Activity–Stability Considerations of Trypsinogen during Spray Drying: Effects of Sucrose

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Abstract □ The preparation and processing of protein pharmaceuticals into powders may impose significant stresses that could perturb and ultimately denature them. In many cases their stabilization through added excipients is necessary to yield native and active proteins. In this study, the effect of spray drying on the structure and activity of a model protein (trypsinogen) was investigated. In the absence of excipients, spray drying resulted in small losses of its enzymatic activity. Protein conformational rearrangements in the solid state (observed via FTIR) and irreversible aggregation (upon reconstitution) constituted the major degradation pathways. The irreversible unfolding in the solid state was also confirmed by solution calorimetric studies that indicated a decreased thermal stability of the spray-dried protein after reconstitution. The presence of sucrose, a thermal and dehydration stress stabilizer, induced a concentration-dependent protective effect. Protein protection was afforded even at low carbohydrate concentrations, while at specific mass ratios (sucrose-to-protein = 1:1) complete activity preservation was achieved. However, at the high end of sucrose concentrations, a small destabilization was evident, indicating that excluded volume effects may be undesirable during preparation of protein microparticles via spray drying. The profile of both the protein conformational changes and thermal stability in the solid state closely followed that of the incurred activity losses, indicating that protein stabilization during dehydration is crucial during processing of these polypeptides.

Introduction

The increased needs for accurate, on-site, and controlled delivery of therapeutic peptides and proteins have led to novel strategies for their release.[1,2] However, their application and successful commercial development may require the formulation of these labile pharmaceutics into alternative forms to improve their bioavalability as well as to achieve stability levels beyond the conventional shelf life requirements. One such approach is their preparation into solid microparticle forms. In the particular case of pulmonary delivery via dry powder inhalers, this requirement becomes even more important since the efficient deposition to the lung, and therefore the therapeutic benefit, requires particles in the range of 1 to 3 μm.[3–5] Further, to minimize physical and chemical degradation reactions and maintain the finished product's physical properties during long-term storage, the protein powders must be produced and maintained at low moisture contents.[6–9]

Several methods have been employed for microparticle production including spray drying,[10,11] spray freeze-drying,[12] ball or jet milling of lyophilized powders, precipitation in supercritical antisolvents,[13] and coprecipitation with water-soluble starch.[14] Of these, spray drying appears to be one of the most promising because of its capacity to produce particles with appropriate characteristics, narrow particle size distribution, process simplicity, and energy efficiency. Moreover, previous efforts have demonstrated the feasibility of its use for processing of pharmaceutical proteins.[11] In contrast to lyophilization, which utilizes freezing and sublimation processes to produce a cake during an energy-intensive and time-consuming process, spray drying is a fast and cost-effective dehydration process. During this operation, heat from a preheated gas stream is used to evaporate microdispersed droplets produced by atomization of a continuous liquid feed.

Among the main drawbacks in the production of protein solid state formulations are the severe stresses that may be imposed during processing. Previous studies have indicated that lyophilization, although it exerts significantly smaller thermal effects than spray drying, can have a significant impact on the structure and stability of several proteins.[15–17] Due to their nature, the processes involved in spray drying impose several stresses that can potentially destabilize labile biological molecules such as proteins. During atomization, proteins are exposed to high pressure and to potentially harmful air–liquid interfaces; both such stresses are known to compromise protein structural integrity[11,18] and decrease their enzymatic or biological activity.[18–20] Previous studies have suggested that heating and subsequent dehydration during the drying step constitute the major degradative stresses.[10] In particular, the outlet temperature during spray drying has been correlated with activity losses of heat-sensitive materials.[10,21,22] Although sucrose would be expected to aggravate interfacial denaturation during atomization via an increase of the solution surface tension,[23,24] it is known to exert an effective baroprotective effect.[25] Finally, well documented is the ability of disaccharides to preserve protein structure and activity in the solution state via their excluded volume effects,[24–26] as well as during thermal and dehydration stresses.[15,16,19,28]

In this effort, the effect of spray drying on the stability of a model protein, trypsinogen, and the impact of a common thermal and dehydration stress stabilizer, sucrose, were investigated. Through a variety of biophysical, chromatographic, and thermal methods of analysis, the degradation pathways of the protein in the presence and absence of sucrose during processing were investigated. Further, through examination of the conformation of spray-dried trypsinogen and its stability both in the solid state and after complete rehydration, we attempted to discern activity–stability relationships for the protein in the sucrose-containing microparticles. The results of the present study are expected to have further implications in production and stabilization of spray-dried pharmaceutical proteins.

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Experimental Section

Chemicals—Trypsinogen (1× crystallized, dialyzed against 1 mM HCl and lyophilized; lot no. 38E273N), trypsin (2× crystallized, dialyzed against 1 mM HCl, and lyophilized; lot no. M5K669) and lysozyme (2× crystallized, dialyzed, and lyophilized) were purchased from Worthington Biochemical Corporation (Freehold, NJ). Myoglobin, ovalbumin (crystallized and lyophilized), and bovine serum albumin (Cohn fraction V, 96% purity) and ultrapure sucrose (>99.5% purity via HPLC, lot no. 35H03582) were purchased from Sigma Chemical Co. (St. Louis, MO). All proteins were used without further purification. Potassium phosphate, calcium chloride, and sodium chloride salts were purchased from J. T. Baker (Phillipsburg, NJ). FTIR grade potassium bromide was purchased from Aldrich Chemicals (St. Louis, MO).

Experimental Design—Protein solutions were spray-dried at different protein-to-sucrose mass ratios in order to examine and compare the processing effect on protein stability as well as to determine the optimum range of sucrose concentrations within which the maximum stabilizing effects are exerted. The carbohydrate-to-protein weight ratios examined in this study were no sucrose, 0.25:1, 0.5:1, 1:1, 2:1, 4:1 and 8:1, which correspond to sucrose concentrations of 0, 0.015, 0.029, 0.058, 0.117, 0.234, and 0.47 M, respectively. All the results in this study are reported in terms of sucrose-to-protein mass ratios.

Spray Drying—Trypsinogen particles were prepared by spray drying in a Yamato mini spray dryer model ADL-31 (Yamato Scientific, Orangeburg, NY). The proteinaceous solutions (at different sucrose mass ratios) were prepared at constant protein concentration of 2% w/v in 1 mM HCl. The pH of all prepared solutions ranged from 3.05 to 3.15; this low pH is necessary to minimize autolysis of the enzyme.[29] Before operation, they were filtered through 0.2-μm nylon filters to remove contaminants or aggregates formed during the solution preparation. The solutions were continuously fed to the spray dryer at an approximate flow rate of 3 mL/min and were dried at an inlet temperature of 120 °C; outlet temperatures ranged from 85 to 90 °C. Both the drying and atomization processes utilized room air with relative humidities (RH) that during the experiments ranged between 35% and 55%. Powders were collected in a collection vessel through a cyclone trap and were then aliquoted in lyophilization vials into a drybox (Ray Products, Inc., El Monte, CA); the latter was operated under constant nitrogen purge, providing an atmosphere of 1% to 10% RH. The vials were then capped, sealed, and stored at −20 °C until further analysis.

Particle Size Analysis—The particle size of the spray-dried trypsinogen−sucrose powders prepared for this study was determined with a Horiba LA-900 (Irvine, CA) laser light size analyzer. Small amounts of powders were dispersed in an excess of poly(ethylene glycol) 400 by overnight stirring. The suspension concentration was adjusted to attain optimal obscuration. The samples were then analyzed in a static mode; measurements were repeated in triplicates over a 20-min period, to ensure that no dissolution or powder agglomeration occurred. The size distribution was expressed in terms of volume median diameter, particle size range, and span. The latter represents a measure of the width of the volume distribution relative to the median diameter and was defined as [D(v, 90)-D(v, 10)]/D(v, 50), where D(v, 90), D(v, 50), and D(v, 10) are the equivalent volume diameters at 90, 50, and 10% cumulative volume, respectively.

Moisture Analysis—The moisture content of the spray-dried trypsinogen powders was analyzed via Karl Fisher titration; assays were performed on an Aquatest 10 Karl Fisher Coulorimetric Titrator (Seradyn, Indianapolis, IN), that was calibrated with anhydrous methanol. The moisture content of the protein microparticles was monitored in triplicate using a methanol-extraction protocol. One milliliter of anhydrous methanol was added in 2-mL lyophilization vials containing known amounts (approximately 10 to 50 mg) of powder. The vials were then subjected to brief sonication and mild shaking, to facilitate the moisture extraction process. For the moisture determinations, 200 to 500 μL of the samples were injected in the Karl Fischer coulorimeter. The moisture content was calculated after subtraction of the background moisture of methanol, and results were expressed on a percent total weight basis.

Protein Concentration Determination—Protein concentration in solution was determined by UV/vis spectroscopy; spectra were recorded on a Beckman model DU 640 (Beckman Instruments Inc., Fullerton, CA) spectrophotometer equipped with an automatic six-cell holder, set up with the appropriate transport mechanism. The cells were thermostated via a constant temperature Neslab circulator, model RTE-111M (Neslab Instruments Inc., Newington, NH). Spectra were recorded at 25 °C between 240 and 400 nm at 50-second intervals with a scan speed of 240 nm/min using 1-cm path length quartz cuvettes. Protein concentrations were determined using the absorbance at 280 nm of the light scattering-corrected spectra and an extinction coefficient of 1.54 mL mg^{-1} cm^{-1};[30] light scattering corrections were performed using a built-in function.

Enzymatic Activity Assay—The proteolytic activity of trypsinogen was determined via an appropriate modification of an established assay.[31,32] Briefly, trypsinogen activity was analyzed by first activation to trypsin by addition of the equivalent of 20 μg of trypsin to a 1 mL solution of 0.5 mg/mL trypsinogen in 10 mM Tris-HCl, 20 mM $CaCl_2$ with 100 mM KCl, pH 8.0. Activation was allowed to reach a maximum by incubating for 4 h at room temperature. The activation process was then stopped by addition of 10 μL of 1 N HCl. The activity determination reaction was initiated by adding 33 μL of an appropriate dilution of the activated trypsinogen to 967 μL of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) (Worthington Biochemical Corp., Freehold, NJ). Enzymatic activity rates were followed by the rate of absorbance increase at 247 nm for 5 min at 25 °C. All activity assays were performed in triplicate, and the reported results were corrected for the intrinsic trypsin activity in the reaction mixture.

Aggregation Status—The presence and amount of soluble (covalent and noncovalent aggregates) in the spray-dried trypsinogen powders were determined via size exclusion HPLC analysis as follows: the stationary phase was a 300 Å, 30 cm × 7.8 mm Progel-TSK Supelco column (molecular weight range 10−120 kDa). The mobile phase consisted of a 10 mM phosphate buffer at pH 7.4 prepared in deionized water; the flow rate was set at 0.5 mL/min. Typically, 10 μL of the spray-dried protein samples reconstituted at approximately 5 mg/mL in 1 mM HCl were loaded onto the column and were eluted with the mobile phase at a flow rate of 0.5 mL/min. Experiments were performed on a Waters (Bedford, MA) system equipped with a 626 model pump and a 996 photodiode array detector. Prior to determination, the samples were loaded to a 717 Plus autosampler (Waters, Bedford, MA), which was thermostated at 4 °C; the column was maintained at room temperature. Trypsin, lysozyme, myoglobin, bSA, and ovalbumin were used as internal standards for molecular weight determination. The relative amount of monomeric trypsinogen was determined by comparison of the percent peak area to that of a standard solution of the protein.

In an effort to examine the presence of insoluble aggregates, 0.5 mL of the reconstituted solutions was centrifuged at 12000 rpm for 20 min in an Eppendorf centrifuge model 5415C (Brinkmann Instruments, Westbury, NY). The supernatant (400 μL) was carefully removed and filtered through 0.2 μm nylon filters; the filtrate was then diluted in 1 mM HCl, and its concentration was determined via UV−vis spectroscopy. The amount of insoluble (covalent and noncovalent) trypsinogen aggregates was determined via mass balance from the protein concentration before and after the centrifugation and filtration steps. All determinations were performed in triplicate.

Protein Secondary Structure—The secondary structure of the spray-dried trypsinogen powders was determined via Fourier transform infrared (FTIR) spectroscopy. FTIR spectra were recorded on a Nicolet Magna 550 spectrophotometer (Madison, WI) equipped with a DTGS detector. The spectrometer and the sample compartment were continuously purged, at a constant rate of 50 ft^3/min, with air that was dried via a Balston air-dryer (Whatman, Pleasanton, CA). Double-sided interferograms (1024) were collected in the 4000−900 cm^{-1} range at 4 cm^{-1} resolution, coadded, apodized with a Happ−Genzel function, and Fourier transformed.

Solution state FTIR spectra were recorded at room temperature in the transmission mode using CaF_2 windows with a 6 μm Mylar spacer (Graseby Specac, Smyrna, GA). The spectral solvent contributions in the solution state were removed by subtraction of a solvent spectrum, which was recorded under identical conditions; spectral subtractions were performed with the OMNIC software (Nicolet, Madison, WI) Version 2.0. The subtractions were performed in order to remove the combination water band at 2150 cm^{-1}. The resulting spectra, as well as the calculated second derivative spectra, were smoothed with a nine-point Savitzky-

Table 1—Particle Size and Size Distribution of Spray-Dried Trypsinogen-Sucrose Powders[a]

sucrose:trypsinogen mass ratio	median diameter[b] (μM)	size range (μM)	span[c]
no sucrose	5.0 ± 2.2	1.1–17.4	1.04 ± 0.02
0.25:1	5.2 ± 2.3	1.3–17.4	1.14 ± 0.03
0.5:1	5.6 ± 2.3	1.5–19.9	1.05 ± 0.03
1:1	5.7 ± 2.8	1.5–26.1	1.06 ± 0.01
2:1	6.4 ± 1.8	1.5–17.3	0.91 ± 0.05
4:1	6.0 ± 1.9	1.5–39.2	1.36 ± 0.04

[a] Values represent averages and standard deviations of triplicate measurements. [b] Volumetric median diameter (D(v, 50)). [c] Span = [D(v, 90) − D(v, 10)]/D(v, 50), where D(v, 90), D(v, 50), and D(v, 10) are the equivalent volume diameters at 90, 50, and 10% cumulative volume, respectively.

Golay convolution window. FTIR spectra of trypsinogen powders were collected in the transmission mode using KBr pellets via a computer-controlled automatic sample wheel (Spectra Tech Inc., Shelton, CT). Both the original absorbance spectra and their second derivatives were smoothed via a nine-point Savitzky-Golay window. The spectral second derivatives in the amide I region (1600−1700 cm^{-1}) were used for the calculation of correlation coefficients (r^2 values).[16] These coefficients provide an estimate of the deviation of the structure of the examined sample from the native state by comparison of the second derivatives of the examined sample with that of the native protein in solution.

Solution State Protein Stability—The thermal stability of the trypsinogen−sucrose solution formulations before and after spray drying followed by reconstitution at 20 mg/mL in 1 mM HCl was examined by differential scanning calorimetry. Approximately 750 μL of protein solutions were loaded on preweighed stainless steel pans, were hermetically sealed, and were loaded on the sample compartment of a model 4207 Calorimetry Sciences DSC (Provo, UT). For each measurement a blank cell was loaded with 750 μL of 1 mM HCl solution at the appropriate sucrose concentration. The samples, after a 30-min equilibration step at 10 °C, were heated to 90 °C at a rate of 60 °C/hr and were subsequently cooled at the same rate down to 10 °C.

The midpoint of temperature denaturation ($T_{unfolding}$) was determined at the peak maximum, while the enthalpies of the transitions ($\Delta H^0_{unfolding}$ and $\Delta H^0_{folding}$ for the unfolding and refolding reaction, respectively) were determined by integration of the heat capacity curves after subtraction of a polynomial baseline drawn between 30 and 75 °C.

Powder Thermal Stability—The thermal stability of trypsinogen in the spray-dried formulations was determined by the endotherm ($T_{melting}$) obtained by solid state differential scanning calorimetry (DSC). The endothermic thermal transitions were assigned to protein denaturation, as per previous literature studies.[33−35] For the data analysis, we also assumed complete miscibility of protein and sucrose in the solid state formulations, as demonstrated in previous studies of lyophilized sucrose-protein mixtures.[36] The experiments were performed on a Pyris 1 Perkin-Elmer DSC (Norwalk, CT) equipped with an intracooler; the instrument was calibrated with indium before sample analysis. Approximately 10−20 mg of the samples was loaded under ambient conditions in sealed aluminum pans. After a short preequilibration at 10 °C, the samples were heated to 200 °C at a heating rate of 5 °C/min. All measurements were performed in triplicate.

Results

Particle Size Analysis—The size distribution analysis of the spray-dried trypsinogen−sucrose particles is given in Table 1. The results indicate that spray drying of trypsinogen produces a homogeneous monomodal distribution of protein particles of an approximate diameter of 5 μm and a narrow size distribution (1.1−17.4 μm). The addition of sucrose appears to have a rather minor effect on the particle dimensions. At the highest sucrose mass ratio examined (total solids loading of 180 mg/mL), the median particle diameter reached 6.6 ± 1.9 μm. The small deviations of the particle size with the addition of sucrose would be expected since the primary determinant of the final particle size is the atomization process, indicating the suitability of this process for microparticle production.

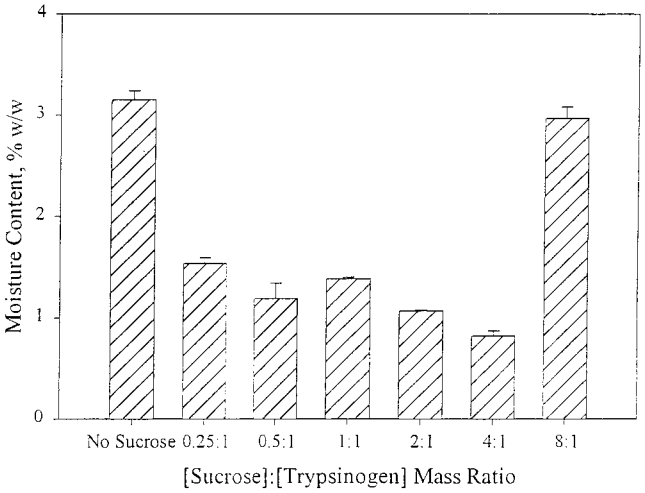

Figure 1—The effect of sucrose on the moisture contents of the spray-dried trypsinogen formulations. Error bars represent (±) one standard deviation of triplicate determinations.

Moisture Content—The moisture content of the spray-dried trypsinogen powders as a function of the protein-to-sucrose mass ratio is shown in Figure 1. The results suggest that even in the absence of secondary drying processes, such as oven drying (which has been employed as a complimentary process following an incomplete drying operation[37]), spray drying can provide powders of low moisture contents.

The final moisture content achieved when trypsinogen was spray-dried in the absence of excipients is 3.1 ± 0.1% w/w (or 3.2 ± 0.2% w/w on a dry protein basis). This amount is even lower than the calculated equivalent water monolayer value of 5.4%; these calculations assume the attachment of one water molecule per charged amino acid.[38] However, this approximation assumes that all charged amino acids are accessible to water vapor. Moreover, spray drying is a flash process, and therefore the obtained moisture contents do not reflect an equilibrium value.

Gradual addition of sucrose progressively decreases the attained moisture content. This is surprising, as one would expect that the presence of a hygroscopic substance like sucrose would increase the amount of residual sorbed moisture. This decrease indicates a possible specific hydrogen bonding interaction between the two substances. At the highest end of sucrose concentrations examined (mass ratio of 8:1), however, the final moisture content increased to 2.9 ± 0.1% w/w; the increased relative humidity during collection and aliquoting may have contributed to this increase.

Activity Profile—The effect of spray drying on the activity profile of trypsinogen at different sucrose mass ratios is shown in Figure 2. When processed in the absence of excipients, trypsinogen loses part of its enzymatic activity, as compared to the unprocessed material (85.1 ± 3.3%). However, the addition of sucrose to the spray-dried solution appears to exert a concentration-dependent stabilizing effect.

Even small amounts of sucrose, sucrose-to-protein mass ratio of 0.25:1, appear to partially stabilize the protein, which recovers part of its initial activity. Increasing concentrations of the carbohydrate further protect trypsinogen in a concentration-dependent fashion. At equal mass ratios of protein and sucrose, the complete restoration of the initial enzymatic activity (99.9 ± 1.5%) was achieved,

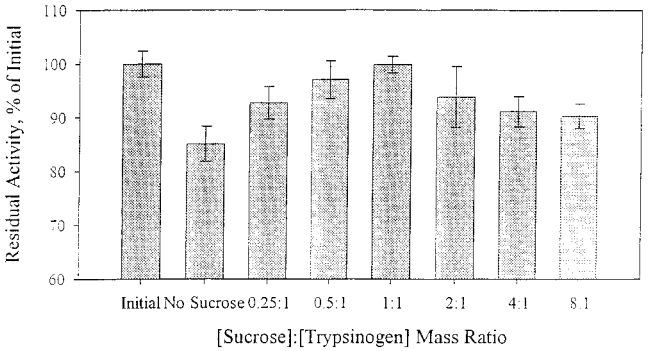

Figure 2—The effect of sucrose on the residual activity of trypsinogen after spray drying. Error bars represent (±) one standard deviation of triplicate determinations.

indicating the complete stabilization of the protein. However, at higher sucrose mass ratios, a small destabilization of trypsinogen is evident. The destabilizing effect is intensified with increasing sucrose concentrations, since the recovered activity of trypsinogen in the spray-dried formulations decreases with increasing sucrose mass ratio. At the high end of the examined carbohydrate concentrations (sucrose-to-protein mass ratios of 4:1 and 8:1), the activity losses approach those observed in the absence of the carbohydrate (91.13 ± 2.82% and 90.33 ± 2.3% of initial, respectively), implying the vast elimination of the protective action of sucrose.

The presence of sucrose in the assay reaction mixture does not have any effect by itself on the determined enzymatic activity of trypsinogen (data not shown). Thus, the observed activity profiles of the spray-dried protein powders truly represent the effects of sucrose on the protein stability during processing rather than an effect of the carbohydrate on the enzymatic activity assay.

Aggregation Profile—The size exclusion chromatography results indicate the presence of high and low molecular weight trypsinogen products in the starting material after reconstitution at pH 3.0; only 89.3 ± 0.3% of the commercial preparation is monomeric trypsinogen. Part of the protein forms an aggregate; the main species appearing to be a dimer (9.6 ± 0.5%). Smaller fragments (approximate molecular weights of 8−12 kDa) account for the remainder of the material. The amount of monomeric and aggregated trypsinogen after reconstitution of the spray-dried powders, relative to the starting material, is shown in Figures 3a,b, respectively. When trypsinogen is spray-dried in the absence of sucrose, it fails to completely recover its initial monomeric state upon reconstitution, as indicated by the decrease of the relative peak area corresponding to the native protein. This loss represents an 8.4 ± 0.3% reduction in the relative amount of the monomeric protein as compared with the unprocessed material; this decline is fully accounted for by an increase of the relative concentration of the dimer (+8.3 ± 0.5%) after spray drying.

Addition of sucrose decreases the extent of aggregation in a concentration-dependent manner. Even small amounts of sucrose partially inhibit dimerization. Increasing concentrations of the carbohydrate progressively diminish the dimer content, up to a sucrose-to-protein mass ratio of 1:1, at which almost complete stabilization of the native protein is achieved. The monomeric state is fully retained at all higher sucrose concentrations examined (up to the highest examined mass ratio of 8:1).

In contrast, formation of insoluble aggregates does not appear to be an operative degradation pathway, as indicated by the small amounts of these species, as shown in Figure 3b. Although some insoluble aggregates were detected when trypsinogen was spray-dried without sucrose and at a sucrose-to-protein mass ratio of 8:1 (1.3 ± 2.0% and 1.64 ± 2.6%, respectively, relative to the unprocessed material) these values are well within the errors of the HPLC analysis method. To further identify the nature of aggregates, we performed SDS−PAGE electrophoresis under both native and reduced conditions. The results (not shown) indicated the absence of covalent disulfide bonds in the high molecular weight species, supporting the formation of hydrophobic aggregates.

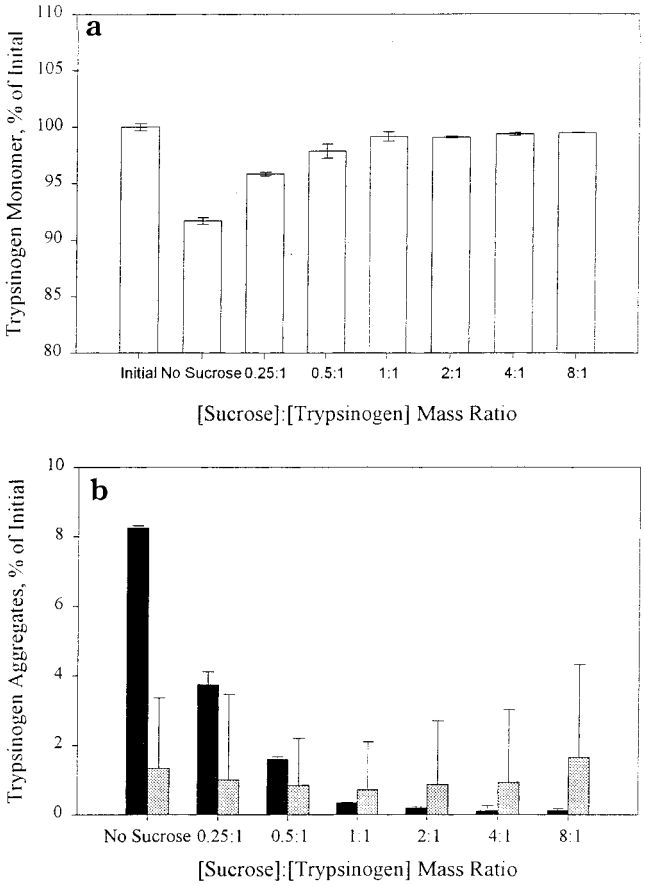

Figure 3—Aggregation status of trypsinogen following spray drying at different sucrose mass ratios and subsequent reconstitution at 5 mg/mL in 1 mM HCl: (a) Relative amount of monomer; (b) Relative amounts of dimer (black bars) and insoluble aggregates (grey bars). Error bars represent (±) one standard deviation based on triplicate determinations.

Solid State Secondary Structure—The second derivative spectra of the amide I band of trypsinogen in the spray-dried powders are displayed in Figure 4, and the calculated correlation coefficients are given in Table 2. The second derivative of the amide I band of native trypsinogen denotes the presence of a major β-sheet band centered at 1636 cm^{-1}, and other components at 1664 and 1685 cm^{-1} signifying primarily β-turn structures. When trypsinogen is spray-dried in the absence of sucrose, it undergoes a significant departure from its native solution structure. As indicated in Figure 4, the second derivative of the amide I band has lost its sharp features as the separation between the bands has diminished. Moreover, the major bands appearing in the native spectrum have all been shifted: the characteristic β-sheet band is shifted approximately 7 cm^{-1} to 1642 cm^{-1}, indicating a rearrangement of the native β-sheet structures of the protein molecule. A similar shift, but of smaller magnitude (+5 cm^{-1}), is evident for the high frequency β-turn band, which is now located at 1689 cm^{-1}. This overall departure from the native state is depicted in the low value of the computed correlation coefficient (0.54 ± 0.05).

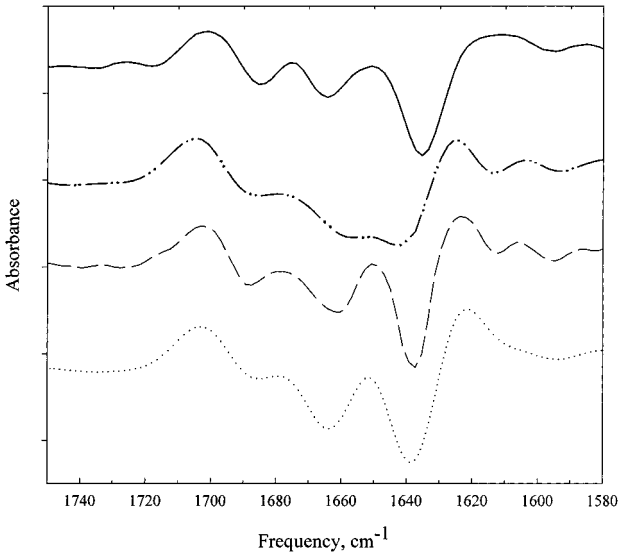

Figure 4—Amide I second derivatives of native trypsinogen in solution (solid line), and in the solid state after spray drying: in the absence of sucrose (– • • –), and with sucrose at 1:1 (– –) and 8:1 (• • • •) mass ratios.

Table 2—Correlation Coefficient Analysis of the Second Derivative Spectra of Spray-Dried Trypsinogen-Sucrose Powders

sucrose:trypsinogen mass ratio	correlation coefficient[a]
no sucrose	0.54 ± 0.05
0.25:1	0.74 ± 0.04
0.5:1	0.88 ± 0.01
1:1	0.93 ± 0.01
2:1	0.87 ± 0.06
4:1	0.78 ± 0.06

[a] Values represent averages and standard deviations of triplicate measurements.

Upon addition of sucrose in the spray-dried trypsinogen formulation (carbohydrate-to-protein mass ratios of 0.25:1−1:1), there is significant recovery of native structural features, as indicated by the increased values of the correlation coefficient. The most remarkable effect is the restoration of the sharpness of the spectral features in the amide I region. Further, the band shifts observed in the absence of sucrose are now, at least partially, reversed. As such, the major β-sheet band has shifted by +3−5 cm^{-1} to 1638 cm^{-1}, closer to its initial position in solution. Likewise, the composite band is centered at 1662 (+2 cm^{-1}) and the high frequency β-turn band at 1688 (+2 cm^{-1}). The observed band shifts occur rather abruptly upon sucrose addition, though a minor improvement appears with increasing sucrose concentration. The profile of the observed spectral restoration of the native structural features is further depicted in the profile of the correlation coefficients (0.8−0.93).

However, upon addition of larger amounts of sucrose in the protein formulation, a small destabilization of the native structure is evident, as indicated by the decreased values of correlation. This observation is in agreement with the determined residual activity results. Moreover, as indicated in Figure 5, the profile of the observed structural alterations of the spray-dried powders follows closely that of the enzymatic activity retention with titrating amounts of sucrose ($r^2 = 0.84$). This correlation provides corroborative evidence that the protein structural deformation in the solid state may be at least partially responsible for the incurred activity losses. Further, it provides indirect evidence that some of the protein molecules may not be

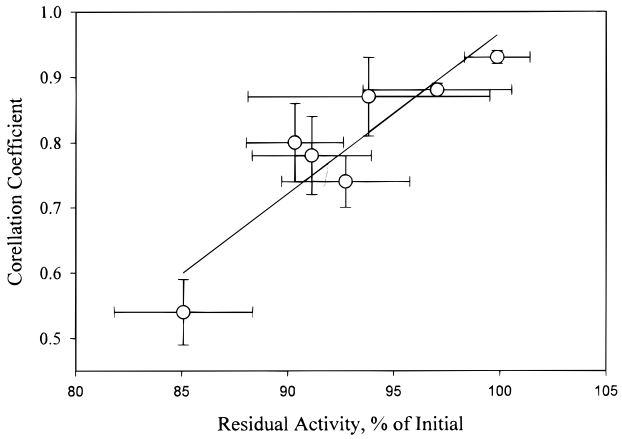

Figure 5—Correlation between the FTIR correlation coefficients and the residual trypsinogen activity after spray drying ($r^2 = 0.84$). Error bars represent (±) one standard deviation based on triplicate determinations.

Table 3—Solid State Thermal Stability of the Spray-Dried Trypsinogen-Sucrose Powders

sucrose:trypsinogen mass ratio	T_{onset}[a] (°C)	$T_{melting}$[a] (°C)	$\Delta H_{melting}$[a] (J/gr)
no sucrose	122.5 ± 3.4	143.5 ± 3.4	54.4 ± 12.5
0.25:1	139.7 ± 2.5	165.7 ± 2.1	92.6 ± 8.9
0.5:1	159.5 ± 1.7	173.7 ± 2.3	139.9 ± 11.5
1:1	182.7 ± 2.5	190.6 ± 3.3	174.8 ± 14.2
2:1	172.1 ± 2.1	180.6 ± 1.1	214.9 ± 16.6
4:1	138.7 ± 4.1	158.6 ± 5.3	214.5 ± 15.3

[a] Values represent averages and standard deviations of triplicate measurements.

able to refold to their native, fully active state upon reconstitution.

Solid State Thermal Stability—The denaturation temperature ($T_{melting}$) of trypsinogen in the spray-dried powders is given as a function of the sucrose mass ratios in Table 3. The results indicate that spray drying induces a significant destabilizing effect on trypsinogen, as indicated by the decrease in the denaturation temperature, which reaches 143.5 ± 3.4 °C in the absence of sucrose. However, in agreement with the activity assays and size exclusion results, the addition of sucrose exerts a concentration-dependent stabilizing effect. When small amounts of sucrose were added to the trypsinogen solution before processing, they induced a structural stabilization of the protein in the solid state, as indicated by the continuous increase of its denaturation temperature. The maximum stabilization occurs, in agreement with the enzymatic activity determinations, at a sucrose-to-protein mass ratio of 1:1, as indicated by the highest denaturation temperature of 190.6 ± 3.3 °C.

However, further addition of sucrose decreases $T_{melting}$, indicating a reduction in the thermal stability of trypsinogen in the spray-dried powders (denaturation temperatures of 158.6 ± 5.3 °C and 161.6 ± 3.3 °C at sucrose-to-protein mass ratios of 4:1 and 8:1 respectively). The overall profile of the thermal stability of the protein in the solid state closely resembles that observed for the activity of the reconstituted protein, indicating again that the mechanism of the incurred enzymatic activity losses proceeds via reduction of the protein stability in the solid state. As indicated in the linear regression shown in Figure 6, there appears to be a good correlation between these two phenomena, as indicated by the high value of the obtained correlation coefficient ($r^2 = 0.88$). In agreement with the similar correlation of the FTIR results, this observation provides additional evidence for the involvement of solid-

Table 4—Effect of Sucrose on the Thermal Stability of Trypsinogen in Solution, before and after Spray Drying and Reconstitution in 1 mM HCl

sucrose:trypsinogen mass ratio	before spray drying			after spray drying		
	$T_{unfolding}$,[a] (°C)	$\Delta H_{unfolding}$a (kcal/mol)	$(\Delta H_{fold}/\Delta H_{unfold})^a$ (×100, %)	$\Delta H_{unfolding}$a (kcal/mol)	$\Delta H_{unfolding}$a (kcal/mol)	$(\Delta H_{fold}/\Delta H_{unfold})^a$ (×100, %)
no sucrose	61.8 ± 0.3	87.2 ± 5.4	86.9 ± 7.5	59.3 ± 0.3	75.4 ± 3.3	52.8 ± 3.9
0.25:1	61.5 ± 0.2	79.4 ± 4.7	78.7 ± 6.5	60.4 ± 0.5	80.3 ± 3.3	63.3 ± 6.1
0.5:1	61.9 ± 0.1	83.3 ± 0.7	78.8 ± 3.8	60.4 ± 0.18	3.9 ± 5.3	63.0 ± 5.7
1:1	62.0 ± 0.2	81.3 ± 2.1	72.0 ± 5.1	61.1 ± 0.3	83.1 ± 9.3	73.5 ± 12.0
2:1	62.3 ± 0.1	82.7 ± 3.3	82.9 ± 11.9	59.7 ± 0.3	77.9 ± 3.4	78.6 ± 1.1
4:1	62.9 ± 0.2	82.4 ± 1.6	75.7 ± 3.0	58.2 ± 0.2	79.0 ± 3.8	75.1 ± 3.8

[a] Values represent averages and standard deviations of triplicate measurements.

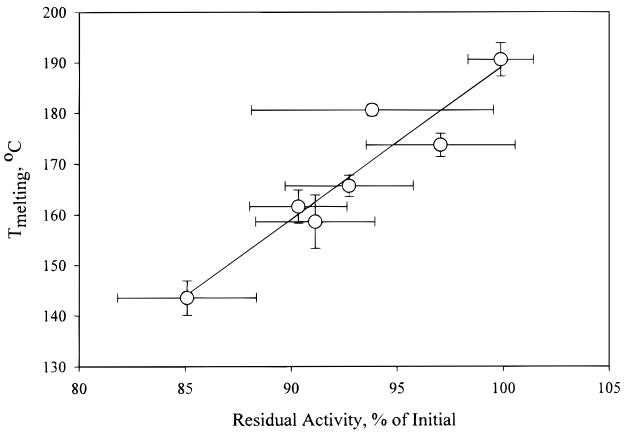

Figure 6—Correlation between the thermal denaturation temperature of trypsinogen in the solid state ($T_{melting}$) and the residual trypsinogen activity after spray drying ($r^2 = 0.88$). Error bars represent (±) one standard deviation based on triplicate determinations.

state destabilization in the mechanism of the induced activity losses of trypsinogen.

Solution Thermal Stability—The above observations prompted the further examination of the possibility of the involvement of irreversible (upon reconstitution) protein denaturation in the activity losses. We examined this hypothesis by determining the solution state thermal stability of trypsinogen at different sucrose mass ratios before spray drying and after reconstitution of the spray-dried powders. The results, which are shown in Figures 7a,b and given in Table 4, indicate the multiple effects of sucrose on the thermal stability of the protein before and after processing and reconstitution.

In the solution state, the midpoint of the thermal unfolding of trypsinogen occurs at 61.8 ± 0.3 °C. Unfolding of this protein appears to be a two-state transition as indicated by the similitude of the experimental enthalpy of the transition and that calculated by the van't Hoff equation: indeed, a second derivative of the thermogram (data not shown) indicates the presence of a single transition. Further, the transition appears to be highly reversible, as indicated by the large value of the ratio of the enthalpies of the unfolding and folding transitions ($\Delta H_{unfold}/\Delta H_{fold}$ = 86.9 ± 7.5%). Addition of small amounts of sucrose (up to a mass ratio of 1:1) does not appear to have a significant impact on the thermal stability of the protein, as indicated by the rather small deviations of both the temperature and enthalpy of unfolding. However, higher concentrations of sucrose appear to exert a protein-stabilizing effect. The magnitude of this phenomenon is largest at the highest sucrose mass ratio (8:1), as indicated by the 2.6 ± 0.1 °C elevation of $T_{unfolding}$.

The thermal stability of trypsinogen when spray-dried without sucrose and reconstituted at the initial conditions was compromised, as indicated by the decreased $T_{unfolding}$ (59.3 ± 0.3 °C). Although the enthalpy of unfolding does not change significantly, spray drying appears to exert a significant effect on the reversibility of the transition: only 52.8 ± 3.9% of the unfolded protein can reversibly refold after thermal treatment. This indicates that a significant proportion of the protein molecules was significantly perturbed from the native state during spray drying. When small amounts of sucrose (mass ratios of 4:1−1:1) were incorporated in the spray-dried formulation however, the reduction of the thermal stability of the protein molecules was alleviated, as indicated by the smaller decrease of trypsinogen's $T_{unfolding}$. The minimal destabilization appears to occur at a sucrose-to-protein mass ratio of 1:1, in agreement with the previous observations with the residual activity and solid state thermal stability analyses. However, higher sucrose concentrations fail to stabilize the protein, as indicated by the decreased values of the unfolding temperatures. Again, minimal stability is observed at the highest sucrose mass ratio, as indicated by the reduction of $T_{unfolding}$ by 5.8 ± 1.6 °C and $\Delta H_{unfolding}$ by 12.9 ± 5.4 kcal/mol relative to the native protein. The destabilization becomes even more profound when we compare it with the $T_{unfolding}$ of the same formulation prior to spray drying.

Discussion

The preparation of solid state protein formulations frequently results in their destabilization due to the stresses imposed on them during processing.[15,16] Trypsinogen appears to follow this trend when processed for microparticle production via spray drying. Protein aggregation, a frequently encountered pathway of protein destabilization during solid-state processing,[39,40] upon reconstitution can account only in part (55.8 ± 2.7%) for the observed activity losses. Another destabilization pathway must be responsible for the unaccounted inactivation. The loss of the native protein structure, as observed by the FTIR correlation analysis, during processing along with the observation of decreased stability of the reconstituted protein offers a potential elucidation of the unaccounted activity losses: it is possible that some of the spray-dried protein has departed from its native state and fails to recover its structural integrity, conformational stability, and enzymatic activity upon reconstitution. This argument is further supported by the decrease of the enthalpy of unfolding of trypsinogen after spray drying: from 87.2 ± 5.4 before to 75.4 ± 3.3 kcal/mol after processing. This represents a 13.6 ± 5.4% decrease of $\Delta H_{unfolding}$, which is, within experimental error, close to the extent of the observed activity losses (14.9 ± 3.2%). Therefore, the amount of native protein available to unfold has decreased by 13.6% after spray drying; part of this reduction must be due to the aggregated protein (8.4 ± 0.3%), while the rest can be assigned to irreversibly unfolded protein (5.5 ± 2.1%). The reason for the irreversibility is rather unclear;

it is possible that a chemical degradation reaction of a folding-crucial residue during spray drying may have impaired the correct folding of the protein upon reconstitution. Alternatively, the protein may have reached a partially unfolded state representing a local energetic minimum along its folding pathway, which cannot be overcome after reconstitution.

The addition of sucrose appears to exert a concentration-dependent stabilization effect. At low to moderate sugar-to-protein mass ratios (up to 1:1), sucrose stabilizes the protein. The stabilization appears to occur through impediment of the aggregation pathway, as indicated by the sucrose concentration-dependent decrease of the aggregated molecules, along with a stabilization of the native structure of the protein, as shown by the increased values of the FTIR correlation coefficients during processing. The stabilizing action of disaccharides has been well documented in studies of protein lyophilization.[15,16,28] In addition, sucrose and trehalose have been shown to efficiently protect hemoglobin (from oxidation via protection of its native structure)[19] and β-galactosidase during spray drying.[20]

The solution calorimetry studies of the commercial protein preparation indicated that at high concentrations, sucrose stabilizes the native state of trypsinogen. This finding is in agreement with previous studies that reported a rise of the thermal transition temperature of α-chymotrypsin, chymotrypsinogen, and ribonuclease-A upon addition of sucrose.[27] Moreover, there appears to be a linear dependence of the transition temperature on the concentration of added sucrose ($r^2 = 0.98$); a similar correlation was also suggested by Lee and Timasheff[27] on the three above-mentioned proteins. Again in agreement with these studies, the observed stabilizing effect of sucrose does not appear to be enthalpic in nature, as indicated by the little variation of the $\Delta H_{\text{unfolding}}$. The same authors[27] concluded that the stabilizing effect of sucrose arises from an increase in the apparent activation energy for the thermal perturbation of the proteins, which in turn reflects changes in the physicochemical properties of the system and in particular of the solvent structure. This is a manifestation of the excluded volume effects exerted by sucrose, which, as a natural consequence of the Le Chatelier principle, become evident at these concentrations of the carbohydrate.

However, at this concentration regime (above 2:1, or 0.117 M), the protective action of sucrose appears to have somewhat dissipated, as there is a substantial reduction of the activity of trypsinogen in the spray-dried formulations. The activity losses cannot be assigned to protein aggregation, since the protein remains essentially monomeric; this provides corroborative evidence that protein dilution in the solid matrix by the addition of sucrose molecules is the aggregation-stabilizing mechanism. Nor is a gross conformational change evident in the FTIR spectra, but rather a small departure from the native state, as indicated by the decreased values of the determined correlation coefficients. Yet, the stability of the protein in the spray-dried formulations has been considerably compromised as indicated by the decreased values of T_{melting}, shown by solid-state DSC. Upon reconstitution of these formulations, the protein fails to recover its conformational integrity, as indicated by the decreased $T_{\text{unfolding}}$ and enthalpies of unfolding. Although the activity losses in this sucrose formulation regime are smaller than those incurred in the absence of sucrose, the $T_{\text{unfolding}}$ results indicate a larger destabilization of the protein when high amounts of sucrose are incorporated in the formulation. Further, the decrease of $\Delta H_{\text{unfolding}}$ after processing provides an additional indication of the extent of the departure of the protein from its native state: at a sucrose-to-protein mass

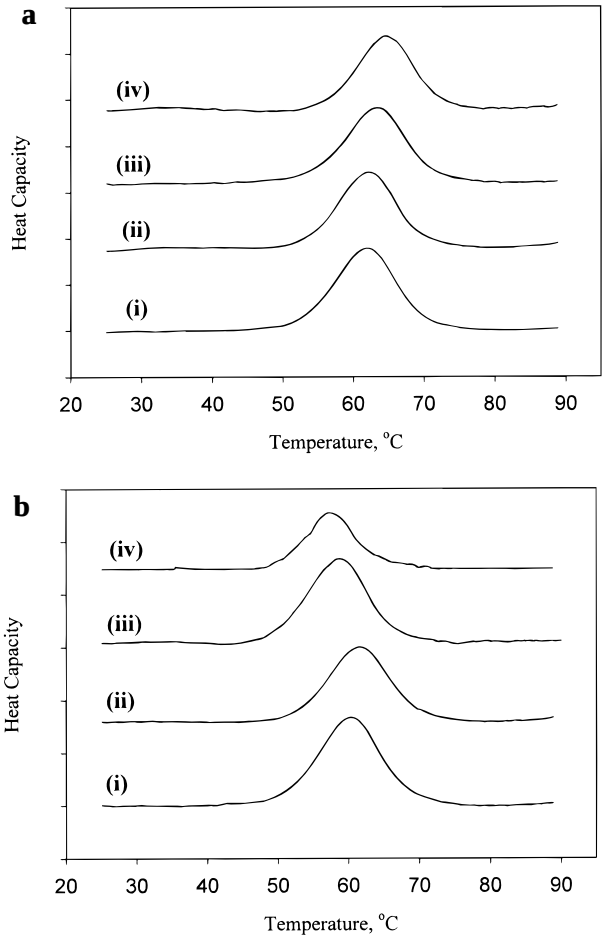

Figure 7—DSC thermograms of trypsinogen–sucrose formulations before (a) and after (b) spray drying: (i) in the absence of sucrose, and at [sucrose]:[trypsinogen] mass ratios of (ii) 1:1, (iii) 4:1 and 8:1.

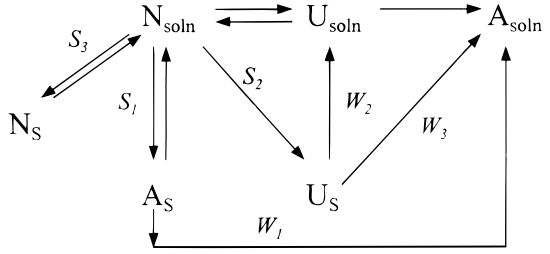

Figure 8—Proposed protein degradation mechanism during spray drying. Subscripts S and soln denote the native (N), unfolded (U), and aggregated (A) protein molecules in the solid and solution states, respectively.

ratio of 8:1 the enthalpy of unfolding has decreased from 86.2 ± 7.3 to 75.7 ± 0.5 kcal/mol, indicating an approximate decrease of 12.2% of the unfolding enthalpy. This value is again in good agreement with the incurred activity losses, indicating that irreversible structural destabilization of trypsinogen in the solid state is the degradation pathway at this end of sucrose concentrations.

Our observations seem to support the destabilization mechanism illustrated in Figure 8. Spray drying of a native protein (N_{soln}) may result in partial protein aggregation (state A_S) and unfolding (state U_S) in the solid state, via pathways S_1 and S_2, respectively. These states are supported by the intermolecular β-sheet band observed in the FTIR spectra and by the departure from the native state into a state of reduced conformational stability (observed by solid-state DSC and FTIR) data, respectively. Alternatively, some protein molecules may still retain their native state in the solid, as illustrated via pathway S_3 (state N_S).

After rehydration, some of the intermolecular contacts persist, leading to formation of soluble, hydrophobic aggregates (state A_{soln}) via pathway W_1. Moreover, some of the unfolded molecules in the solid state fail to adopt their native structure and stability and remain partially unfolded in solution (state U_{soln}) via pathway W_2. Alternatively, some unfolded molecules may participate in self-association reactions via pathway W_3. Finally, molecules that retained their native conformation and structural integrity are able to return in their native solution state (N_{soln}). Addition of sucrose appears to stabilize the protein by blocking both pathways S_1 and S_2, as indicated by both decreased aggregation, nativelike conformationally stable structures in the solid state. However, high concentrations of sucrose, while still effective in impeding aggregation, fail to efficiently stabilize the protein structure in the solid state, rendering pathway S_2 operative.

A consideration of the nature of the destabilizing stresses is particularly interesting. Protein denaturation at the air−liquid interface has been previously considered as the potential degradation pathway of human growth hormone (hGH) during spray drying.[11] Since it is well established that sucrose increases the surface tension of water,[23,24,27] one would expect that its presence would lead to preferentially increased interfacial interactions and consequent destabilization. Yet, the observed stabilizing action of sucrose contradicts this hypothesis, thereby discounting the presence of interfacial inactivation phenomena. Neither heat appears to be the destabilizing stress. If thermal stresses were responsible for the observed destabilization, then, based on the solution DSC results, it would be expected that addition of sucrose at 1:1 mass ratio would not significantly impact the stability of trypsinogen during processing. However, this is contrast with our observations, which indicated complete stabilization of the protein during spray drying at this mass ratio of sucrose, thereby ruling out the involvement of thermal stresses on trypsinogen destabilization. Finally, pressure-induced denaturation during atomization does not appear to be an operative destabilization pathway, since the addition of high concentrations of sucrose, a well-known baroprotectant,[25] would be expected to provide adequate stabilization, which is inconsistent with our observations. The elimination of these potential stresses provides corroborative evidence that dehydration is the major stress responsible for the observed structural destabilization of trypsinogen during spray drying. These observations are in agreement with studies of protein lyophilization, which indicated that the dehydration was the major stress responsible for protein denaturation.[16,41]

Protein destabilization in the solid state in the presence of large amounts of protectants has been previously observed during lyophilization of β-galactosidase with mannitol.[39] The observed activity losses were assigned to excipient crystallization in the solid state. It was thought that the decreased stabilizing effect of crystallized protectants was due to the preferential replacement of the protein−excipient with excipient−excipient interactions. Moreover, this process frequently results in increased water content of the protein-rich phase arising from its exclusion from the excipient crystals, eventuating increased protein molecular mobility and therefore a higher propensity for degradation and inactivation.

The results further indicate that spray drying of trypsinogen under conditions where excluded volume effects dominate results in compromised protein stability and resultant reduction of its enzymatic activity. If crystallization or phase separation were responsible for the protein destabilization, as was previously observed with lyophilized β-galactosidase,[39] these must arise from the exclusion of sucrose from the protein surface. As a result of this phenomenon, the sucrose molecules preferentially interact with themselves (presumably via hydrogen bonding) rather than with the protein surface, leading to the formation of separate phases: a protein-rich and a sucrose-rich phase. Indeed, exclusion is a form of phase separation at the molecular level; this separation precedes the processing step. Despite the high surface area produced during atomization, the solution distribution of each excipient in the droplets should not be expected to alter dramatically, and the microphase separation should remain. This implies that during the spray drying process, the sucrose molecules are not available to hydrogen bond efficiently with the protein, since they remain in their carbohydrate-rich regions, resulting in insufficient protection of the protein. We therefore hypothesize that it is the solution separation that is responsible for the departure from the native state and the decreased stability patterns in the solid state. This hypothesis suggests that when the dispersed droplets are dried, a similar molecular-level separation must exist in each dried particle. These observations indicate that, at high sucrose concentrations, excluded volume effects are destabilizing during protein processing in microparticles via spray drying. These are phenomena that warrant further exploration.

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