Stability and Surface Activity of Lactate Dehydrogenase in Spray-Dried Trehalose

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Abstract □ The stability of the model protein lactate dehydrogenase (LDH) during spray-drying and also on subsequent dry storage was examined. Trehalose was used as a carrier. The spray-drying temperatures T_{inlet} and T_{outlet} have a measurable effect on LDH inactivation. Low T_{inlet} produced the least process inactivation, but gave a high residual moisture content making the protein's storage stability poor. High T_{inlet} reduced residual moisture and improved storage stability, but at the cost of high process inactivation. As already found for other systems, addition of a surfactant (in this case polysorbate 80) could ameliorate process inactivation of LDH at T_{inlet} = 150 °C. Surfactant had, however, a deleterious effect on storage stability of LDH, the vital factor being the molar ratio of surfactant/protein in the dried product. By using electron spectroscopy it was shown that LDH has a 10 times higher surface concentration in the dried trehalose particles than expected for a homogeneous distribution. Surface tension measurements at the water/air interface proved that LDH is surface active, although the Gibbs equation appeared to be inapplicable. Calculations of spray-droplet formation time and drying time indicate than the extent of diffusion-driven LDH adsorption to the liquid/air interface is sufficient to account for the measured amount of LDH inactivation during spray-drying. The presence of 0.1% polysorbate 80 to the spray solution prevents LDH from appearing at the surface of the dried particles. As a negative control, the phosphatide Lipoid E 80 does not prevent the appearance of LDH in the surface according to electron spectroscopy and does not therefore prevent LDH inactivation during spray-drying at T_{inlet} = 150 °C.

Introduction

It is just 6 years since Broadhead et al.[1] concluded in their review article that the full potential of spray-drying of pharmaceutical proteins had yet to be exploited. Since then, a number of papers and patents have addressed the question of spray-drying to produce protein formulations suitable for pulmonary delivery. In most cases, spray-drying of an aqueous solution of the bulk protein led to substantial protein aggregation or inactivation.[2] The addition of stabilizing adjuvants as carriers, typically carbohydrates or amino acids, reduces the extent of protein inactivation during spray-drying. Sucrose (0.25 M), for example, reduced the formation of methemoglobin on spray-drying hemoglobin at an inlet air-temperature of 100 °C from 50% to <4%.[3] Trehalose, arginine, and sucrose completely ameliorated the activity loss of spray-dried β-galactosidase.[4] Both trehalose and sucrose have, however, been reported to be difficult to spray-dry, at least in the placebo state. The latter is extremely hygroscopic[5] whereas the former has been reported to form fused, sticky aggregates at an inlet air-temperature of 90 °C, unsuitable as a protein carrier.[6]

This paper presents part of our work investigating the relationship between process stability, storage stability, and surface activity of proteins in spray-dried powders. The question of process stability of proteins during spray-drying has already been addressed in the papers cited above. The problem of subsequent storage stability of the proteins has, however, received less attention. Broadhead et al.[4] reported that trehalose as a carrier produced the best storage stability of β-galactosidase at ambient temperatures. We wished to reconcile this report with the above-cited difficulty in producing dry spray-dried trehalose particles.[6] For pulmonary application, where the maximum powder-dose is severely limited,[7] the spray-drying of pure proteins appears attractive. Recombinant human growth hormone could, for example, be successfully spray-dried at an inlet air-temperature of 90 °C without a carrier, although only with addition of polysorbate 20 and/or $ZnCl_2$ to the spray solution.[8] The storage stability of such spray-dried proteins having no carrier has, however, yet to be addressed and will certainly be protein-specific. The use of a glass-forming carrier will surely be necessary for many proteins.

We selected lactate dehydrogenase (LDH) as a model protein. It is known to be sensitive to thermal treatment and has the distinct advantage that its enzyme *activity* can be measured directly and not just its degree of aggregation. To reconcile the conflicting reports in the literature regarding its suitability,[4,6] we used trehalose as a carrier. Its high glass transition temperature in the dried state renders trehalose an efficient stabilizer of proteins on storage,[9] even when containing relatively high residual moisture contents. We examined the influence of spray-drying temperatures on both the process and storage stability of LDH in spray-dried trehalose. The results are discussed in terms of the residual water contents and glass transition temperatures of the spray-dried powders. As has been already reported for other proteins,[8] surfactant could reduce loss of LDH activity occurring during the process of spray-drying. We show, however, that polysorbate 80 has an unexpectedly deleterious effect on storage stability of LDH. Surface tension measurements show that LDH is strongly surface-active. A particular feature of this work is the use of electron spectroscopy to assay the surface layer of the spray-dried LDH/trehalose particles. Using this method we find the first unequivocal evidence of protein exclusion from an interface on spray-drying with surfactant. The addition of lecithin did not induce protein exclusion from the interface and did not therefore prevent loss of LDH activity during spray-drying.

Materials and Methods

Materials—Porcine lactate dehydrogenase (LDH) of molecular weight approximately 144 kDa was obtained from Boehringer Mannheim (Mannheim, Germany). This suspension in 3 M $(NH_4)_2$-

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SO_4 was dialyzed immediately before use at 4 °C against 0.1 M phosphate buffer of pH 7 using a regenerated cellulose membrane (MW cutoff 12000−14000). The LDH activity of the dialysate was determined by the assay described later. Trehalose dihydrate was used as received from Sigma Chemicals (Munich, Germany). Polysorbate 80 of MW approximately 1300 and HLB value 15 was obtained from ICI (Sandhofen, Germany). Lipoid E 80 is an egg yolk lecithin obtained from Lipoid (Ludwigshafen, Germany) and was used without further refinement. Miglyol 812 came from Pharma Zentrale (Herdeke, Germany), and Span 85 from ICI. Water was double distilled from an all-glass apparatus.

Spray-Drying—Trehalose dihydrate was dissolved either in water or the diluted, dialyzed LDH solution in pH 7 phosphate buffer. Polysorbate 80 was added as appropriate. Fifty milliliters of the resulting trehalose or trehalose/LDH solution (with or without polysorbate 80) was spray-dried on a Büchi model 190 laboratory spray-dryer operated in the cocurrent mode. The liquid feed rate was 4 mL/min ($0.24 \times 10^{-3} m^3/h$) through a pneumatic nozzle (0.7 mm diameter) driven at 6 bar air pressure. Cooling water was circulated through a jacket around the nozzle. The atomizing-air flow rate was 0.7 m^3/h, and the aspirator vacuum was 38 mbar. It was possible to control only the inlet air-temperature (T_{inlet}) on the machine we used; the outlet air-temperature (T_{outlet}) could only be measured. Spray-drying was performed under the following four sets of conditions: T_{inlet}/T_{outlet} = 90 °C/60 °C; T_{inlet}/T_{outlet} = 110 °C/70 °C; T_{inlet}/T_{outlet} = 130 °C/80 °C; T_{inlet}/T_{outlet} = 150 °C/95 °C. Unless otherwise stated, the spray solution contained 10% w/w total solids (trehalose + LDH + surfactant), giving a maximum theoretical yield of 5 g of powder per 50 mL of spray solution used in one experiment.

Characterization of Spray-Dried Powders—The residual moisture contents of the spray-dried powders were determined by Karl Fischer Titration (Schott Instruments). Powder samples (50−100 mg) were dissolved in water-free methanol/formamide (1:1) prior to titration. The thermal transitions of the powders were examined on a Polymer Laboratories differential scanning calorimeter. Samples (10 mg) were first sealed in Al pans and then repeatedly cooled and heated between −20 °C and 150 °C at 10 °C/min. Each sample's glass transition temperature, T_g, was determined from the midpoint of the endothermic shift on the DSC trace. Wide-angle X-ray diffraction was performed at 25 °C ± 1 °C on a Phillips Model TW 1730, with Cu Kα radiation of λ = 0.15418 nm at 40 kV/30 mA. The external morphology of the spray-dry powders was examined using scanning electron microscopy (SEM) on an Amray 1810 T microscope. The powders were gold sputtered on an Al sample holder.

Laser Diffractometry—The particle size distributions of the spray-dried powders were determined using a Coulter Model LS 130 Laser Diffractometer. Each dry powder sample was initially deaggregated by dispersion in a 1% w/w solution of Span 85 in Miglyol 812 using an ultrasonic bath for 4 min. This dispersion was placed in the small volume accessory of the diffractometer, and the laser light scattering intensity was evaluated by Fraunhofer analysis.[10]

Electron Spectroscopy for Chemical Analysis (ESCA)—This technique was used to quantify the surface coverage of the spray-dried powders with LDH. Using ESCA it is possible to identify quantitatively the elements present in the surface of dried particles.[11] Briefly, the powder sample is exposed to an X-ray beam, and electrons contained in the near-surface region of the solid (approximately 10 nm) that have a binding energy less than the photon energy will be ejected from the atom. The kinetic energy of the ejected electrons will be equal to the difference between photon energy and binding energy, allowing for an instrument response function. As the binding energy is characteristic of the atom from which it is ejected, the elements present in the specimen can be identified quantitatively.[12]

To determine the surface coverages of the spray-dried powders with LDH, we analyzed the surface atomic concentrations of the elements C, O, and N. We assume only that the ratio of elements found in a dried powder sample is a linear combination of the ratio of elements occurring in the pure components comprising the sample.[11] In this fashion the concentration of LDH in the outermost 10 nm-thick layer of the spray-dried powder can be calculated. A photoelectron spectrometer (Physical Electronics) was used with an Al Kα X-ray source. The pressure in the sample chamber was reduced to below 0.1 μbar bar. The detector (electron kinetic energy analyzer) was operated with a pass energy of 188 eV, and the ESCA spectra were determined using a step size of 1.0 eV. Each spray-dried powder sample was placed in the indentation of the machine's sample holder. The result of each ESCA analysis is expressed as a spectrum of counts/s at the detector versus binding energy in eV.

Table 1—Properties of Trehalose Powders Spray-Dried under Different Conditions of T_{inlet}/T_{outlet}[a,b]

T_{inlet} [°C]	T_{outlet} [°C]	yield [%]	water content [% w/w]	T_g [°C]
110	70	58	3.5	72
130	80	65	2.4	86
150	95	78	2.6	84

[a] Spray solution contained 10% w/w total solids. [b] Liquid feed rate was 4 mL/min, and atomizing air flow rate was 0.7 m^3/h.

Process and Storage Stability of LDH—The residual LDH activity in the trehalose powders was determined immediately after spray-drying and also after various storage times at 4 °C, 25 °C, 40 °C, and 60 °C in sealed glass vials over P_2O_5. The LDH activity assay was conducted as follows: 2.5 mL of phosphate buffer (0.1 M, pH 7), 0.1 mL of pyruvate (0.02 M), and 0.05 mL of NADH (11 mM) were first mixed in a quartz cuvette at 25 °C. LDH solution (0.05 mL) to be tested was then added with mixing. The extinction of the solution was measured at λ = 365 nm over 5 min using a Kontron Uvikon 810 UV−vis photometer with water bath for temperature control. The enzymatic activity [units/mL] could be calculated from the measured rate of change in extinction per min [$\Delta E/\Delta t$]. The residual LDH activities were expressed on a scale between 0 and 100% of the LDH activity of the spray solution determined immediately before spray-drying. Both trehalose and polysorbate strongly influenced the LDH assay and were accounted for by running appropriate blank references.

Surface Tension Measurements—The surface tension of LDH in water was measured at 25 °C ± 0.1 °C and at various concentrations using a thermostated Krüss K 10 Tensiometer equipped with a Wilhelmy plate. The surface tension was measured at various time points up to 60 min. The LDH concentrations were determined from UV-extinction of the solutions at λ = 280 nm by using $E^{0.1\%}_{280nm}$ 1.40 cm^{-1} mL mg^{-1}.[13]

Results and Discussion

Spray-Drying of Protein-Free Trehalose—The reported "sticky" nature of trehalose spray-dried at T_{inlet}/T_{outlet} = 90 °C/60 °C[6] could be overcome by the simple expedient of increasing T_{inlet} to improve product quality, as can be deduced from Broadhead's earlier work.[4] At T_{inlet}/T_{outlet} = 150 °C/95 °C a respectable yield (for the Büchi) of almost 80% is obtained, and the product has a residual moisture content of 2.6% with a T_g of > 80 °C (Table 1). Clearly, use of a low T_{inlet} = 90 °C[6] appears intuitively attractive to reduce possible protein inactivation by thermal stress during spray drying. The consequences of increasing T_{inlet} to 150 °C to obtain a drier product on LDH's process and storage stability will be seen presently. The advantage of using trehalose compared with other nonreducing sugars such as sucrose is also evident from Table 1. A residual moisture of 2.6% is still high compared with freeze-drying the same product, and this value would depress the T_g of sucrose down to approximately 40 °C.[9] By virtue of its high T_g in the dried state (T_g = 115 °C),[14] the relatively high residual moisture contents achievable with spray-drying still yield T_g's of >80 °C. This could be of advantage for the process stability of a protein and certainly will be propitious for its storage stability.[9]

Hancock and Zografi[15] showed that the plasticizing effect of water on various amorphous pharmaceutical solids could be accurately described by the simple Gordon−Taylor model of perfect volume-additivity and no interactions between water and the amorphous solid. Figure 1 shows the T_g's of the spray-dried trehalose powders plotted versus weight fraction residual moisture in the range 0 to ap-

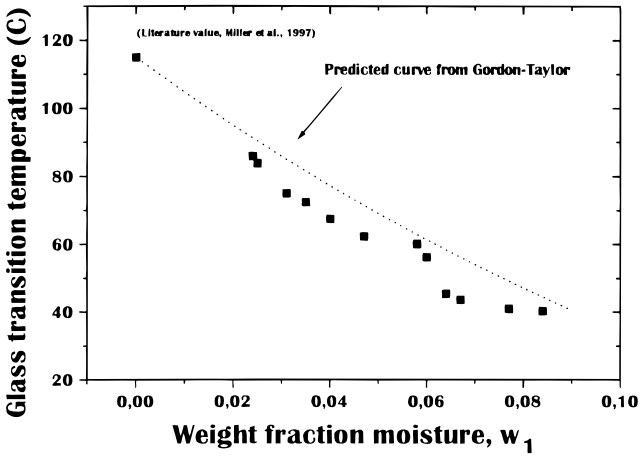

Figure 1—Variation in glass transition temperature (T_g) with weight fraction residual moisture content (w_1) of the spray-dried trehalose powders. Spray solutions contained 10% w/w total solids (trehalose dihydrate). Liquid feed rate was 4 mL/min, and atomizing air flow rate was 0.7 m^3/h. The solid squares are the individual results from the spray-dried powders. The dotted line is the curve calculated from the Gordon–Taylor equation for the water/trehalose system: $T_{g,mix} = (w_1T_g^1 + Kw_2T_g^2)/w_1 + Kw_2$, $K = (T_g^1\rho_1/T_g^2\rho_2)$. The following values were used: water $T_g^1 = 135$ K (–138 °C) $\rho_1 = 1.00 \times 10^3$ kg/m^3; trehalose $T_g^2 = 388$ K (115 °C) $\rho_1 = 1.47 \times 10^3$ kg/m^3; $K_{water/trehalose}$: = 0.237.

proximately 9% w/w water. These different residual water contents were obtained by varying T_{inlet}/T_{outlet} accordingly. The experimentally determined T_g's lie consistently below the curve predicted from the Gordon−Taylor equation (Figure 1). This deviation from ideality is, however, less than that observed for the sucrose−water system.[15] We expect the system trehalose−water to be very nonregular in terms of solution theory by virtue of hydrogen bonding. Bearing this in mind, the relatively small negative deviation from ideality in Figure 1 is surprising. As noted by Hancock and Zografi,[15] however, such nonideality obviously does not have a strong influence on the free volume of the water−glass mixtures.

The scanning electron micrograph (SEM) for trehalose spray-dried at T_{inlet}/T_{outlet} = 150 °C/95 °C (Figure 2a) shows almost perfectly spherical particles with smooth surfaces. This is the typical picture for other spray-dried sugars such as lactose and mannitol[6,7] that are not loaded with higher molecular weight ingredients such as proteins or polymers. No change in particle morphology could be observed with change in T_{inlet}/T_{outlet} in the range examined here. In a literature report, partly fused agglomerates were found with trehalose prepared at T_{inlet}/T_{outlet} = 90 °C/60 °C.[6] Although the product at T_{inlet}/T_{outlet} = 110 °C/70 °C showed no indication of particle fusion, any fused aggregates formed during the process were retained on the inner walls of the cyclone, accounting for the small yield of 58% at this T_{inlet}. Under all spray-drying conditions examined here the trehalose particles were fully amorphous, the wide-angle X-ray diffractograms showing the typical amorphous halo (Figure 2b). Although the particle size determined by laser diffraction was independent of T_{inlet}/T_{outlet}, decreasing the total solids concentration of the spray solution led to reduced particle size of the dried product (Figure 2c). Total solids (10% w/w) give, for example, an average particle diameter of 3.5 μm. If the fine particle dose of a powder is defined as that ≤ 6 μm,[7] it is approximately 72% with 10% w/w solids content. Such spray-dried powders are, therefore, attractive for pulmonary application, as noted before.[7] This would need to be confirmed, however, by measurements of the aerodynamic particle size using a suitable impactor. If the spray droplet size is independent of the total solids content of the spray solution in the range used

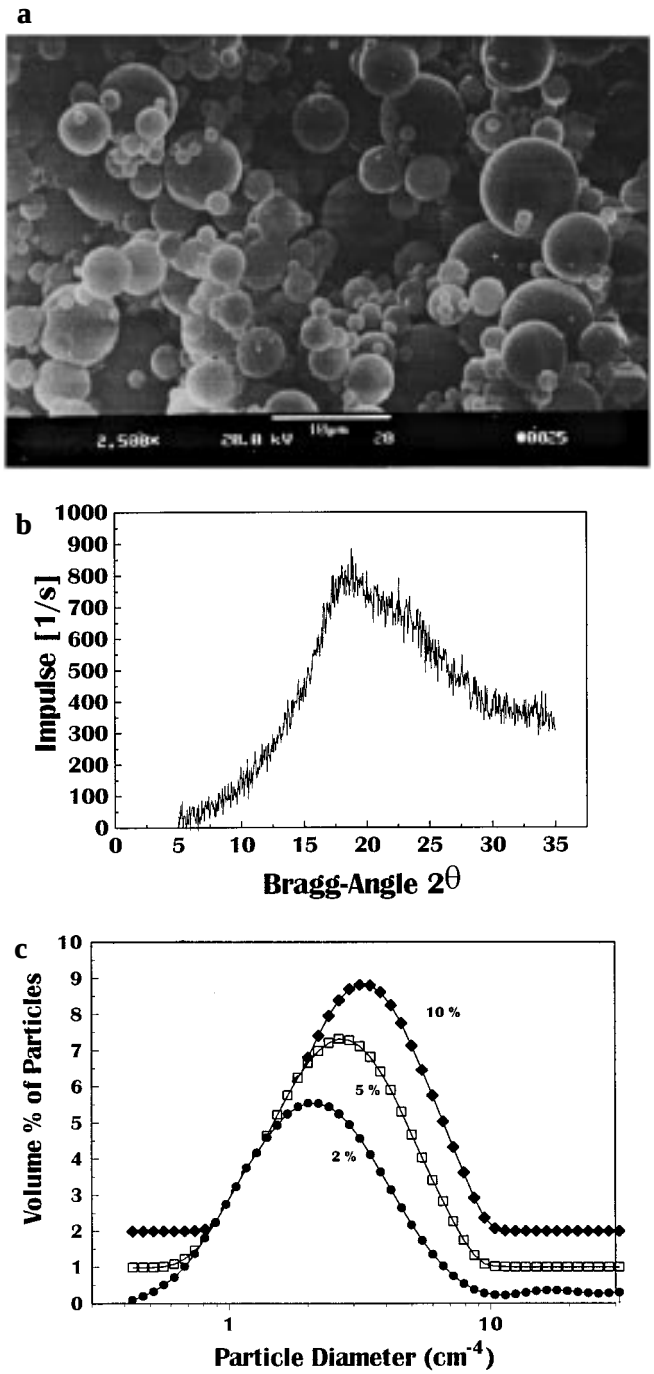

Figure 2—Spray-dried trehalose prepared at T_{inlet}/T_{outlet} = 150 °C/95 °C. (A) Scanning electron micrograph of magnification 2500×. (B) Wide-angle X-ray scattering diffractogram showing characteristic amorphous halo. (C) Influence of total solids content of spray solution on particle size distribution determined by laser diffraction. With decreasing total solid content from 10 through 5 to 2%, the size distribution is shifted to lower diameters.

here, then reducing the total solids content should produce smaller particles, as also observed in Figure 2c.

On dry storage (over P_2O_5) at room temperature, the residual moisture content of the spray-dried particles prepared at T_{inlet}/T_{outlet} = 150 °C/95 °C remained constant over at least 43 days (Table 2). Accordingly, T_g also remained unchanged. The powder remained fully amorphous (X-ray diffractogram not shown, but identical to Figure 2b), indicating that these spray-dried trehalose particles retain their glass properties on suitable dry storage. Uptake of moisture into spray-dried lactose particles[7] induced massive particle aggregation with subse-

Table 2—Properties of Spray-Dried Trehalose Powders on Dry Storage over P_2O_5[a]

storage time [days]	water content [% w/w]	T_g [°C]
0	2.6 ± 0.2	84
1	2.5 ± 0.1	83
8	2.5 ± 0.2	84
15	2.6 ± 0.1	84
43	2.4 ± 0.1	85

[a] Powder prepared at T_{inlet}/T_{outlet} = 150 °C/95 °C and as given in Table 1. n = 3.

quent major reduction in fine particle dose. When used as a protein carrier, it is vital, however, that adjuvents such as trehalose retain their amorphous glassy character on storage.

Spray-Drying of LDH in Trehalose—We distinguish between process stability and storage stability of the model protein LDH in the spray-dried trehalose. *Process stability* defines the extent of inactivation of LDH during spray-drying and can be quantified by measuring residual LDH activity in the powder immediately after spray-drying. Possible causes for protein unfolding and inactivation during spray-drying are shearing stress in the nozzle, thermal stress during droplet drying in the spray-drying tower, and adsorption at the greatly expanded liquid/air interface of the spray solution on atomization.[8] Recent work by Maa et al.[16] indicates that unfolding and aggregation of recombinant human growth hormone (rhGH) during spray-drying was induced primarily by adsorption at the liquid/air interface created during spraying; shearing and thermal stresses were considered to be of minor importance. Figure 3 shows the behavior of the spray-dried LDH/trehalose powders. A low dry-particle protein loading of 0.3% w/w LDH was purposefully chosen to maximize the possible stabilizing effects of the trehalose. Under the mild process conditions of T_{inlet}/T_{outlet} = 90 °C/60 °C (Figure 3a), a loss of approximately 11% LDH activity is caused by the spray-drying process. More vigorous process conditions increase this activity loss up to approximately 25% with T_{inlet}/T_{outlet} = 150 °C/95 °C (Figure 3b–d). It follows that thermal stress must play some part in LDH inactivation, since shearing stress and, presumably, liquid/air interfacial adsorption during the short time required for atomization will be independent of T_{inlet}/T_{outlet}. Only droplet drying time will decrease at higher T_{inlet}/T_{outlet}.[17] The spherical smooth particles of pure trehalose (cf. Figure 2a) are changed with the 0.3% dry-particle LDH loading to show a slightly dimpled appearance (Figure 4a). This is typical for the effects of a high molecular weight additive such as a polymer[18] or a protein.[6] Increasing the dry-particle loading to 5% LDH for T_{inlet}/T_{outlet} = 150 °C/95 °C reduces the percent inactivation of LDH during spray-drying from approximately 25% with 0.3% LDH to approximately 11% (Figure 5a). This represents, however, an increase in the absolute amounts of LDH inactivated from 0.075 mg LDH/mL of spray solution (with 0.3% LDH dry-particle loading) to 0.55 mg LDH/mL of spray solution (with 5% LDH dry-particle loading). Also, the SEM of 5% LDH dry-particle loading shows an extremely wrinkled appearance (Figure 4b). If the primary cause of this LDH inactivation during spray-drying is adsorption at the liquid/air interface of the nebulized spray solution, then the amount adsorbed evidently increases with greater LDH concentration in the spray solution, indicating a diffusion-controlled process.

Some light can be shed on the likelihood of adsorption-induced inactivation by comparing the rates of droplet formation, droplet drying, and LDH adsorption. As shown in the Appendix, the spray droplets formed under the conditions used in these experiments had an average diameter of 8.5 μm and an almost instantaneous formation time, t_f, at the tip of the pneumatic nozzle of 5×10^{-6} ms. The subsequent droplet drying process is slower and occurs in two periods:[19] a constant-rate period until solid phase

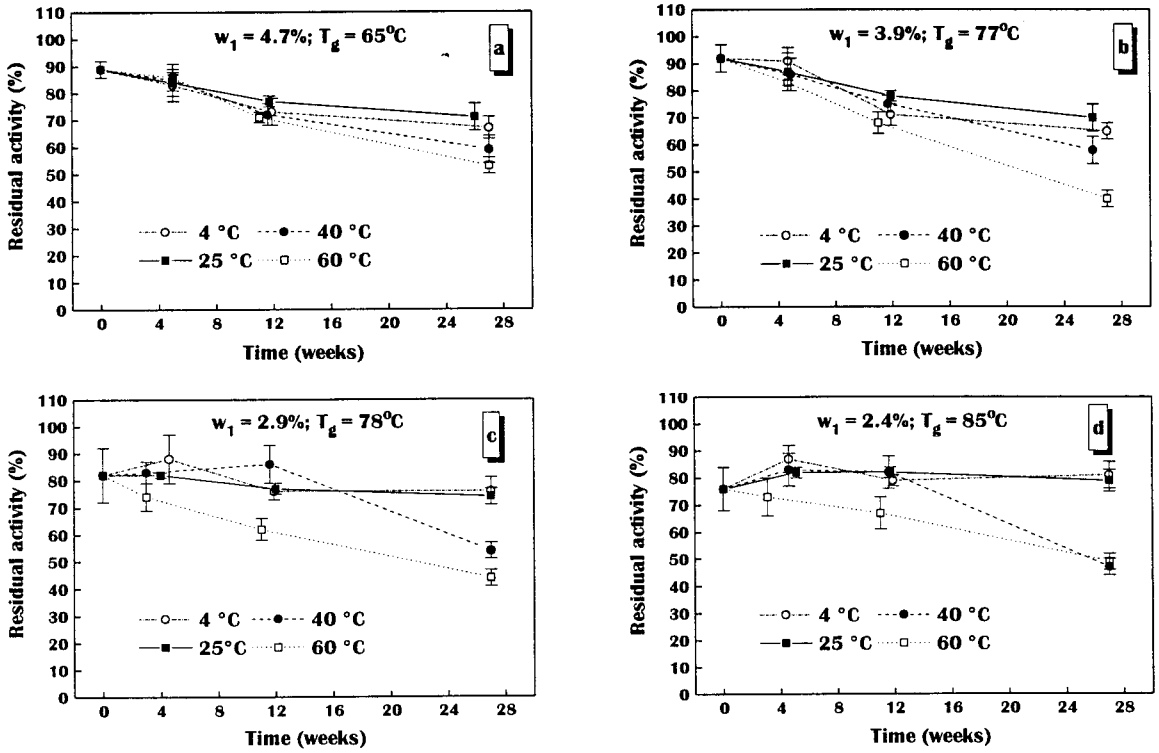

Figure 3—Effect of process temperatures (T_{inlet}/T_{outlet}) on process and storage stability of LDH in spray-dried trehalose powders. The trehalose was initially dry-particle loaded with 0.3% w/w LDH. The spray solution contained 10% total solids (trehalose dihydrate). Liquid feed rate was 4 mL/min, and the atomizing air flow rate was 0.7 m³/h. In each figure the corresponding values for residual water content (w_1) and glass transition temperature (T_g) are given. (A) T_{inlet}/T_{outlet} = 90 °C/60 °C; (B) T_{inlet}/T_{outlet} = 110 °C/70 °C; (C) T_{inlet}/T_{outlet} = 130 °C/80 °C; (D) T_{inlet}/T_{outlet} = 150 °C/95 °C.

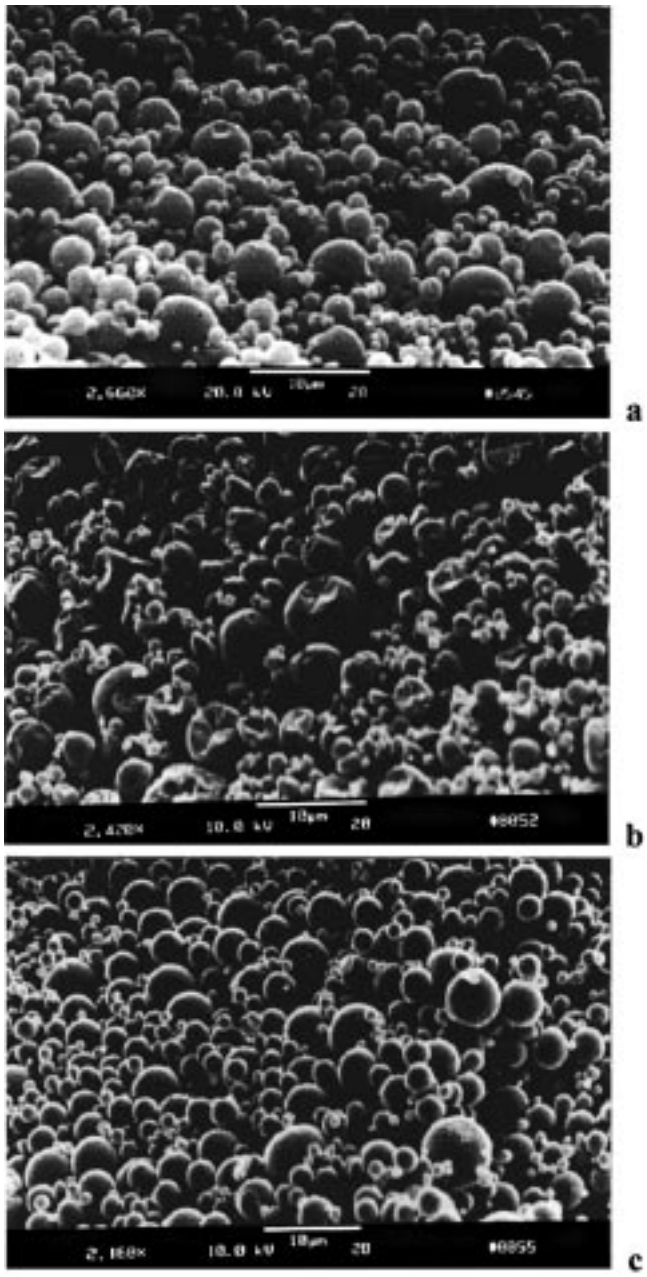

Figure 4—Scanning electron micrographs of trehalose powders spray-dried at T_{inlet}/T_{outlet} = 150 °C/95 °C. The spray solutions contained 10% w/w total solids. (A) Trehalose dry-loaded with 0.3% LDH. (B) Trehalose dry-loaded with 5% LDH. (C) Trehalose dry-loaded with 5% LDH; spray solution also contained 0.1% w/w polysorbate 80.

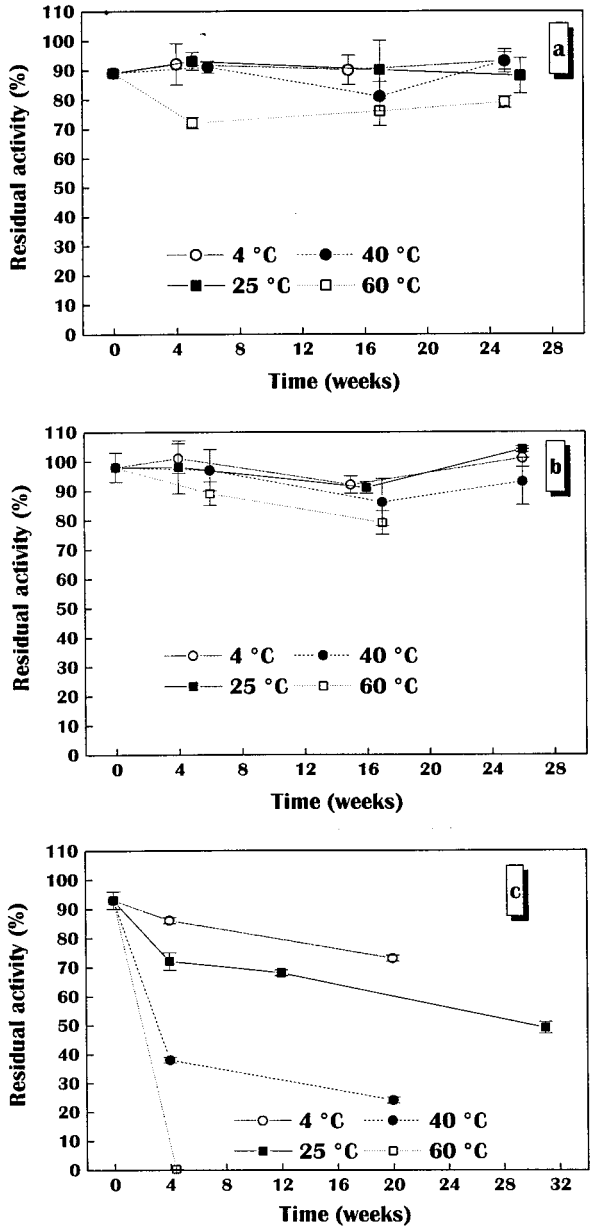

Figure 5—Process and storage stability of LDH in various spray-dried trehalose powders. The process temperatures were T_{inlet}/T_{outlet} = 150 °C/95 °C. The liquid feed rate was 4 mL/min, and the atomizing air flow rate was 0.7 m³/h. (A) 5% w/w dry-particle loading of LDH in trehalose, 10% total solids in spray solution, 0% polysorbate 80 (P 80); (B) 5% w/w dry-particle loading of LDH in trehalose, 10% total solids in spray solution, 0.1% w/w P 80 solution concentration = P 80/LDH molar ratio (27:1); (C) 0.3% w/w dry-particle loading of LDH in trehalose, 2% total solids in spray solution, 0.1% w/w P 80 solution concentration = molar ratio P 80/LDH (1890:1).

starts to form at the droplet surface, followed by a falling-rate period where particle diameter does not change further. As calculated in the Appendix, the droplet evaporation times for an 8.6 μm diameter water droplet containing 10% w/w dissolved solid in the constant-rate period, t_{cr}, and the falling-rate period, t_{fr}, are 0.4 and 1.1 ms, respectively (see Table 3). These process times can be used to calculate the amounts of LDH which could be adsorbed at the liquid/water interface [mg/m²] during droplet formation and drying. Figure 6a shows the air/water surface tension (γ) plot for LDH determined after 0.5 h and 1 h equilibration time. It is clear that this protein is surface active with a surface excess of Γ = 221 mg/m² calculated from the slope using the Gibbs equation. This value is 2 orders of magnitude larger than the saturated monolayer surface concentrations of β-casein (MW = 24000), lysozyme (MW = 14500), and bovine serum albumin (MW = 67000)

Table 3—Results of Calculated Times for Spray Droplet Formation, t_f, and Drying Times in Constant-Rate, t_{cr}, and Falling-Rate, t_{fr}, Periods (see Appendix for calculations)

time	calculated value, ms
t_f	5×10^{-6}
t_{cr}	0.41
t_{fr}	1.07

determined by a radio-tracer method.[20] Despite the higher molecular weight of LDH (144000 for the tetrameter), a Γ of 221 mg/m² is unreasonable. As previously found with human serum albumin, the Gibbs equation evidently cannot be applied to γ versus log C_B data for proteins in this concentration region.[21] It is more useful to examine

Table 4—Calculation of Diffusivity, D, and Surface Excess Concentration, Γ, for LDH in Water from Plot of Surface Pressure, $\Pi(t)$, versus $\sqrt{t}$ According to eq 1

LDH bulk concentration [mg/mL]	slope $d\Pi(t)/dt^{1/2}$ [N/m·s$^{1/2}$]	D [m^2/s]	Γ_f [mg/m^2]	Γ_{cr} [mg/m^2]	LDH adsorbed up to critical point [mg/mL solution]
0.43	1.2×10^{-4}	2.1×10^{-10}	5×10^{-4}	0.14	0.1

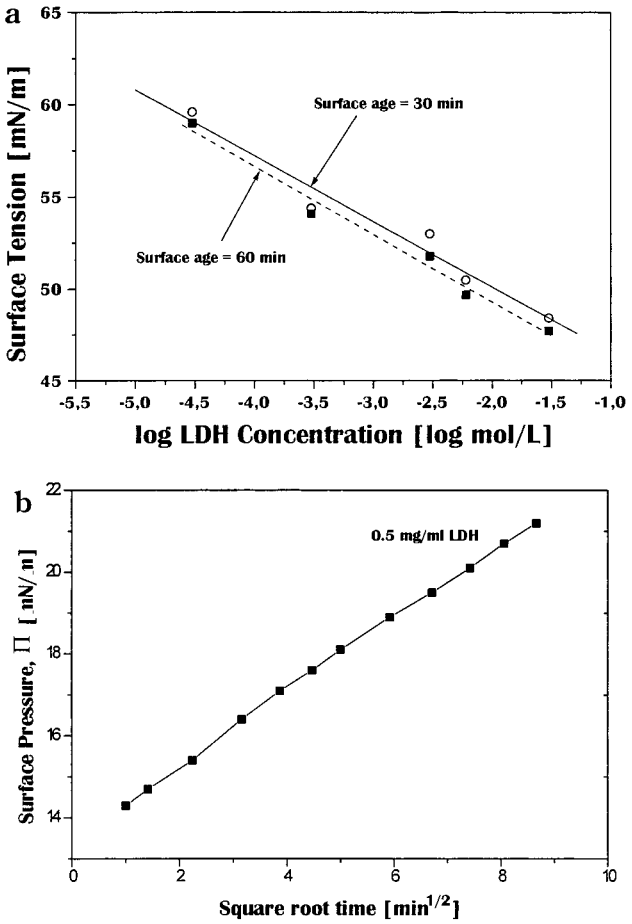

Figure 6—(A) Plot of surface tension versus log LDH concentration in water. From the Gibbs equation, $\Gamma = -[\, d\gamma/d \ln c] \times 1/RT$, the surface excess, Γ, is 221 mg/m^2. (B) Change in surface pressure (Π) with time for 0.43 mg/mL LDH (■) solution in water determined with Wilhelmy plate.

the time-dependence of γ. Adsorption of protein molecules at a liquid/air interface is diffusion-controlled up to approximately 10% surface saturation:[22]

$$\Pi(t) = \Gamma(t)RT = 2RTC_B\sqrt{\frac{Dt}{\pi}} \qquad \Gamma \le 0.1\Gamma_{sat} \quad (1)$$

where Π is the surface pressure ($= \gamma_{solvent} - \gamma_{solution}$), C_B is the protein's bulk concentration, and D is its diffusivity. Figure 6b shows a plot of Π versus $\sqrt{t}$ for an LDH solution in water of $C_B = 0.43$ mg/mL, this being approximately that LDH concentration in the spray solutions used to produce a dry-particle protein loading of 0.3%. The values for $\Pi(t)$ fit well to a straight line in accordance with eq 1 and yield calculated diffusivity of 2.7×10^{-10} m^2/s (see Table 4). This is in the same order of magnitude as found for β-casein and lysozyme.[22] If LDH can diffuse to the liquid/air interface only until the critical point is reached where solid forms at the droplet surface, the total time available for protein adsorption on spraying will be: $t_f + t_{cr}$ (see Table 3). The surface excess concentrations of LDH at the end of droplet formation, Γ_f, and at the critical point, Γ_{cr}, can now be calculated directly from eq 1 and are given in Table 4.

Table 5—Influence of T_g on % LDH-Inactivation Loss on Storage after 27 Weeks at 4 °C, 25 °C, 40 °C, and 60 °C[a]

storage temp [°C]	% LDH-inactivation loss at T_g [°C]: 65 °C	77 °C	78 °C	85 °C
4	25	29	7	0
25	20	24	10	0
40	37	37	34	38
60	40	57	46	36

[a] Values calculated from results in Figure 3.

The calculated total excess amount of LDH adsorbed at the liquid/air interface up to the critical point is clearly more than sufficient to account for the measured LDH-inactivation during spray-drying. With 0.3% LDH dry-particle loading, an LDH-inactivation of 0.075 mg LDH/mL spray solution was measured (Figure 3d), whereas 0.1 mg LDH/mL spray solution could be adsorbed at the spray droplet surface according to this calculation (Table 4). Thus approximately 75% of the theoretical amount of excess adsorbed LDH is therefore inactivated during spray-drying. The interfacial adsorption and inactivation of LDH during spray-drying thus appear feasible.

The *storage stability* of a protein in an amorphous solid will depend in a complex fashion on residual moisture content and T_g.[23] In an early study[3] it was found that those carriers which gave the best protein process stability also gave the best storage stability. The effect on T_{inlet}/T_{outlet} on the balance between process and storage stability has, however, not yet been clarified. Figure 3 shows that the influence of T_{inlet}/T_{outlet} on storage stability is the opposite of that seen with process stability. The high residual moisture content and low T_g of the dried product obtained with T_{inlet}/T_{outlet} = 90 °C/60 °C are associated with a continual decline in LDH activity at all four storage temperatures (Figure 3a). At 25 °C, for example, the LDH loses approximately 15% activity during 26 weeks of storage. Raising T_{inlet}/T_{outlet} reduces the residual moisture content, increases T_g, and improves the storage stability of the LDH, with no loss of LDH activity being seen after 26 weeks of storage at 25 °C for the product spray-dried at T_{inlet}/T_{outlet} = 150 °C/95 °C (Figure 3d). Degradation of proteins in amorphous solids is thought to follow fast kinetics (VTF or WLF) in the rubbery state and slow kinetics (Arrhenius) in the glassy state.[23] Only the samples stored at 4 °C and 25 °C show, however, a trend to less LDH-inactivation with increasing T_g of the carrier (Table 5). Those stored at 40 °C and 60 °C show no dependence of LDH-inactivation on the T_g. These inconsistencies underline the complexity of protein stability in a solid carrier. Protein degradation at storage temperatures below a formulation's T_g can, for example, depend on the presence of non-native protein structure in the dried state,[24] as has been demonstrated for freeze-dried LDH.[25] Also, the mechanism of protein degradation may be temperature dependent, making application of Arrhenius redundant. These aspects were not, however, considered further in this work.

Process and storage stability of LDH in the spray-dried powders are thus seen to be at odds. By decreasing T_{inlet}/T_{outlet} to improve the process stability of LDH, its storage stability (at least at 4 °C and 25 °C) is worsened. This is a result of either reduced T_g[23] or reduced degree of native

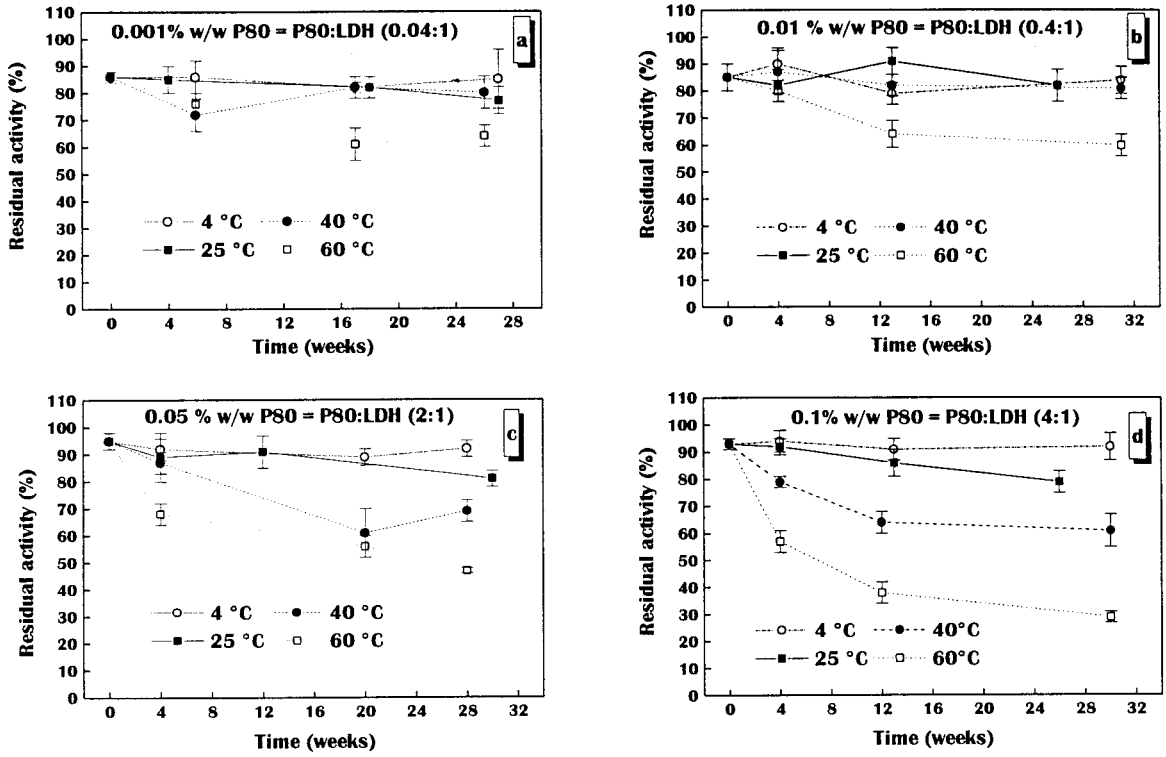

Figure 7—Influence of added Polysorbate 80 (P 80) concentration on process and storage stability of LDH in spray-dried trehalose powders. The trehalose was initially dry-particle loaded with 0.3% w/w LDH. The spray solution contained 10% total solids and was dried at T_{inlet}/T_{outlet} = 150 °C/95 °C. The liquid feed rate was 4 mL/min, and the atomizing air flow rate was 0.7 m³/h. (A) 0.001% w/w P 80 solution concentration = molar ratio P 80/LDH (4.4:1); (B) 0.01% w/w P 80 solution concentration = molar ratio P 80/LDH (44:1); (C) 0.05% w/w P 80 solution concentration = molar ratio P 80/LDH (222:1); (D) 0.1% w/w P 80 solution concentration = molar ratio P 80/LDH (444:1).

structure in the solid immediately after drying.[24] The physicochemical state of the spray-dried powders was stable at all four storage temperatures (over P_2O_5) examined here. There was no change in either residual moisture content (± 0.3% w/w), T_g (± 2 °C), or the X-ray diffractogram up to 27 weeks of storage (cf. Table 2). This was also the case for the powder whose T_g = 65 °C lies only marginally above the highest storage temperature of 60 °C. The incipient formation of a rubbery state without the additional uptake of moisture was evidently insufficient to promote crystallization of the trehalose after 27 weeks. Although not examined in this study, we expect storage under humid conditions to produce major changes in stability of both carrier[7] and protein.[26]

Influence of Polysorbate 80 on LDH Inactivation—It is known that nonionic surfactants can reduce aggregation of proteins during spray-drying at or above the critical micelle concentration of the surfactants in solution.[8,16] The addition of polysorbate 20 to spray solutions of rhGH reduced the total extent of rhGH aggregation during spray-drying at T_{inlet}/T_{outlet} = 90 °C/60 °C from approximately 45% to <18%.[8] Both of these values were reduced at higher protein concentrations in the spray solution. A similar effect is seen with polysorbate 80 on the process stability of LDH in the spray-dried trehalose powders (Figure 7). These results were all obtained for the powder spray-dried at T_{inlet}/T_{outlet} = 150 °C/95 °C. Recall that in the absence of polysorbate 80 some 25% loss in LDH activity occurred during spray-drying under these conditions (cf. Figure 3d). Again we must distinguish between *process stability* and *storage stability* of the LDH. Increasing polysorbate 80 solution-concentration in the spray solution progressively ameliorates the inactivation of LDH during the spray-drying process. At and above a polysorbate 80 solution-concentration of 0.05% w/w, the LDH inactivation has been reduced to approximately 5% during spray-drying (Figure 7c,d). The value of 0,05% w/w surfactant is the same critical surfactant concentration for protein stability found previously for polysorbate 20 and rhGH.[8] The current opinion is that the liquid–air interface of the spray-droplets is preferentially occupied by the surfactant rather than the protein,[8,16] reducing protein unfolding and aggregation during spray-drying. In accord with this idea, the addition of 0.1% polysorbate 80 to the spray solution (Figure 4c) changes the dimpled appearance of trehalose particles dry-loaded with 0.3% LDH (cf. Figure 4a) to the perfect sphericity and smoothness seen with pure trehalose (cf. Figure 2a). This can be explained by a reduction in surface tension of the spray droplets containing surfactant, which alters the balance of surface-to-viscous forces influencing droplet shape during drying.[18]

The effect of polysorbate 80 on the storage stability of LDH in the spray-dried trehalose is unexpected, although its presence in the concentrations examined here influenced neither residual moisture content nor T_g. Recall from Figure 3d that with a T_{inlet}/T_{outlet} = 150 °C/95 °C and a resulting T_g of 84 °C, LDH was storage stable at 4 °C and 25 °C up to 27 weeks. This behavior is retained with 0.001% polysorbate 80 in the spray solution (Figure 7a). Increasing polysorbate 80 solution concentration produces, however, a change in protein stability behavior. The good storage stability at 4 °C remains unaltered; at higher storage temperatures the presence of polysorbate 80 causes a reduction in stability. At polysorbate 80 solution concentrations ≥ 0.05%, we find a clear temperature-dependence of the LDH storage stability (Figure 7c,d). The higher concentrations of polysorbate 80 evidently promote inactivation of LDH within the trehalose glass on storage. The illuminating factor here is the molar ratio of polysorbate 80/LDH present in the dried particles. The low dry-particle LDH loading (0.3% w/w) of trehalose used for the data in Figure 4 makes this ratio large at the higher polysorbate

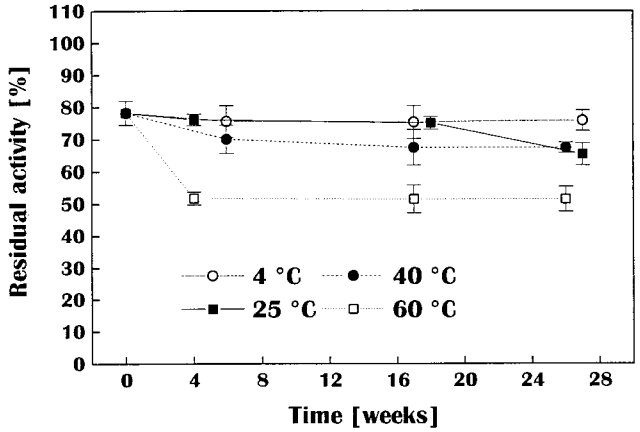

Figure 8—Influence of 0.01% w/w added Lipoid E 80 on process and storage stability of LDH in spray-dried trehalose powders. The trehalose was initially dry-loaded with 0.3% w/w LDH. The spray solution contained 10% total solids and was dried at T_{inlet}/T_{outlet} = 150 °C/95 °C.

80 concentrations. A high molar ratio of polysorbate 80/LDH of 444:1 exists, for example, in the dry product obtained with 0.1% polysorbate 80 in the spray solution, and LDH shows poor storage stability (Figure 7d). By increasing the dry-particle LDH loading to 5% (Figure 5b), 0.1% polysorbate 80 in the spray solution is now equivalent to a mole ratio of 27:1, and LDH is storage stable again. The opposite effect is achieved by reducing the total solids content of the spray solution to 2% with a dry-particle LDH loading of 0.3% and 0.1% polysorbate 80 in the spray solution. This yields the very high molar ratio of polysorbate 80/LDH of 1890:1 in the dry product, which causes extreme LDH instability on storage (Figure 5c). It is conceivable that this instability on storage is a result of peroxide contaminants known to be present in surfactants. These would be present in sufficient quantities to react with LDH in the solid trehalose particles. Care needs therefore to be exercised when adding surfactants to spray-drying solutions. The dependence on the molar ratio of surfactant/protein in the dried product means, however, that protein storage instability caused by the surfactant will not be noticeable with spray-dried pure proteins containing surfactant[8] where the surfactant/protein molar ratio will be smaller than here.

As a negative control we also examined the effect of egg lecithin added to the spray solution on LDH process and storage stability. Lipoid E 80 contains approximately 80% phosphatidylcholine and forms liposomes in water. We chose this material assuming that the single unilamellar vesicles formed would not be adsorbed at the liquid/air interface of the spray droplets and could not therefore stabilize LDH during spray-drying. Subsequent measurements showed, however, that Lipoid 80 reduced the γ of water to approximately 26 mN m^{-1} in the concentration range used in the spray solutions. We attribute this to interfacial adsorption of more water-soluble lyso-derivatives present within the Lipoid E 80. Figure 8 shows how the addition of ≥0.01% w/w solution concentration of Lipoid E 80 indeed fails to ameliorate the approximately 25% loss in LDH activity seen during spray-drying at T_{inlet}/T_{outlet} = 150 °C/95 °C (cf. Figure 3d without colloid). We conclude that the liposomes are not adsorbed at the liquid/air interface of the spray droplets sufficiently to prevent LDH being inactivated. The water-soluble lyso-derivatives evidently also do not stabilize LDH, although it is not clear why this is so. There is a reduction in storage stability of the dry product on addition of Lipoid E 80, but this negative effect (Figure 8) is less pronounced than with polysorbate 80 (cf. Figure 7b).

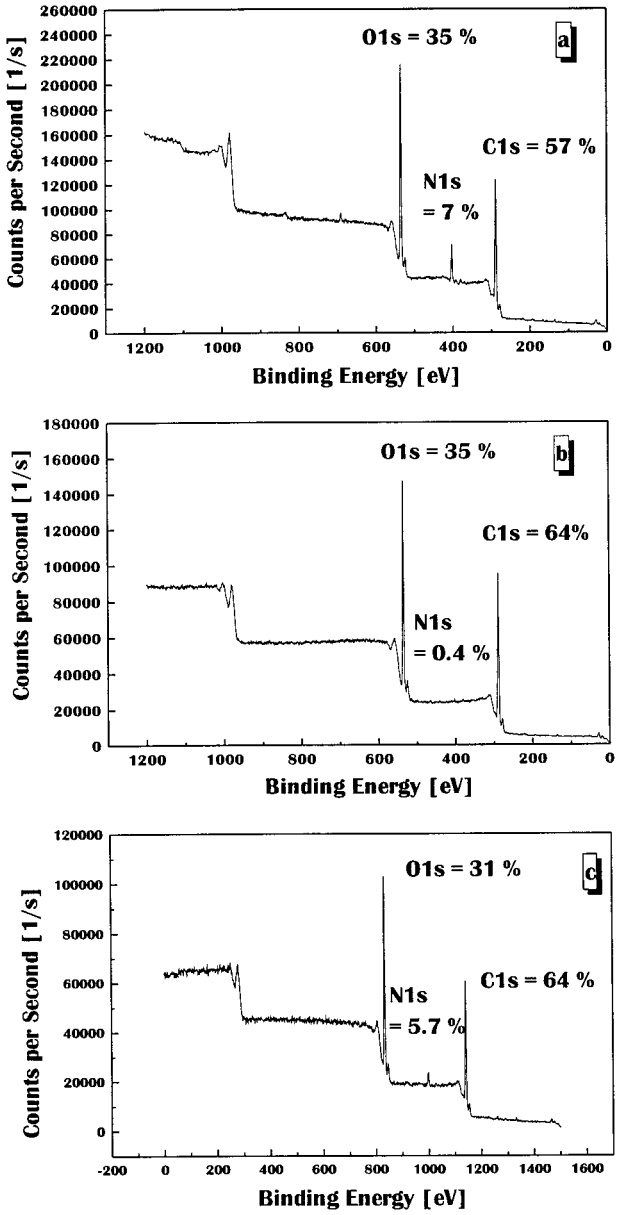

Figure 9—ESCA results for spray-dried trehalose powders dry-loaded with 5% w/w LDH. (A) 0% added polysorbate 80; (B) 0.1% added polysorbate 80 solution concentration; (C) 0.1% added Lipoid E 80 solution concentration.

ESCA Examination of LDH in Trehalose (5:95)—All ESCA measurements had to be carried out with 5% LDH dry-particle loading of the trehalose to ensure sufficient sensitivity to the elements being sought. Figure 9a shows the result for spray-dried LDH/trehalose (T_{inlet}/T_{outlet} = 150 °C/95 °C) in the absence of any added surfactant or colloid. The binding energy profile shows three elemental peaks that are of interest, viz. the 1 s electrons of O, N, and C. From the integrated peak heights the percentage surface-layer coverage of the dried product with each of these elements was calculated and is shown in the figure. The N peak is unequivocally indicative of the presence of LDH in the outer 10 nm of surface of the spray-dried particles. According to the ESCA result, 7 atomic weight % of this surface layer is composed of N (Figure 9a). LDH contains approximately 15 wt % N, although this could not be measured directly by ESCA, since pure solid LDH is not readily obtainable from the LDH-suspension used here. The spray-dried powder is loaded with 5 wt % LDH, so it follows that the surface-layer should contain approximately 0.75 wt % N, if the LDH were homogeneously distributed

throughout each uniformly solid particle. The result is, however, 10 times higher than this value and means that LDH is concentrated within the outer surface-layer of the spray-dried trehalose particles. This cannot be an artifact resulting from the formation of hollow particles, since this would not alter the concentration ratio of trehalose to LDH in the solid phase. The calculations presented above showed that there is sufficient time during droplet formation and drying to allow diffusion-mediated adsorption of LDH to the liquid/air interface. There seems no reason to believe that the rapidly *contracting* liquid/air interface during spray droplet drying up to the critical point would further alter the concentration of LDH at the resulting particle surface. As a result of this process we find the substantial inactivation of LDH during spray-drying seen in Figure 3.

The addition of 0.1% polysorbate 80 to the spray solution reduces the surface coverage with N to <0.4 atomic weight % (Figure 9b). The LDH present in the surface-layer of the spray-dried particles is thus greatly reduced in the presence of the surfactant. The N-coverage of 0.4% is indeed less than that value of 0.75% expected for a homogeneous distribution of LDH throughout a uniformly solid particle. It could also be a result of trace amounts of atmospheric N_2 adsorbed to the particle surface. The presence of surfactant at the liquid/air interface of the spray droplets thus hinders adsorption of LDH there. Owing to its much smaller molecular weight, the polysorbate 80 will diffuse faster to the liquid/air interface than does the LDH. Thus, in that range between 0% and 0.1% solution-concentration where polysorbate 80 progressively reduces LDH inactivation during spray-drying (cf. Figure 7), it also has to a great extent prevented the concentration of LDH in the surface-layer of the dried product. This result provides the first direct and quantitative evidence of the previously accepted stabilizing mechanism of the surfactant on proteins during spray-drying[8,16]

The ESCA result with Lipoid E 80 and LDH is also satisfactory. Recall that addition of Lipoid E 80 to the spray solution in the concentration ≥ 0.01% w/w did not influence inactivation of LDH during spray-drying (Figure 5b). The ESCA result in Figure 9c shows also no reduction in surface coverage of the spray-dried LDH/trehalose particles with N on the addition of 0.1% solution concentration of Lipoid E 80. Lipoid E 80 does not therefore prevent adsorption of LDH at the liquid/air interface of the spray solution and cannot therefore protect LDH against inactivation at T_{inlet}/T_{outlet} = 150 °C/95 °C. This is seen despite the aforementioned reduction in γ of the spray solution with Lipoid E 80. Neither liposomes nor surface-active impurities lead to LDH protection. Whatever the cause, the ESCA results for polysorbate 80 and Lipoid E 80 confirm that surfactants reduce the presence of proteins at the solid particle surface on spray-drying.

Conclusions

The main object of this work was to distinguish between process stability and storage stability of LDH in spray-dried trehalose. The influence of process conditions run contrary to one another: those process conditions that produce good process stability yield poor storage stability. The same behavior is found with addition of surfactant: this improves process stability but is deleterious to storage stability. A particular feature of this work was the use of ESCA to prove that polysorbate 80 prevents LDH appearing in the surface of the spray-dried particles. This could be correlated with a reduction in LDH inactivation during spray-drying. The negative result with Lipoid E 80 confirms this stabilizing mechanism. The relevant equations indicate that the concentrated presence of LDH in the surface of surfactant-free trehalose particles is a result of diffusion-driven adsorption of LDH at the liquid/air interface during spray droplet formation and drying.

Table 6—Comparison of Spraying Conditions and Resulting Spray Droplet Diameter

	from ref 6	in this work
liquid feed rate [m^3/h]	3×10^{-4}	2.4×10^{-4}
air flow rate [m^3/h]	0.9	0.7
air/liquid mass ratio[a]	3.61	3.35
spray droplet diameter [μm]	7.5 (measured)	8.6 (calcd)

[a] $= \dfrac{\text{air flow rate [m}^3\text{/h]} \times \text{air density (1.204)[kg/m}^3\text{]}}{\text{liquid feed rate [m}^3\text{/h]} \times \text{liquid density (1000 or 1407)[kg/m}^3\text{]}}$

Appendix

1. Calculation of Average Spray Droplet Volume—The relation between the diameter of a spray droplet, D_w, and the diameter of the resulting solid particle after drying, D_d, is given by:[27]

$$D_w = 2\left(\frac{\left(\frac{D_d}{2}\right)^3 \rho_2}{W_2}\right)^{1/3} \quad \text{(A1)}$$

where ρ_2 is the density of the solid and W_2 is the total solids' content of the spray solution. For the 10% w/w trehalose solution spray-dried at T_{inlet}/T_{outlet} = 150 °C/95 °C, D_d = 3.5 μm (from Figure 2c), and ρ_2 = 1470 kg/m^3 and W_2 = 100 kg/m^3. Equation A1 predicts a value of 8.6 μm for D_w, which agrees closely with measured values of droplet size at the same air/liquid mass ratio using laser diffraction (see Table 6).[6]

2. Calculation of Spray Droplet Formation Time—The formation time of a single spray droplet, t_f, at the tip of a pneumatic nozzle is given by:

$$t_f = \frac{\text{droplet volume [m}^3\text{]}}{\text{feed rate [m}^3\text{/h]}} \quad \text{(A2)}$$

For a droplet volume of $^4/_3\pi(4.3 \times 10^{-6})^3 = 3.33 \times 10^{-16}$ m^3 and a feed rate of 0.24×10^{-3} m^3/h, eq A2 gives a value for t_f of 5×10^{-6} ms, illustrating the almost instantaneous nature of droplet formation.

3. Calculation of Spray Droplet Drying Time—The drying of spray droplet containing dissolved trehalose in water can be divided into two phases. In the initial constant-rate period, the water concentration at the droplet surface and hence the rate of evaporation, dW/dt, are constant:[19]

$$\frac{dW}{dt}\Big|_{cr} = \frac{2\pi k_d D_{av} \Delta T}{\lambda} \quad \text{(A3)}$$

where ΔT is the log mean temperature difference:

$$\Delta T = \frac{(T_{inlet} - T_{wb}^{inlet})(T_{outlet} - T_{wb}^{outlet})}{\ln(T_{inlet} - T_{wb}^{inlet})/(T_{outlet} - T_{wb}^{outlet})} \quad \text{(A4)}$$

with T_{wb}^{inlet} being the wet-bulb temperature of the spray droplet surface and T_{wb}^{outlet} is the wet-bulb temperature of the dried particle surface. For the spray-dried trehalose, T_{inlet} = 150 °C, T_{wb}^{inlet} = 55 °C (from enthalpy/humidity chart), T_{outlet} = 95 °C, and T_{wb}^{outlet} = 55 °C, giving a value for ΔT of 64 °C. k_d is the thermal conductivity of the water vapor in the stagnant layer around the droplet = 0.567 kcal/m h °C at 64 °C. D_{av} is the average droplet/particle

diameter = 6.05 μm, and λ is the latent heat of vaporization of water = 540 kcal/kg. For these values, $dW/dt = 2.56 \times 10^{-6}$ kg/h according to eq A3. The total mass of water removed in the constant-rate period, ΔW_{cr}, is given by:

$$\Delta W_{cr} = \frac{4}{3}\pi(r_w^3 - r_d^3)\rho W_1 \quad \text{(A5)}$$

where r is droplet/particle radius, ρ is the density of the trehalose/water solution (1047 kg/m^3), and W_1 is the water content of the spray solution (0.9 kg/kg). Equation A5 gives for ΔW_{cr} a value of 2.93×10^{-13} kg. The drying time of the droplet in the constant-rate period, t_{cr}, is then given by:[19]

$$t_{cr} = \frac{\Delta W_{cr}}{dW/dt|_{cr}} = 0.41 \text{ ms} \quad \text{(A6)}$$

In the following falling-rate period, solid is present in the surface and the particle size is assumed not to change further. The rate of evaporation, $dW/dt|_{fr}$, decreases and is given by

$$\frac{\Delta W}{dt}|_{fr} = \frac{dW'}{dt} \times \text{wt of dry particle} \quad \text{(A7)}$$

where:

$$\frac{dW'}{dt} = \frac{12 k_d \Delta T}{\lambda D_c^2 \bar{\rho}_s} \quad \text{(A8)}$$

k_d is the thermal conductivity of the drying air around the particle = 0.0238 kcal/h m °C at approximately 64 °C. D_c is the critical particle diameter = D_d. $\bar{\rho}_s$ is the average particle density during the falling-rate period = 1374 kg/m^3. For these values eqs A7 and A8 give $dW/dt|_{fr} = 6.48 \times 10^{-8}$ kg/h. The mass of moisture removed in the falling-rate period, ΔW_{fr}, is given by the difference between moisture content at the critical point and the end moisture content:

$$\Delta W_{fr} = (2.00 \times 10^{14}) - (8.25 \times 10^{-16}) = 1.92 \times 10^{-14} \text{ kg} \quad \text{(A9)}$$

The drying time of the particle in the falling-rate period, t_{fr}, is then given by:

$$t_{fr} = \frac{\Delta W_{fr}}{dW/dt|_{fr}} = 1.07 \text{ ms} \quad \text{(A10)}$$

The results are summarized in Table 3. The extremely short drying times are a consequence of the small droplet size and high T_{inlet}. As a comparison, the drying time of a pure water droplet of the same diameter[17] yields:

$$t = \frac{\lambda \rho_1}{r k_d \Delta T} \int_0^{D_w} D\,dD = 0.5 \text{ ms} \quad \text{(A11)}$$

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