Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 346 (2008) 102–108
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Stable sugar-based protein formulations by supercritical fluid drying

Nataša Jovanović [a], Andréanne Bouchard [b], Marc Sutter [c], Michiel Van Speybroeck [a], Gerard W. Hofland [b], Geert-Jan Witkamp [b], Daan J.A. Crommelin [a], Wim Jiskoot [a,c,*]

[a] *Department of Pharmaceutical Sciences, Utrecht Institute for Pharmaceutical Sciences (UIPS), P.O. Box 80082, 3508 TB Utrecht, The Netherlands*
[b] *Delft University of Technology, Laboratory for Process Equipment, Leeghwaterstraat 44, 2628 CA Delft, The Netherlands*
[c] *Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

Received 20 February 2007; received in revised form 11 June 2007; accepted 13 June 2007
Available online 19 June 2007

Abstract

The aim of this work was to produce stable, sugar-containing protein formulations by supercritical fluid (SCF) drying. Lysozyme solutions with and without added sucrose or trehalose were dried by spraying them in an SCF composed of CO_2 and ethanol or CO_2 only. The protein-to-sugar ratio was varied between 1:0 and 1:10 (w/w). Dried formulations were stored at 4 °C for three months and analyzed by Karl Fischer titration, scanning electron microscopy, X-ray powder diffraction, differential scanning calorimetry and Fourier transform infrared spectroscopy. Lysozyme stability after reconstitution was determined by an enzymatic activity assay, UV/Vis spectroscopy, and SDS-PAGE. Smooth, spherical particles of 1–25 μm size were obtained. All formulations were initially amorphous. Crystallization during storage only occurred with a protein-to-sugar ratio of 1:10 and could be avoided by performing SCF drying without ethanol. Absence of residual ethanol in dried trehalose formulations increased the glass transition temperature up to 120 °C. Lysozyme in dried formulations was structurally stable, with exception of the 1:0 and 1:1 protein-to-sugar ratios, where reversible protein aggregation occurred. The results show that by avoiding ethanol, which up to now has been considered mandatory for efficient drying of aqueous solutions, and by choosing the proper protein-to-sugar ratio, it is possible to obtain stable, sugar-based protein formulations through SCF drying.

Keywords: Supercritical fluid drying; Protein stabilization; FTIR; Protein structure; Sucrose; Trehalose; Lysozyme

1. Introduction

Supercritical fluid (SCF) drying offers the possibility to quickly obtain large amounts of protein powders with controlled particle morphology, which makes SCF drying an attractive alternative to existing drying techniques for the stabilization of pharmaceutical proteins. However, literature data on protein stabilization by this technique are limited (Nesta et al., 2000; Jovanovic et al., 2004). Therefore, further studies on the effects of operating conditions and formulation parameters on protein stability are necessary to obtain better insight into the potential of SCF drying for the production of solid protein formulations.

In a previous study, we compared the preservation of lysozyme and myoglobin structure by sucrose and trehalose during SCF drying and lyophilization (Jovanovic et al., 2006). The selected formulations with low protein-to-sugar ratio (1:100, w/w) worked well for freeze-drying but were not optimal for SCF drying. Freeze-drying was superior to SCF drying with regard to the preservation of protein structure upon reconstitution and the prevention of sugar matrix crystallization. It was shown that the composition of protein solutions needs to be optimized for SCF drying, and that long-term stability of both the dried matrix and the incorporated protein should be tested.

The present study focuses on optimization of the protein-to-sugar ratio and the SCF composition for obtaining dried powders with favorable physicochemical properties for ensuring the stability of encapsulated protein. Several protein-to-sugar ratios (1:1, 1:4 and 1:10 (w/w)) were tested and solutions were dried with or without ethanol in the SCF phase. Lysozyme was used as a model protein and sucrose and trehalose as commonly used

* Corresponding author at: Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands. Tel.: +31 71 527 4314; fax: +31 71 527 4565.
E-mail address: w.jiskoot@lacdr.leidenuniv.nl (W. Jiskoot).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.06.013

sugar stabilizers. Storage stability of the dried formulations was assessed by measuring crystallization of the amorphous sugar matrices and aggregation of encapsulated lysozyme.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (~7×10^4 U/mg) was purchased from Fluka (Buchs, Switzerland). Trehalose (crystalline, dihydrate from *Saccharomyces cerevisiae*) and Micrococcus lysodeikticus (*Micrococcus luteus*) were acquired from Sigma–Aldrich (Steinheim, Germany). Sucrose (crystalline, European Pharmacopeia grade) was purchased from Riedel-de Haën (Honeywell, Germany). All other chemicals were obtained from different commercial suppliers and were of analytical grade, unless mentioned otherwise.

2.2. SCF drying

Protein solutions, excipient-free (only containing protein) or with sugars (sucrose or trehalose) in a 1:1, 1:4 and 1:10 (w/w) protein-to-sugar ratio, were dried in a type SFP4 (Separex, Champigneulles, France) supercritical drying apparatus as described previously (Jovanovic et al., 2006). Protein solutions were sprayed into an SCF mixture containing CO_2 and ethanol or only CO_2. In the first case (further referred to as condition A), the flow rates used were 0.5 ml/min for the protein solution, 250 g/min for the supercritical CO_2 (SC-CO_2) and 25 ml/min for the ethanol. When no ethanol was used during SCF drying (condition B), the CO_2 flow rate was doubled (500 g/min) and the protein solution flow rate decreased (0.25 ml/min) to compensate for the decrease in water extraction capacity of the supercritical phase. The pressure and temperature were maintained at 100 bar and 37 °C for all components, before and after drying. After completion of the spraying process, the ethanol flow was stopped and the vessel was flushed for 16 min with SC-CO_2 to remove residual solvents (water and/or ethanol) before depressurization and product recovery. The dry powder was recovered from the filter on the bottom of the vessel. For selected samples, part of the powder was immediately placed into vacuum oven for additional drying at 55 °C for 24 h. All dried products were stored under nitrogen in air-tight containers at 4 °C.

2.3. Physical properties of SCF dried powders

2.3.1. Residual water content

Samples (ca. 30 mg) of the SCF dried formulations were dispersed in methanol and the residual water content of the formulations was measured by the Karl-Fischer method using a Metrohm 756 KF instrument (Metrohm, Herisau, Switzerland) as described in the manufacturer's manual. Methanol was used as a blank.

2.3.2. Residual ethanol content

Quantification of the residual ethanol in the powder was done by gas chromatography (GC) (Chromopack CP9002, Bergen op Zoom, The Netherlands). Approximately 20 mg of powder (SCF dried lyzozyme trehalose 1:10, w/w, formulations), accurately weighed, was dissolved in 1 g of water and the solution was placed into a 2-ml GC vial before capping it. Standards, freeze-dried lysozyme trehalose (1:10, w/w) containing known amounts of added ethanol, were prepared in the same way as SCF dried powder. A volume of 0.1–0.5 μl of standard solution was then injected directly into the column (Varian, CP SIL 5CB, 25 m, 0.53 mm, film thickness 5 μm) at a 120 °C isotherm. Data were analyzed using Galaxie Chromatography Workstation (Varian Inc.). A blank solution containing standard without ethanol was injected as well to check background signal. A 0.3 μl of each SCF dried sample was injected. A calibration curve was made by plotting the AUC of the ethanol peak against the ethanol content of the standards and linear regression ($r^2 = 0.9993$).

2.3.3. Scanning electron microscopy (SEM)

SEM (JSM-5400, Jeol, Peabody, USA) images were used to examine the morphology of the dried particles. Conductive double-sided tape was used to fix the particles to the specimen holder before covering them with a thin layer of gold.

2.3.4. Modulated differential scanning calorimetry (MDSC)

MDSC was performed on a Q-1000 calorimeter (TA instruments, New Castle, Delaware, USA). Dry powder samples (5–10 mg) in aluminum hermetic sealed pans (TA Instruments, New Castle, Delaware, USA) were heated from 0 to 170 °C at a rate of 1 °C/min and a modulation of ±1 °C/30 s. MDSC enabled to distinguish reversible thermal events such as glass transition temperature (T_g) from irreversible thermal events. Temperature calibration of the instrument was performed with Indium (melting point 156.60 °C).

2.3.5. X-ray powder diffraction (XRPD)

XRPD was performed using a D8 Discover X-ray diffractometer with a bi-dimensional area detector (Bruker AXS, Madison, USA) according the procedure described earlier (Jovanovic et al., 2006).

2.4. Protein structure in the solid state

Protein structure in the solid state was studied by Fourier transform infrared (FTIR) spectroscopy using a Bio-Rad FTS6000 FTIR spectrometer with Win-IR Pro software, version 2.95 (Cambridge, USA). KBr pellets were prepared by mixing 5–10 mg of SCF dried formulation with approximately 150 mg of spectroscopy grade KBr and pressing the mixture into a pellet (diameter 13 mm) at a pressure of 260 MPa. The number of scans per experiment was set to 256, the scan speed to 0.16 cm/s and the resolution to 2 cm^{-1}. The spectra were corrected for water vapour and smoothed with a 7-point Savitsky-Golay smoothing function (van de Weert et al., 2005). The second derivative spectra were smoothed using a 7-point Savitsky-Golay smoothing function and subsequently inverted. The amide I region of the spectra (1720–1600 cm^{-1}) was used for analyzing protein secondary structure. To compare spectra and to quantify changes in protein secondary structure, second derivative spectra were

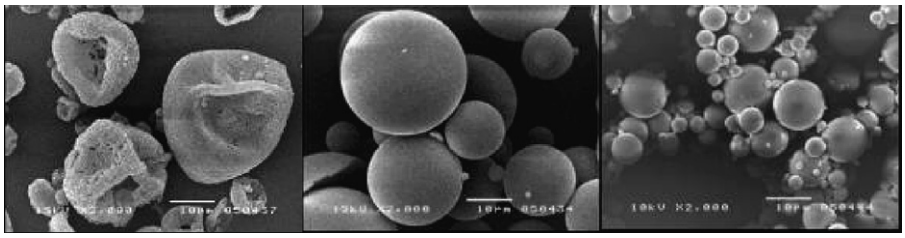

Fig. 1. SEM images of SCF dried lysozyme formulations. From left to the right: excipient-free lysozyme, 1:1 (w/w) lysozyme:sucrose and 1:1 (w/w) lysozyme:trehalose formulations. SCF drying condition A: protein solution flow rate: 0.5 ml/min, CO_2 flow rate: 250 g/min, ethanol flow rate: 25 ml/min.

truncated between 1720 and 1600 cm^{-1}, baseline-corrected and area-normalized to unity (Kendrick et al., 1996).

2.5. Protein structure after reconstitution

2.5.1. UV/Vis spectroscopy

Absorption spectra between 240 and 350 nm were acquired at room temperature using a Lambda 2 UV/Vis spectrophotometer (Perkin-Elmer, Ueberlingen, Germany). Optical density (OD) above 320 nm was taken as an indication for the presence of aggregates (Kueltzo and Middaugh, 2005). In absence of aggregates, absorption at 280 nm was used for protein concentration determination (Jovanovic et al., 2006).

2.5.2. Enzymatic activity

Lysozyme enzymatic activity was determined using a bacteriolysis assay based on the decrease of optical density at 450 nm of a *M. luteus* suspension in presence of lysozyme (van de Weert et al., 2000). Enzymatic activity is expressed as percentage of activity with respect to a reference lysozyme solution.

2.5.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed under reducing (sample buffer containing β-mercaptoethanol) and non-reducing (without β-mercaptoethanol) conditions using a Biorad Protean III system (Biorad, Veenendaal, The Netherlands) as described before (Jovanovic et al., 2006).

3. Results and discussion

3.1. Particle morphology and size

The scanning electron microscopy images in Fig. 1 show that the addition of sugars affected particle morphology and resulted in smooth, spherical particles with sizes ranging from 1 to 25 μm, whereas agglomerated structures and collapsed spheres were obtained when pure lysozyme solution was dried. Particle morphology and size were independent of sugar type and composition of the SCF (not shown).

3.2. Sugar matrix crystallization upon SCF drying with a CO_2/ethanol mixture

The addition of ethanol to SC-CO_2 is generally considered necessary for an efficient drying of aqueous solutions by SCF techniques, because it enhances the solubility of water in the SCF phase (York et al., 2004). Accordingly, at first an SCF composed of CO_2 and ethanol was used for drying aqueous solutions of lysozyme and sucrose or trehalose. Table 1 shows the physicochemical properties of SCF dried formulations with different protein-to-sugar ratios. The residual water content was between 2.5 and 5%. All formulations were amorphous upon SCF drying, as confirmed by XRPD (see Fig. 2a for sucrose formulations) and by the presence of a glass transition in MDSC scans (Table 1). It can be seen from Table 1 that the glass transition temperature (T_g) of all formulations was fairly low, between 15 and 45 °C. Since this is in vicinity of the storage

Table 1
Water content, glass transition temperature (T_g) and lysozyme enzymatic activity of SCF dried formulations

Formulation	Water content[a] (%)	T_g[b] (°C)	Enzymatic activity (%)[c]	
			Immediately after drying	After 1 month storage
Lysozyme (excipient-free)	4.2 ± 1.8		99.6 ± 3.6	97.6 ± 4.4
Lysozyme:sucrose				
1:10 (w/w)	2.4 ± 1.4	15.2–31.0	96.6 ± 2.2	94.0 ± 2.9
1:4 (w/w)	4.7 ± 0.8	14.2–38.0	98.7 ± 4.8	98.5 ± 5.3
1:1 (w/w)	2.8 ± 0.7	31.0–34.0	101.9 ± 6.7	100.6 ± 4.0
Lysozyme:trehalose				
1:10 (w/w)	2.6 ± 0.8	36.9–46.0	99.9 ± 8.2	101.5 ± 6.8
1:4 (w/w)	4.0 ± 0.3	25.8–32.2	100.2 ± 2.1	96.7 ± 4.8
1:1 (w/w)	3.5 ± 1.5	34.1–40.8	101.8 ± 6.4	105.3 ± 9.2

[a] Average ± S.D. of three independent batches.
[b] Minimum and maximum value of two independent batches.
[c] Percentage ± S.D. of three independent batches relative to unprocessed lysozyme.

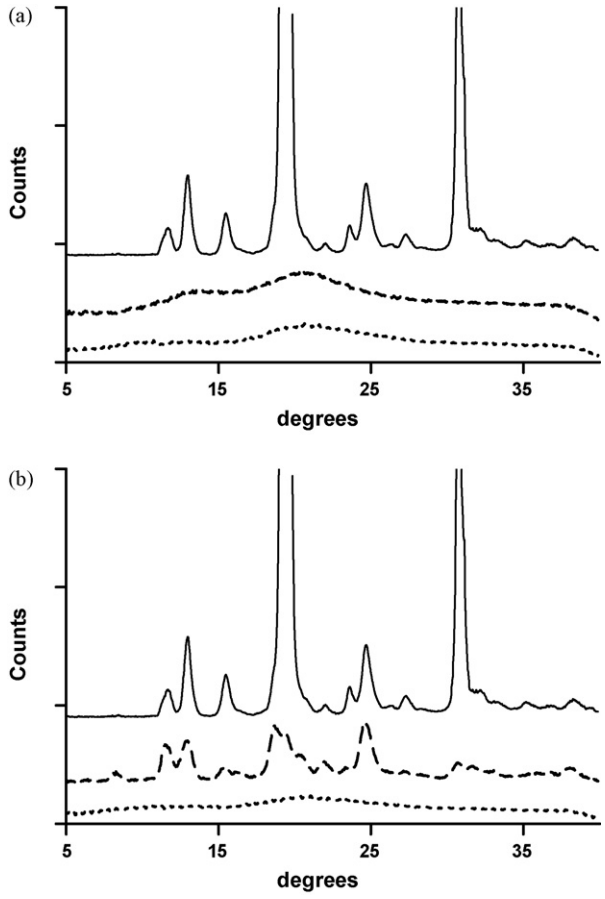

Fig. 2. XRPD scans of SCF dried lysozyme:sucrose formulations in comparison with crystalline sucrose. From bottom to the top: SCF dried 1:1 (w/w) lysozyme:sucrose formulation, 1:10 (w/w) lysozyme:sucrose formulation and crystalline sucrose. (a) Scans taken immediately after SCF drying. (b) Scans taken after 1 month of storage at 4 °C.

temperature of 4 °C, crystallization of the sugar matrix is theoretically possible (Frokjaer and Hovgaard, 2000; Carpenter and Manning, 2002; Costantino and Pikal, 2004) and indeed occurred with the lysozyme-sucrose formulation (1:10, w/w) already after 1 month (Fig. 2b) and lysozyme-trehalose formulations with a protein-to-sugar ratio of 1:10 during three months storage (Table 2). Crystallization during storage did not occur with protein-to-sugar ratios of 1:4 and 1:1. This shows that sugar crystallization can be prevented using a minimum fraction of protein, as has been reported for other protein–sugar matrices obtained by different drying techniques (Costantino et al., 1998; Tzannis and Prestrelski, 1999; Sharma and Kalonia, 2004). A possible explanation for this is the formation of hydrogen bonds between sugars and protein molecules (Frokjaer and Hovgaard, 2000; Carpenter and Manning, 2002; Costantino and Pikal, 2004), which prevent interactions between sugar molecules that lead to nucleation and crystallization. The mechanism of preventing crystallization by hydrogen bond interactions has also been proposed for other systems, like mixtures of indomethacin and polyvinylpyrrolidones (Matsumoto and Zografi, 1999). In addition, the presence of other molecules can prevent sugar crystallization by dilution of the sugar molecules and reduced

Table 2
Protein aggregation and sugar crystallization in SCF dried formulations

Time (days)	Lysozyme:sucrose (w/w)						Lysozyme:trehalose (w/w)					
	1:1		1:4		1:10		1:1		1:4		1:10	
	Protein aggregation	Sugar crystallization	Protein aggregation	Sugar crystallization	Protein aggregation	Sugar crystallization	Protein aggregation	Sugar crystallization	Protein aggregation	Sugar crystallization	Protein aggregation	Sugar crystallization
0	–	–	–	–	–	–	–	–	–	–	–	–
30	+	–	–	–	–	+	+	–	–	–	–	–
90	Not studied		–	–	Not studied		Not studied		–	–	–/+	+

Aggregation as detected by FTIR. Crystallization as detected by XRPD. (–, not detected; +, detected; –/+, detected in one out of three formulations).

molecular motions in the dried matrix (Saleki-Gerhardt and Zografi, 1994).

3.3. Protein stability upon SCF drying with a CO_2/ethanol mixture

The structural stability of lysozyme in SCF dried formulations was studied by FTIR spectroscopy. The main peak at 1657 cm^{-1} in the spectra (Fig. 3a and b) was associated to α-helix, which is the dominant secondary structure in unperturbed lysozyme (van de Weert et al., 2005). Increased intensity of two peaks at 1695 and 1625 cm^{-1} indicated the formation of intermolecular β-sheets, i.e. aggregation of lysozyme in the sugar matrices (Dong et al., 1995; van de Weert et al., 2005). Lysozyme dried without sugars contained aggregated protein as indicated by the intense peaks at 1695 and 1625 cm^{-1} (Fig. 3a). Lysozyme dried with sugars contained much less aggregated protein. As can be seen from Fig. 3a, the addition of sucrose or trehalose resulted in an increase of the peak representing α-helical structure and a concomitant decrease of the peaks representing aggregated protein. Immediately after SCF drying, no significant differences between formulations with different protein-to-sugar ratios were observed for sucrose (see Fig. 3b) and trehalose formulations (not shown).

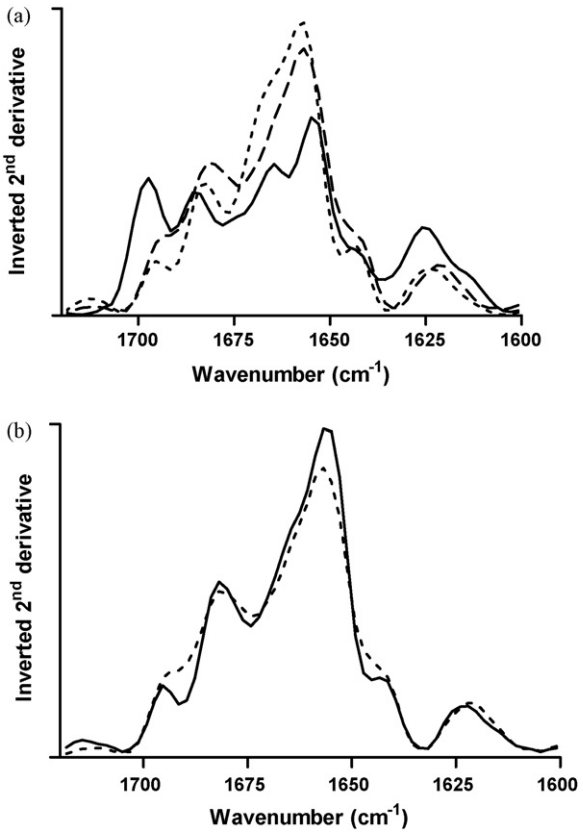

Fig. 3. Second derivative FTIR spectra of freshly prepared SCF dried lysozyme formulations. (a) Effect of sugars: excipient-free lysozyme (solid line), 1:10 (w/w) lysozyme:sucrose (dashed line), 1:10 (w/w) lysozyme:trehalose (dotted line). (b) Comparison of different protein-to-sugar ratios: 1:1 (w/w) lysozyme:sucrose (solid line), 1:10 (w/w) lysozyme:sucrose (dotted line).

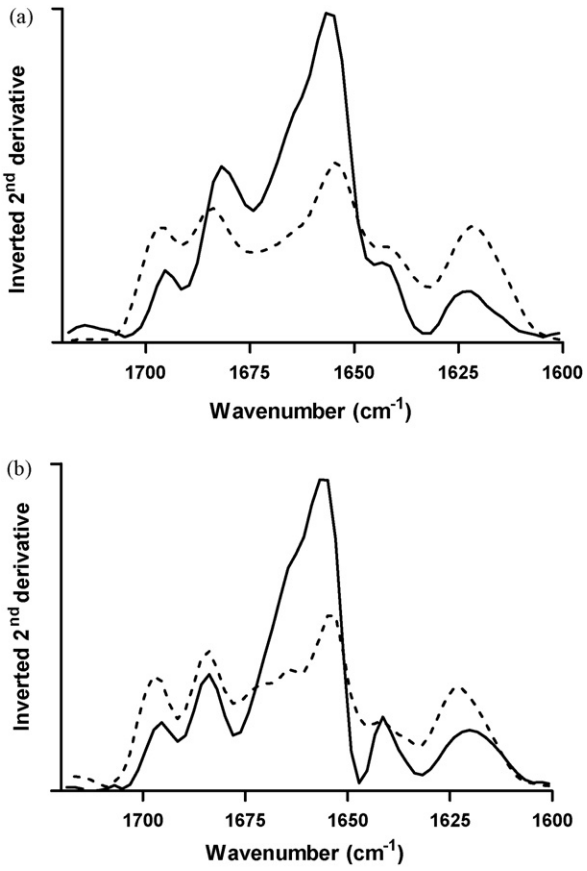

Fig. 4. Second derivative FTIR spectra of SCF dried formulations with 1:1 (w/w) lysozyme:sugar ratio, right after the drying (solid line) and after 1 month of storage at 4 °C (dotted line). (a) Formulation containing sucrose. (b) Formulation containing trehalose.

During storage for up to three months at 4 °C, no structural change of lysozyme was observed by FTIR in sucrose and trehalose formulations with protein-to-sugar ratios of 1:10 and 1:4 (not shown). However, both sucrose and trehalose formulations with a protein-to-sugar ratio of 1:1 showed enhanced aggregation of lysozyme after already 1 month storage (Table 2; Fig. 4a and b), indicating that in these formulations, the amount of sugar is insufficient to prevent protein–protein interactions (Carpenter and Manning, 2002; Andya et al., 2003; Costantino and Pikal, 2004). This aggregation was reversible upon reconstitution, as confirmed by the absence of a scattering signal above 320 nm in UV spectroscopy and the absence of aggregate bands in reducing and non-reducing SDS-PAGE. Furthermore, the enzymatic activity upon reconstitution of aggregated lysozyme was between 97 and 100%. Nevertheless, it is important to choose protein-to-sugar ratios where structural alteration and aggregation in the solid state do not occur, because these processes are irreversible for many proteins (Dong et al., 1995; Allison et al., 2000; Carpenter and Manning, 2002; Moshashaee et al., 2003; Hermeling et al., 2004; Sane et al., 2004) and may lead to immune reactions in patients upon administration (Carpenter and Manning, 2002; Hermeling et al., 2004).

Table 3
Effect of SCF processing conditions on physical properties of dried lysozyme-trehalose (1:10, w/w) formulations

Formulation containing lysozyme trehalose in 1:10 (w/w) ratio			Water content (%)[a]	Ethanol content (%)[a]	T_g (°C)[a]	Three months storage (crystallization)
	Ethanol	Secondary drying[b]				
SCF condition A[c]	+	–	4.7–5.4	8.7–10.9	22.1–29.2	+
	+	+	2.0–2.5	2.2–2.9	52.3–57.0	+
SCF condition B[c]	–	–	3.3–4.4	n.d.	52.0–70.0	–
	–	+	1.6–1.8	n.d.	119.6–121.5	–

[a] Lower-upper values of two formulations tested.
[b] Secondary drying: vacuum oven, 55 °C for 24 h.
[c] SCF drying condition A: protein solution flow rate: 0.5 ml/min, CO_2 flow rate: 250 g/min, ethanol flow rate: 25 ml/min; SCF drying condition B: protein solution flow rate: 0.25 ml/min, CO_2 flow rate: 500 g/min, ethanol flow rate: 0 ml/min.

3.4. Effect of residual water and ethanol

The above results show that an optimal protein-to-sugar ratio (in this case a 1:4 ratio) leads to mutual stabilization of protein and sugar molecules in the sense that aggregation of the former and crystallization of the latter are prevented. However, absolute values of T_g between 15 and 45 °C (Table 1) are clearly too low to be considered safe in the case of accidental warming to higher temperatures. These low T_g values could not be explained solely by a residual water content of the SCF dried powders between 2.5 and 5% (Table 1). For example, we reported previously for freeze-dried lysozyme-trehalose formulations with similar water content (ca. 4%) T_g value of around 70 °C (Jovanovic et al., 2006). Furthermore, dried trehalose with comparable water content is expected to have higher T_g of 80–90 °C based on Fox equation (Saleki-Gerhardt and Zografi, 1994; Jovanovic et al., 2006). It was hypothesized that residual ethanol, which had partitioned from the SCF into the microspheres during drying, could be responsible for the unexpected decrease of T_g, and this hypothesis was evaluated with lysozyme-trehalose formulations with a protein-to-sugar ratio of 1:10. Table 3 confirms that in addition to a residual water content of around 5%, microspheres contained a substantial fraction of residual ethanol of around 10%. Subjecting these microspheres to an additional drying step in a vacuum stove (see Section 2) decreased the residual water and residual ethanol contents to values between 2 and 3% (Table 3). Although this resulted in an increase of T_g from about 25 °C to around 55 °C, crystallization of the sugar matrix during three months storage at 4 °C still occurred.

In order to completely avoid residual ethanol, SCF drying without addition of ethanol to the SC-CO_2 was performed (see Section 2). This gave microspheres with smooth surfaces. Their sizes were similar as when ethanol was added to the SC phase (not shown). As can be seen from Table 3, the residual water content of these microspheres was about 4%. The T_g was at about 65 °C, which is comparable to the T_g of about 70 °C reported for freeze-dried trehalose formulations with a similar water content (Jovanovic et al., 2006). Crystallization of the sugar matrix during three months storage at 4 °C did not occur. Even higher T_g values of about 120 °C, at a residual water content of 1.5–2%, were achieved by subjecting the microspheres to secondary drying in the vacuum stove. Also after this treatment, no crystallization of the sugar matrix during three months storage at 4 °C was observed (Table 3).

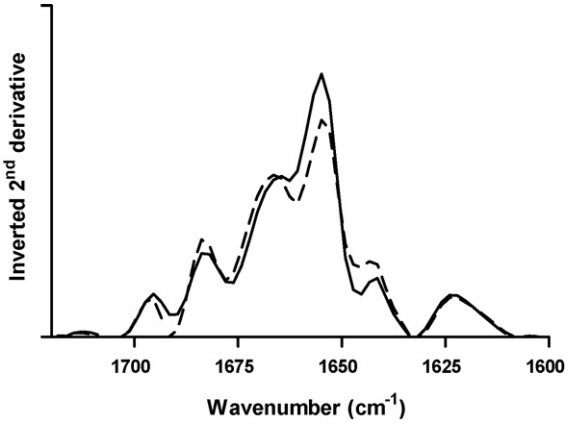

Fig. 5. Second derivative FTIR spectra of SCF dried formulations with 1:10 (w/w) lysozyme:trehalose: dried under condition A, 0.5 ml/min protein solution, 250 g/min SC-CO_2 and 25 ml/min ethanol flow rate (solid line); dried under condition B, 0.25 ml/min protein solution, 500 g/min SC-CO_2 and 0 ml/min ethanol flow rate (dashed line).

These results clearly demonstrate that residual ethanol in the microspheres has a strong plasticizing effect, and that avoiding ethanol entirely during SCF drying is an effective way of obtaining stable, amorphous sugar matrices with high T_g. Importantly, the change in the SCF drying procedure did not have a negative effect on lysozyme structural stability, as can be seen from the FTIR spectra in Fig. 5. This was also confirmed by analysis upon reconstitution: UV spectroscopy showed no scattering above 320 nm, indicating the absence of aggregates in solution, and the enzymatic activity of lysozyme upon reconstitution was fully preserved (data not shown). Recent results obtained at our laboratory show that by the ethanol-free SCF drying procedure, it is also possible to stabilize other proteins than lysozyme. This could be demonstrated for polyclonal human serum IgG (Jovanovic et al., in press).

4. Conclusions

The present work demonstrates that residual ethanol acts as a plasticizer in SCF dried powders, leading to decreased T_g values and increased tendency of amorphous sugar matrices to crystallize. In contrast to previous reports (Nesta et al., 2000;

Moshashaee et al., 2003; Jovanovic et al., 2004; York et al., 2004), SCF drying of aqueous solutions without the addition of ethanol proved to be feasible. With the studied formulations, this resulted in high T_g values of the amorphous sugar matrices. Crystallization of these matrices during storage at 4 °C was not observed, and the encapsulated protein remained structurally stable.

Optimization of the protein-to-sugar ratio may be an additional strategy for improving the stability of SCF dried protein formulations. Our results show that when the glass transition temperature is close to the storage temperature, an optimal protein-to-sugar ratio prevents both crystallization of the sugar matrix and structural alteration and aggregation of encapsulated protein. Finding this optimal protein-to-sugar ratio is therefore important for the stability of a formulation in case of accidental heating to high temperatures.

Overall the present work demonstrated that stable, sugar-based protein formulations can be obtained by SCF drying. As compared to freeze-drying, applying SCF drying for this purpose brings along a substantial increase of production speed and the possibility to produce free-flowing particles of a defined size.

Acknowledgments

This research was supported by the Technology Foundation STW, applied science division of NWO and the technology program of the Dutch Ministry of Economic Affairs. We thank Marco van de Weert from the University of Copenhagen for his helpful suggestions with respect to the FTIR technique and Eduardo Mendes from Delft University of Technology for his help with the XRPD measurements.

References

Allison, S.D., Manning, M.C., Randolph, T.W., Middleton, K., Davis, A., Carpenter, J.F., 2000. Optimization of storage stability of lyophilized actin using combinations of disaccharides and dextran. J. Pharm. Sci. 89, 199–214.

Andya, J.D., Hsu, C.C., Shire, S.J., 2003. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. AAPS Pharmsci. 5, E10.

Carpenter, J.F., Manning, M.C., 2002. Rational Design of Stable Protein Formulations, Theory and Practice. Kluwer Academic/Plenum Publishers, New York.

Costantino, H.R., Pikal, M.J., 2004. Lyophilization of Biopharmaceuticals. American Association of Pharmaceutical Sciences, Arlington.

Costantino, H.R., Andya, J.D., Nguyen, P.A., Dasovich, N., Sweeney, T.D., Shire, S.J., Hsu, C.C., Maa, Y.F., 1998. Effect of mannitol crystallization on the stability of aerosol performance of a spray-dried pharmaceutical protein, recombinant humanized anti-IgE monoclonal antibody. J. Pharm. Sci. 87, 1406–1411.

Dong, A., Prestrelski, S.J., Allison, S.D., Carpenter, J.F., 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J. Pharm. Sci. 84, 415–424.

Frokjaer, S., Hovgaard, L., 2000. Pharmaceutical Formulation Development of Peptides and Proteins. Taylor & Francis, London.

Hermeling, S., Crommelin, D.J., Schellekens, H., Jiskoot, W., 2004. Structure-immunogenicity relationships of therapeutic proteins. Pharm. Res. 21, 897–903.

Jovanovic, N., Bouchard, A., Hofland, G.W., Witkamp, G.J., Crommelin, D.J., Jiskoot, W., 2004. Stabilization of proteins in dry powder formulations using supercritical fluid technology. Pharm. Res. 21, 1955–1969.

Jovanovic, N., Bouchard, A., Hofland, G.W., Witkamp, G.J., Crommelin, D.J., Jiskoot, W., 2006. Distinct effects of sucrose and trehalose on protein stability during supercritical fluid drying and freeze-drying. Eur. J. Pharm. Sci. 27, 336–345.

Jovanovic, N., Bouchard, A., Hofland, G.W., Witkamp, G.J., Crommelin, D.J., Jiskoot, W. Stabilization of IgG by supercritical fluid drying: optimization of formulation and process parameters. Eur. J. Pharm. Biopharm., in press.

Kendrick, B.S., Dong, A., Allison, S.D., Manning, M.C., Carpenter, J.F., 1996. Quantitation of the area of overlap between second-derivative amide I infrared spectra to determine the structural similarity of a protein in different states. J. Pharm. Sci. 85, 155–158.

Kueltzo, L.A., Middaugh, C.R., 2005. Ultraviolet absorption spectroscopy. In: Jiskoot, W., Crommelin, D.J. (Eds.), Methods for Structural Analysis of Protein Pharmaceuticals. AAPS, Arlington, pp. 1–25.

Matsumoto, T., Zografi, G., 1999. Physical properties of solid molecular dispersions of indomethacin with poly(vinylpyrrolidone) and poly(vinylpyrrolidone-co-vinyl-acetate) in relation to indomethacin crystallization. Pharm. Res. 16, 1722–1728.

Moshashaee, S., Bisrat, M., Forbes, R.T., Quinn, E.A., Nyqvist, H., York, P., 2003. Supercritical fluid processing of proteins: lysozyme precipitation from aqueous solutions. J. Pharm. Pharmacol. 55, 185–192.

Nesta, D.P., Elliott, J.S., Warr, J.P., 2000. Supercritical fluid precipitation of recombinant human immunoglobulin from aqueous solutions. Biotechnol. Bioeng. 67, 457–464.

Saleki-Gerhardt, A., Zografi, G., 1994. Non-isothermal and isothermal crystallization of sucrose from the amorphous state. Pharm. Res. 11, 1166–1173.

Sane, S.U., Wong, R., Hsu, C.C., 2004. Raman spectroscopic characterization of drying-induced structural changes in a therapeutic antibody: correlating structural changes with long-term stability. J. Pharm. Sci. 93, 1005–1018.

Sharma, V.K., Kalonia, D.S., 2004. Effect of vacuum drying on protein-mannitol interactions: the physical state of mannitol and protein structure in the dried state. AAPS Pharmscitech. 5, E10.

Tzannis, S.T., Prestrelski, S.J., 1999. Activity-stability considerations of trypsinogen during spray drying: effects of sucrose. J. Pharm. Sci. 88, 351–359.

van de Weert, M., Hoechstetter, J., Hennink, W.E., Crommelin, D.J., 2000. The effect of a water/organic solvent interface on the structural stability of lysozyme. J. Control. Release 68, 351–359.

van de Weert, M., Hering, J.A., Haris, P.I., 2005. Fourier transform infrared spectroscopy. In: Jiskoot, W., Crommelin, D.J. (Eds.), Methods for Structural Analysis of Protein Pharmaceuticals. AAPS, Arlington, pp. 131–166.

York, P., Kompella, U.B., Shekunov, B.Y., 2004. Supercritical Fluid Technology for Drug Product Development. Marcel Dekker, New York.