid/air interface of droplets during atomization into air. It follows from Figure 10c that progressive exclusion of the protein from the solid, and hence, also from the spray droplet surface occurs between 1 and 10 mg/mL polysorbate 80 concentration in the spray solution. Ten milligrams per milliliters polysorb-
bate 80 is sufficient to reduce the relative atomic nitrogen to 1.0%, which is only marginally higher than the 0.75% for a homogeneous distribution of protein between bulk and surface, i.e., $G = 0$. It is not enough, however, to cause any measurable reduction in activity loss of the same concentration of trypsinogen (50 mg/mL) during atomization into the glass beaker (cf. Figure 7d).

Recall that $G = 0$ means that the same concentration of dissolved protein molecules exists in the bulk liquid and at the surface. Intuitively it is unlikely that such a substantial reduction in $\Gamma$ would be accompanied by no improvement in protein stability if adsorbed protein was the source of inactivation on atomization into air. We conclude that inactivation of trypsinogen during atomization into air (cf. Figure 7b) cannot be attributed to the substantial positive $\Gamma$ of the protein at the air/liquid interface of the spray droplets.

The surface of the surfactant-free SFD trypsinogen/trehalose (5:95) particles, and hence, also the spray droplets from which it was formed, contains 20 parts protein to 80 parts trehalose (cf.

![Figure 10](image1.png)

**Figure 10.** Results of electron scattering for chemical analysis (ESCA) of some SFD powders and starting materials. (a) ESCA spectrum of untreated pure trypsinogen. Note strong N1s-peak equivalent to approximately 15.1% relative atomic concentration of nitrogen; (b) ESCA spectrum of SFD trypsinogen/trehalose (15:85). The N1s relative atomic concentration of 6.6% should be compared with 2.25% expected if the protein were homogeneously distributed throughout the particles, i.e., if $\Gamma$ were zero; (c) effect of increasing concentration of polysorbate 80 in spray solution on N surface coverage of SFD trypsinogen/trehalose (5:95).
Table 2). This formulation shows no loss in protein activity during SFD/rehydration \((n = 4\) determinations, Table 1). We have already seen that the trehalose does not prevent inactivation of the protein during atomization into air, but only during FD (Table 1). This result further supports the argument that inactivation of trypsinogen during SFD does not occur during atomization, but rather during the FD step.

**CONCLUSIONS**

This first part of this work presents some possibilities for tailoring the properties of SFD particles used as protein carriers. We have demonstrated that the use of variation in formulation and/or process conditions modifies particle properties. We could identify a trehalose/mannitol/dextran (0.4:0.4:0.2) formulation giving SFD particles prepared using annealing during the freeze-drying step, which shows homogeneous external and internal morphology.

The behavior of our model protein, trypsinogen, during the sequential steps of SFD is complex. Taken together, the results suggest that irreversible, partial unfolding of the trypsinogen occurs during SFD, i.e., a fraction of molten globules is formed. This destabilization is not related to protein adsorption at the liquid/air interface of the spray droplets, but occurs during the FD step.

Of particular interest is the finding that atomization of an aqueous trypsinogen solution into LN2 reverses protein inactivation shown to occur on atomization into air. Further research is necessary to examine and explain further this quite unexpected behavior.

**REFERENCES**

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