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The Stability of Insulin in Solid Formulations Containing Melezitose and Starch. Effects of Processing and Excipients

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ABSTRACT Solid insulin formulations obtained by different methods of preparation were compared with respect to chemical stability and morphology. Spray- and freeze-drying, solution enhanced dispersion by supercritical fluids (SEDS) and precipitation into starch microspheres were the methods used for preparation of solid powders. The excipients applied were melezitose, starch, and sodium taurocholate. The stability of the samples was evaluated after storage in open containers at 25°C and 30% RH for 6 months.

All samples were amorphous after processing and storage as detected by XRD, except for the starch microspheres which were semi-crystalline. The spray- and freeze-dried samples containing melezitose and sodium taurocholate experienced a significant water uptake during storage, resulting in changes in morphology and disappearance of Tg. However, the chemical stability of these samples did not seem to be affected by the water uptake. Changes in morphology were not observed for the SEDS powders and the starch microspheres.

The chemical stability of the samples was assessed by HPLC. In general, conventional spray- and freeze drying resulted in samples with higher chemical stability compared to SEDS powders and starch microspheres. Nevertheless, the excipients applied were observed to be of major importance, and further optimization of the formulation as well as processing conditions may lead to slightly different conclusions.

KEYWORDS Insulin, Spray-drying, Lyophilization, Freeze-drying, Chemical stability, SEDS, Starch microspheres

INTRODUCTION

Inadequate stability of pharmaceutical proteins in aqueous solution often necessitates the application of solid formulations to attain acceptable shelf-life. Solid formulations may be reconstituted in aqueous solvent before injection or be administered in solid form, e.g., by pulmonary delivery. Due to the lower

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molecular mobility in the solid state, which reduces the rate at which degradation reactions take place, solid formulations are in general considered to be more stable than proteins in solution (Randolph, 1997; Chang, 1996b). Nevertheless, several stability issues are still to be encountered, since proteins are sensitive to the stresses imposed during processing and drying to form the solid. Many of these stresses are similar in different methods of preparation of solid formulations, e.g., the dehydration of the protein and the presence of certain interfaces.

Preservation of the native conformation of the protein and obtainment of a high glass-transition temperature (Tg) of the formulation are believed to be of central importance for achieving optimal storage stability (Randolph, 1997; Chang, 1996a; Carpenter, 2002). Therefore, excipients that may result in a high Tg are often added to the formulation, due to the lower molecular mobility below Tg. However, it has been discussed by Crowe et al. that a high Tg is not sufficient to obtain a stable product (Crowe, 1998). The dehydration of the protein during removal of water from the formulation may induce conformational changes in the protein, which can lead to the exposure of sites more sensitive to chemical degradation (Carpenter, 2002). Thus, excipients that reduce changes in the protein structure should also be included in the formulation. Nonreducing disaccharides are known to increase protein stability in lyophilized formulations, probably by acting as hydrogen donors for the protein (Arakawa, 1993; Carpenter and Crowe, 1989; Kreilgaard, 1999).

The stability of solid formulations during storage is highly affected by the storage conditions, with moisture and temperature as some of the important factors. Decreased chemical stability has been observed in solids with increasing water content due to the uptake of moisture (Strickley & Anderson, 1996). Here, water is thought to act as a plasticizer, resulting in a reduced Tg and thus an increased molecular mobility (Ahlneck & Zografi, 1990). The reduced Tg after water uptake may also lead to crystallization of excipients, resulting in phase separation and loss of the stabilizing effect of the excipients on the protein. Furthermore, the presence of water may catalyze chemical reactions (Shalaev, 1996).

The aim of this work is to compare different methods of particle formation in order to identify key factors in relation to protein stability in solid insulin formulations. The methods of preparation investigated were spray- and freeze-drying as well as solution-enhanced dispersion by supercritical fluids (SEDS) and precipitation into starch microspheres.

Powders prepared by SEDS may potentially be used for pulmonary delivery, while starch microspheres potentially can be used in controlled-release formulations of proteins. These methods of preparation are compared to the more common spray- and freeze-dried powders with respect to particle morphology and chemical stability of insulin.

The excipients used are sodium taurocholate, melezitose, and starch (amylopectin). Sodium taurocholate may act as an absorption enhancer in pulmonary delivery of insulin (Johansson, 2002) and was included in the formulations obtained by SEDS as well as spray- and freeze-dried powder. Furthermore, sodium taurocholate is a surfactant and can potentially stabilize insulin against surface adsorption (Collins, 1987).

The nonreducing tri-saccharide, melezitose has a high Tg and can be expected to form amorphous powders and thereby possibly stabilize the native conformation of insulin. However, the effect of melezitose on the chemical stability of insulin has to our knowledge not been studied previously.

In order to compare excipient effects in the preparation of starch microspheres with other methods of preparation, spray- and freeze-dried formulations containing starch are also included in the study.

MATERIALS AND METHODS

Materials

Freeze-dried bulk preparations of recombinant human insulin containing 2 Zn^{2+} per hexamer were kindly donated by Novo Nordisk A/S (Bagsvaerd, Denmark) and used as received. PEG 20000 with an average molecular weight of 20 kDa was obtained from Clariant, USA. Acid hydrolyzed amylopectin starch (waxy maize) with an average molecular weight of 222 kDa was obtained from Cerestar, Belgium. Melezitose was purchased from Ferro Phannstiel, USA, and sodium taurocholate was obtained from Fluka (Buchs, Switzerland).

All other chemicals were of analytical grade.

Preparation of Samples

The preparation of samples is described under the individual methods of preparation. The composition

TABLE 1 Method of Preparation and Formulation of Samples

Preparation		Excipients (content in w %)			
Method	Batch	Insulin	Melezitose	NaTC	Starch
Spray drying	SD-1	11	89		
Spray drying	SD-2	11	86.25	2.75	
Spray drying	SD-3	11			89
Freeze drying	FD-1	11	89		
Freeze drying	FD-2	11	86.25	2.75	
Freeze drying	FD-3	11			89
SEDS*	SEDS-1	100			
SEDS*	SEDS-2	50	50		
SEDS*	SEDS-3	80		20	
Starch spheres (lyophilized)	SMS-1	8			92
Starch spheres (air-dried)	SMS-2	8			92

*Solution enhanced dispersion by supercritical fluids.

of dry material in the samples is listed in Table 1. However, some presence of water after processing must be expected in all samples. It was not possible to obtain acceptable powders by SEDS that were directly comparable to the spray- and freeze-drying formulations with respect to the composition, and thus the compositions differs somewhat.

Preparation of Solutions for Spray-Drying and Freeze-Drying

Insulin (0.22 g) was suspended in 10 mL of 5 mM NaCl solution, and acetic acid was added to dissolve the insulin. Thereafter the pH was adjusted to 7.5 with NaOH. Excipients (1.78 g) were dissolved in 20 mL 5 mM NaCl solution. The insulin solution was mixed with the appropriate excipient solution, and 5 mM NaCl was added to give a final weight of 40 g. An aliquot (10 mL) of each solution was divided in three glass vials and frozen at –80°C for freeze-drying. The remaining solution (30 mL) was spray-dried.

Preparation of Spray-Dried Samples

The samples were spray-dried in a laboratory spray-dryer built at the Institute for Surface Chemistry (Stockholm, Sweden) resulting in 3 g of solid material. The dimensions of the drying chamber are 0.75×0.15 m. The spray dryer operates in a co-current mode and with a jacketed two-fluid spray-nozzle is equipped with an orifice diameter of 1.5 mm and a cyclone for powder collection. The inlet gas temperature was 180°C, whereas the outlet gas temperature was kept at 70°C. The jacket temperature was 25°C and the liquid feed to the dryer was 5 mL/min. The flow of drying air was 0.8 m^3/min at 180°C.

Preparation of Lyophilized Samples

The vials with solutions to be freeze-dried were frozen at –80°C. The vials were placed on a shelf at –20°C, thereafter no temperature control was applied to the shelf. The pressure during freeze-drying was $7.5 \cdot 10^{-2}$ mbar, corresponding to a sample temperature of –43°C during primary drying. The samples were freeze-dried for at least 16 h.

Preparation of Samples By Solution Enhanced Dispersion by Supercritical Fluids (SEDS)

Preparation of small particles by Solution Enhanced Dispersion by Supercritical fluid (SEDS) has been described previously (Palakodaty, 1998; Moshashaée, 2003). In the present study triethylcitrate (TEC) is used as a clouding agent, which enables formation of protein particles with a particle size suitable for inhalation (Sundholm, 2003).

The composition of the batches produced is given in Table 1 and the batch size was 200 mg. Insulin and excipients were dissolved in 10 mL of 1% formic acid containing 6% TEC. After particle formation, the TEC was fully distributed to the supercritical phase.

The solutions were pumped at a rate of 0.05 mL/min through a modified two-channel coaxial nozzle (nozzle tip diameter 0.1 mm) into a 100 mL high-pressure vessel (Thar Designs, USA) where the particle formation took place. The high-pressure vessel was kept at 50°C, and the pressure inside the vessel was controlled by a back pressure regulator (Thar Designs, USA) to 125 bar. Liquid CO_2 was cooled and pumped with a CO_2 pump (Thar Designs, USA) with a rate of 18 g/min as controlled with a mass flow meter (Micro motion, USA). Just before entering the coaxial nozzle, the supercritical CO_2 was modified by adding 99.5% ethanol at a flow rate of 2 mL/min. This was done in order to extract the water to the supercritical phase. After the process was completed, the system was depressurized, and the particles were collected from the filter placed at the end of the particle-formation vessel.

Preparation of Starch Microspheres

The technique for preparation of starch microspheres relies on the formation of a water-in-water emulsion, where the dispersed phase (starch) can precipitate and form (close to) spherical particles. The two-phases system is formed spontaneously due to the unfavorable free energy of mixing (due to mainly the enthalpic effect) (Flory, 1953). The interfacial tension is very low (Forciniti, 1990), and thus a water-in-water emulsion is easily formed by merely stirring the system. Insulin, like many other proteins, can be partitioned to one of the two phases, here the starch-rich phase (Albertsson, 1986). When the protein resides in the dispersed starch-rich phase, the protein will be encapsulated as starch precipitates.

Starch microspheres with encapsulated insulin were prepared in an aqueous two-phase system at pH 8.6 using 50 mM sodium carbonate buffer. Two grams of 40% solution of an acid hydrolysed starch were dissolved by heating and cooled to about 55°C before mixing with $ZnCO_3$ (11% final concentration) and 2 mL 5.25% insulin solution.

Fifteen grams of a 40% PEG-solution were added slowly in about 3 min under stirring. The obtained water-in-water emulsion was first kept at 4°C for 1 day with appropriate stirring and secondly at 30°C for about 6 h.

The micro-spheres were washed three times at room temperature by centrifugation with 10 mM sodium acetate buffer pH 6.0, containing 2 mM Zn acetate. One batch was lyophilized (SMS-1) and one batch was air-dried (SMS-2).

Storage Conditions

The samples were stored protected from light in open containers for 6 months in climate facilities at 25°C ± 2°C and 30% RH ± 5% RH. Samples were withdrawn and analyzed after 0, 1, 3, and 6 months. The storage conditions applied were intended to represent worst-case humidity for dry storage conditions and resulted in measurable degradation of the insulin formulations during storage. During shipment for analysis the samples were stored in desiccated containers at ambient temperatures.

Scanning Electron Microscopy (SEM)

Samples to be investigated with scanning electron microscopy (SEM) were mounted on double-sided adhesive carbon tabs mounted on SEM stubs and were coated with Au/Pd in a BAL-TEC SCD 050 sputter coater (Balzers Union AG, Liechtenstein). Thereafter, the samples were examined with an JEOL JSM – 5200 Scanning Electron Microscope (JEOL, Japan) operated in high vacuum mode.

X-ray Diffraction (XRD)

A PanAlytical X'Pert Pro MPD X-ray powder diffraction instrument (PanAnalytical, The Netherlands), equipped with X'Celerator detector was used to generate X-ray diffractograms of the samples. A copper X-ray source operated at 45 kV and 40 mA provided Kα radiation of a wavelength of 1.5418 Å. All data were collected between 2θ of 2 and 40. The step width was 0.017 and the counting time per step 32 sec.

Approximately 2 mg of sample was gently smeared out on zero background holders (rotating silicon wafers). The samples were measured under ambient conditions. The result for the 6 months samples was checked by measurements using a counting time per step of 96 sec to obtain an improved signal-to-noise ratio.

Differential Scanning Calorimetry (DSC)

An 822^e STAR^e System (Mettler Toledo, USA) was used to obtain solid-state DSC thermograms. Samples (1–3 mg) were weighed into 40 µl Al pans with perforated lids, to allow escape of water vapor. The

temperature was raised from 20°C to 250°C at 10°C/min. Nitrogen was used as purge gas at 40 mL/min. An empty pan with perforated lid was used as reference.

HPLC

HPLC analysis was performed as described in (Hvass, 2003). Covalent-bound dimers and high molecular weight polymers (HMWP) were separated from monomeric derivatives using size exclusion (SE) chromatography. Reversed-phase (RP) chromatography (pH 3.6) with gradient elution was used in the separation of chemically and structurally modified degradation products of human insulin. The desamido forms of insulin reported in the study are the sum of A21 desamido insulin, B3 desamido insulin, and B3 isoasp insulin. Other degradation products as well as the polymers coelute in RP-HPLC and are referred to as related substances. For samples without starch an amount corresponding to 5 mg of insulin was suspended in 400 µl Milli-Q water. Then 32 µl of 0.2 N HCl was added to dissolve insulin, where after 150 µl 0.5 M phosphate buffer was added and the pH was adjusted to 8.0 ± 0.15. A final volume of 2 mL was obtained by addition of Milli-Q water. If insulin was not dissolved upon addition of HCl, a larger volume was added. For samples containing starch an amount corresponding to 5 mg human insulin was extracted to 1200 µl 30 mM phosphate buffer with pH 7.4, containing 0.5 mM $CaCl_2$, 82 mM NaCl, and 0.1 % poloxamer 188. The extraction proceeded by incubation at 37°C for 6 h on a Gyro Rocher (STR 9) at lowest speed. Thereafter the samples were centrifuged at 14000 rpm for 10 min, to obtain starch-free HPLC samples.

RESULTS

Morphology of Solid Formulations

SEM Micrographs

All powders were imaged by SEM after production and after storage for six months. The particle size and structure, which depend both on the drying method and the formulation, are indicated in Table 2, and some of them are shown in Fig. 1.

The spray-dried powders had particle sizes in the range of 1–10 µm after processing for primary particles. However, in the powders containing melezitose (SD-1 and SD-2) the primary particles were agglomerated into larger structures. SD-1 and SD-3 shared the wrinkled particle morphology that is typical of spray-dried particles containing protein (Fig. 1a). SD-2, on the other hand, showed indications of melting and fusion of the material, resulting in smooth particles (Fig. 1c). After storage, SD-1 and in particular SD-2 had experienced water uptake, and the primary particles had fused to form large lumps (Fig. 1b, d). SD-3, with starch as the excipient, did not exhibit any morphological change during storage.

The freshly produced lyophilized powders all had brittle flake-like structures (Fig. 1e). After storage, FD-1 and FD-2 had formed large lumps with a much smoother surface, due to water uptake and fusion of primary particles (Fig. 1f), whereas the structure of FD-3 appeared intact.

The powders produced by SEDS all consisted of agglomerates of very small particles, in the submicron range for SEDS-1 and SEDS-3 (Fig. 1g), and slightly larger for SEDS-2 (up to 5 µm). No change in

TABLE 2 **Scanning Electron Micrographs of Samples After Preparation and After 6 Months Storage at 25°C/30% RH**

Samples	After preparation	After storage (6 months)
SD-1	Raisin-like spheres 1–10 µm	Fused spheres 20 µm
SD-2	Spheres 10–20 µm	Molten particles 100–500 µm
SD-3	Raisin-like spheres <10 µm	Raisin-like spheres <10 µm
FD-1	Flake-like structures 50–100 µm	Molten particles 100–500 µm
FD-2	Flake-like structures 50–100 µm	Molten oblong particles 50–200 µm
FD-3	Flake-like structures 50–100 µm	Flake-like structures 50–100 µm
SEDS-1	Very small particles <5 µm	Very small particles <5 µm
SEDS-2	Very small particles <5 µm	Very small particles <5 µm
SEDS-3	Very small particles <5 µm	Very small particles <5 µm
SMS-1	Spheres 50–200 µm	Spheres 50–200 µm
SMS-2	Spheres 50–200 µm	Spheres 50–200 µm

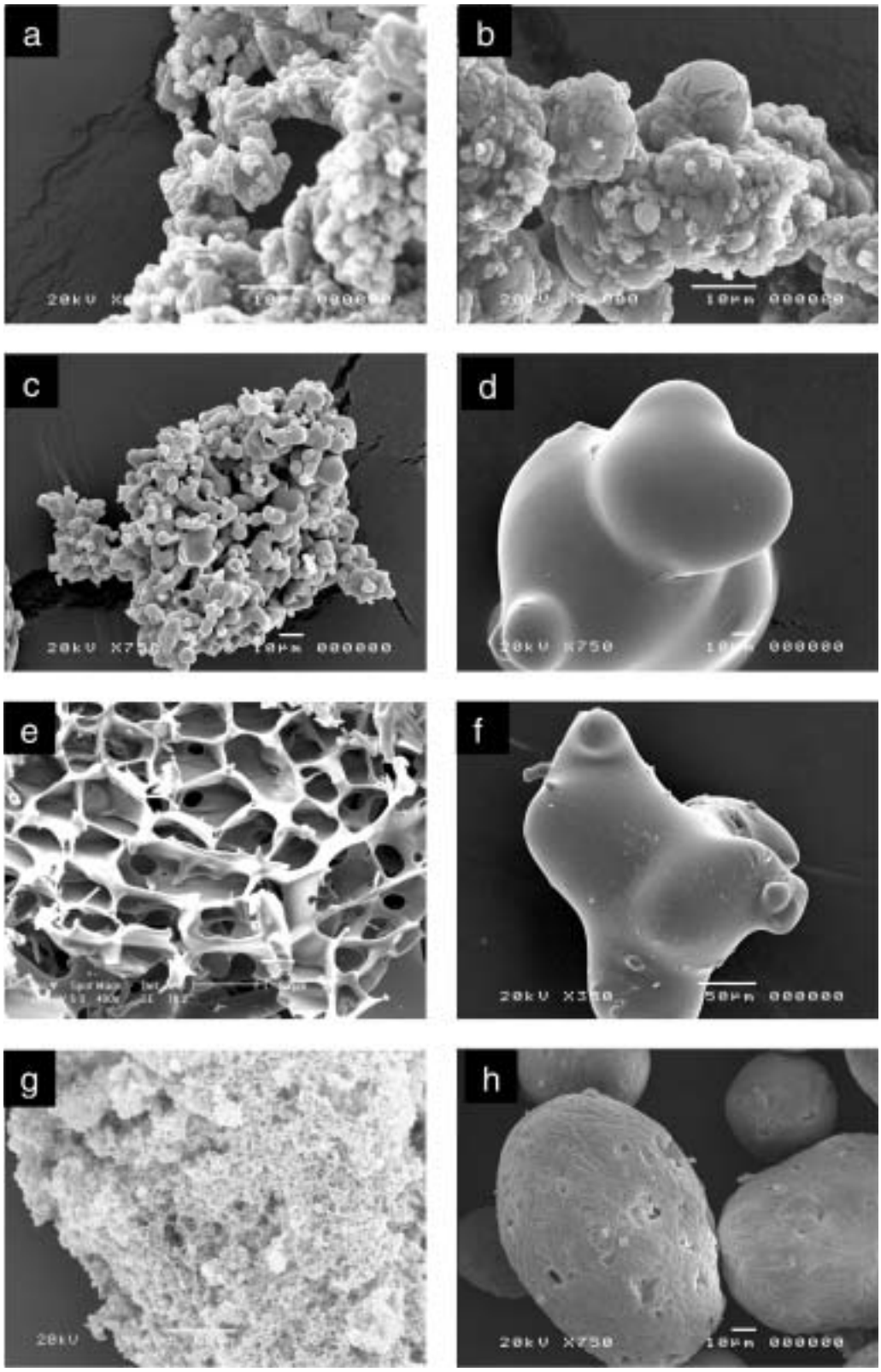

FIGURE 1 SEM images of the particles formed. (a) SD-1 after processing. (b) SD-1 after 6 months storage. (c) SD-2 after processing. (d) SD-2 after 6 months storage. (e) FD-1 after processing. (f) FD-1 after 6 months storage. (g) SEDS-1 after processing. (h) SMS-2 after 6 months storage.

morphology of the SEDS powders was observed after 6 months storage. The starch microspheres were spherical or somewhat elongated spheres in the size range 50–200 μm. They were relatively smooth but with a fairly rough surface structure. No change in morphology was observed after 6 months storage (Fig. 1h).

X-Ray Diffraction

The crystallinity of the excipients and formulations was analyzed by X-ray diffraction. For the unprocessed excipients the crystallinity of human insulin, sodium taurocholate, and melezitose was determined to be approximately 10%, 95%, and 60%, respectively. The starch excipient was predominantly amorphous with some diffuse peaks, and is here referred to as semicrystalline

All samples, except for the starch microspheres, were fully amorphous after processing. Furthermore, this did not change during storage. Regarding the starch microspheres, some diffuse peaks and one sharp peak were seen. The latter was not consistent with peaks found for insulin and starch excipients that constitute the main components of the microspheres, and was also observed in placebo starch microspheres. This peak is most likely due to $ZnCO_3$ crystals formed during the microsphere processing, while the diffuse peaks relate to the semicrystalline structure of starch as suggested earlier by Elfstrand et al. (2004).

Solid-State DSC

The unprocessed excipients were analyzed by DSC, the results being displayed in Table 3. Sodium taurocholate and melezitose both displayed distinct melting transitions, whereas starch only showed a broad endothermic transition that peaked at ≈100°C.

The data of the fresh and stored formulations are summarized in Table 4.

Samples Containing Melezitose and Sodium Taurocholate

Transitions were observed at around 60°C in samples SD-1, SD-2 (Fig. 2a), FD-1 and FD-2 (Fig. 2c), corresponding to the glass transition of melezitose. This transition disappeared over time in all samples, whereas a transition at 150–160°C developed over time in the SD-2 and FD-2 samples, both containing

TABLE 3 Solid-state DSC on Excipients

Sodium taurocholate	endotherm 112°C endotherm 190°C exotherm 200°C endotherm 241°C
Melezitose	Tg = 160°C exotherm 240°C
Starch bulk	-
Placebo microspheres	-

Tg: glass transition temperature, taken as the midpoint of the glass transition.

TABLE 4 Solid-state DSC on Formulations

Sample	t = 0	t = 1 month	t = 3 months	t = 6 months
SD-1	Tg ≈ 60°C	Tg ≈ 57°C	Tg ≈ 56°C	n.t.
SD-2	Tg ≈ 62°C	Tg ≈ 57°C	Tg≈57°C	T≈160°C T≈200°C
SD-3	n.t.	n.t.	n.t.	n.t.
FD-1	Tg ≈ 58°C	Tg ≈ 56°C	n.t.	n.t.
FD-2	Tg ≈ 57°C	T≈150°C	T≈150°C	T≈150°C
FD-3	n.t.	n.t.	n.t.	n.t.
SEDS-1	n.t.	n.t.	n.t.	n.t.
SEDS-2	Tg ≈ 58°C	n.a.	Tg ≈ 69°C	n.a.
SEDS-3	n.t.	broad peak≈80°C small peak≈195°C	broad peak≈80°C small peak≈195°C	small peak≈195°C
SMS-1	n.t.	n.t.	n.t.	n.t.
SMS-2	small peak ≈60°C	small peak ≈60°C	small peak ≈60°C	small peak ≈60°C

Tg: glass transition temeperature, taken as the midpoint of the glass transition.
T: peak temperature.
n.a. not analyzed, insufficient material.
n.t. no transition observed.

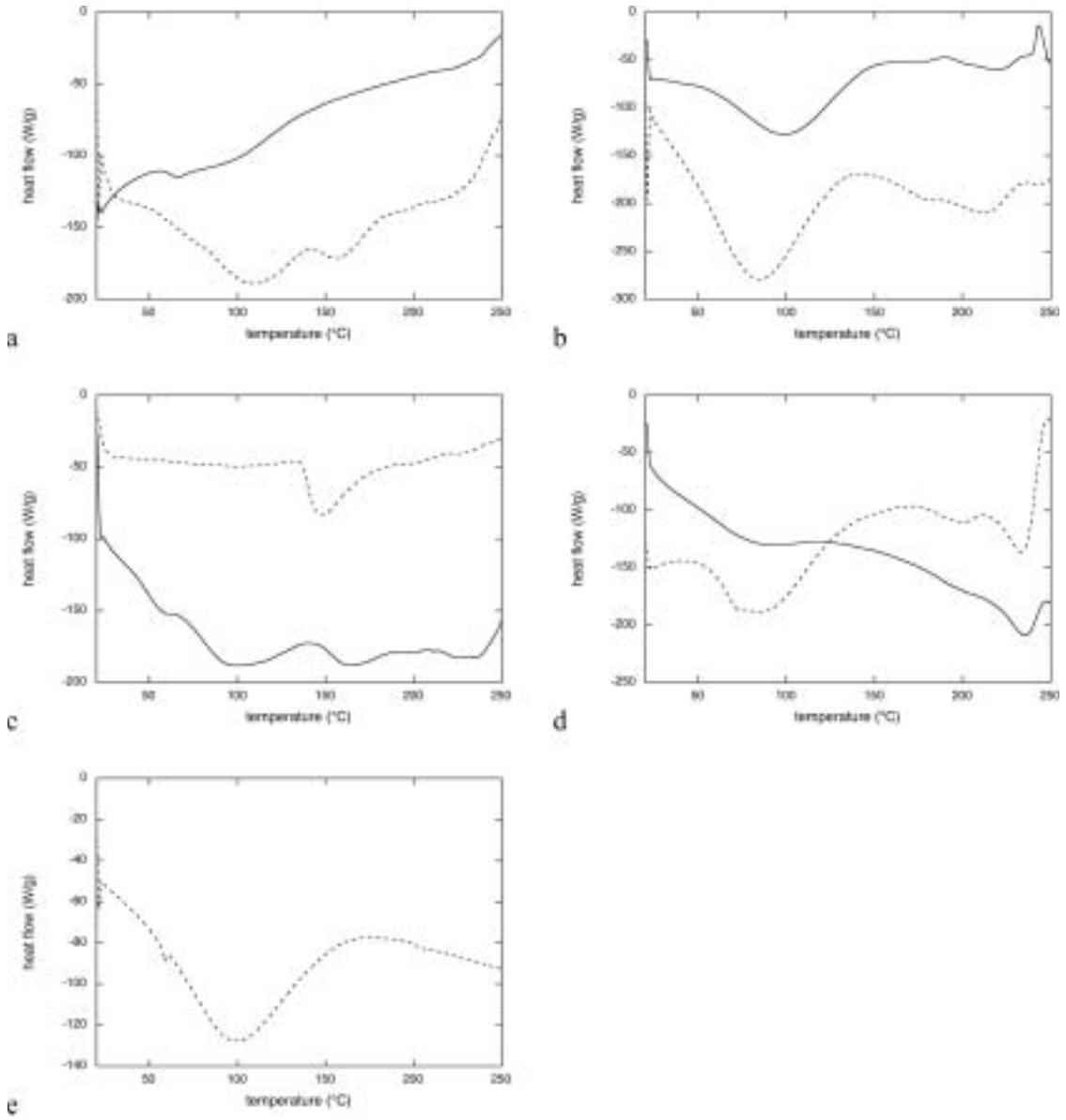

FIGURE 2 **Solid-state DSC thermograms. (a) SD-2, (b) SD-3, (c) FD-2, (d) SEDS-2, and (e) SMS-2. Solid lines correspond to samples after processing and dashed lines to samples stored for 6 months (SEDS-2: stored for 3 months).**

sodium taurocholate. In addition, the freeze-dried and spray-dried samples display a broad endothermic transition with a peak around 80–100°C, most likely corresponding to loss of adsorbed water (e.g, SD-3 in Fig. 2b). The SEDS particles with melezitose displayed Tg in the same region as the spray-dried and freeze-dried samples, but here there was no change in Tg during storage for the period tested (Fig. 2d). For SEDS particles prepared from insulin without excipients no transitions were observed. In SEDS samples containing sodium taurocholate as excipient, very small endothermic transitions occurred at about 190°C, corresponding to one of the melting peaks of the pure sodium taurocholate.

Samples Containing Starch

In all samples containing starch a broad endothermic transition was observed, with a peak around 100–120°C, which can be attributed to bulk water. The broad transition shifted to somewhat lower temperatures during storage in SD-3, FD-3, and SMS-1, and the enthalpy of the transition appeared to increase over time, indicating increased uptake of water.

In SMS-2 microspheres a small peak was observed at 60°C (Fig. 2e), and no significant change in this transition was seen during the storage period. The transition was not seen in unprocessed starch and placebo microspheres, as well as spray- and freeze-dried samples. At present it is not known what the peak corresponds to.

Chemical Stability of Insulin

The aggregation and degradation of insulin was monitored by Size Exclusion HPLC and Reversed Phase HPLC, respectively.

Aggregation of Insulin

Samples Containing Melezitose and Sodium Taurocholate

The total amount of polymer, dimer, desamido forms and related substances formed after manufacture and after 6 months storage of the samples is shown in Fig. 3a–d. As can be seen from the figure, no polymers were observed in samples formulated without starch. Also the dimer formation was low, especially for the spray- and freeze-dried samples without starch containing less than 2% of dimer after 6 months of storage. The dimer content in the SEDS samples after storage was generally higher than for spray- and freeze-dried formulations, with the highest amount (4.5%) formed in SEDS-2 containing melezitose, although the initial level was comparable to the spray- and freeze-dried formulations. The increase in soluble aggregated forms (polymer + dimer) of insulin in the samples during storage after subtraction of the initial content is shown in Fig. 4. Apparently, only a minimum of soluble aggregates are formed in the spray- and freeze-dried formulations without starch, whereas there is some formation of soluble aggregates in the SEDS samples during storage.

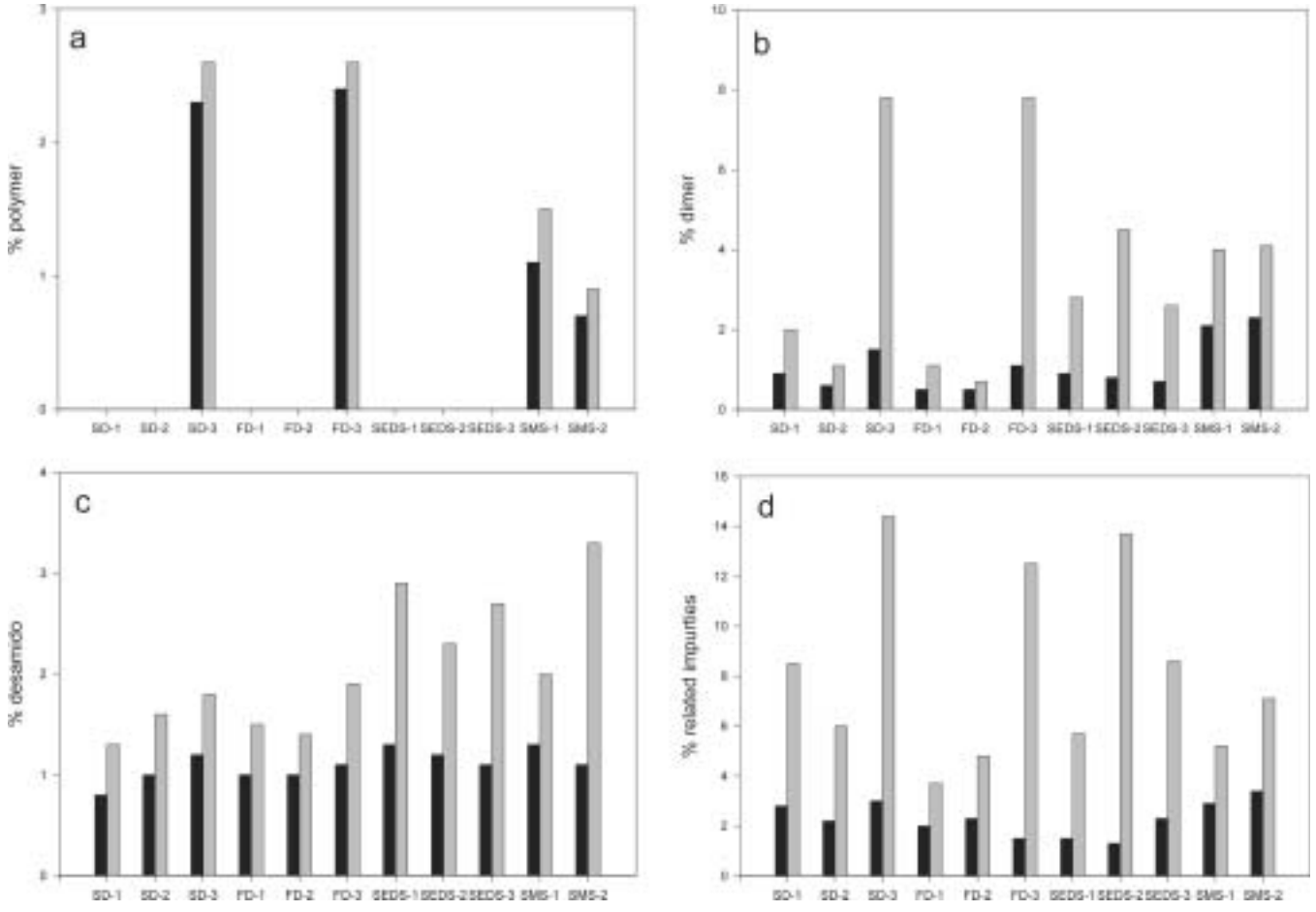

FIGURE 3 **Chemical stability of insulin. (a) amount of polymers formed. (b) amount of dimer formed. (c) amount of desamido forms formed. (d) amount of related substances formed. Black columns indicate samples after processing. Gray columns indicate samples after 6 months storage.**

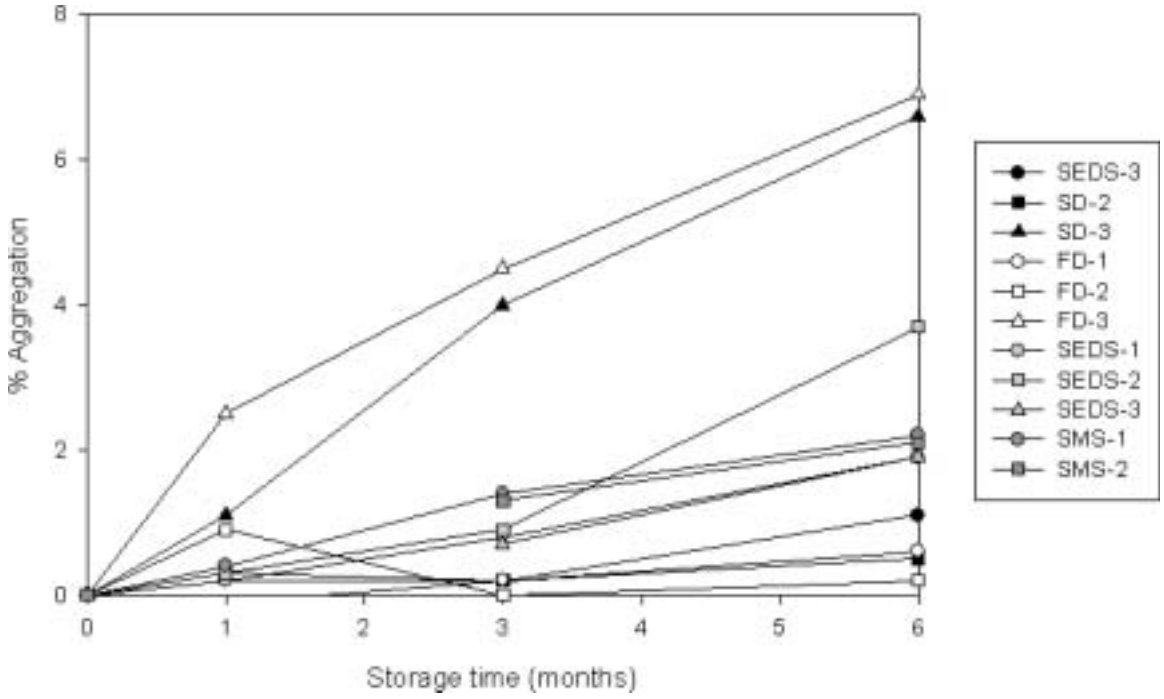

FIGURE 4 Increase in insulin aggregation during storage, (n = 2).

Samples Containing Starch

In samples containing starch, the formation of polymer and dimer after processing was higher than for the other formulations. The amount of insulin polymers formed in spray- and freeze-dried samples after preparation was around 2.3%, whereas the amount in the air- and freeze-dried starch microspheres was 1.2 and 0.8%, respectively. Thus, a considerable fraction of the polymers was present immediately after manufacture.

The dimer content in the spray- and freeze-dried formulations after processing was a little higher than for formulations without starch. However, a major difference is observed during storage, where the total amount of soluble aggregates in SD-3 and FD-3 increases significantly (Fig. 4). As can be seen in Fig. 3, this is primarily due to the formation of dimers.

The formation of dimers was less pronounced in the starch microspheres, where the level after storage was similar to the SEDS samples of approximately 4%.

Degradation of Insulin

Samples Containing Melezitose and Sodium Taurocholate

The content of desamido forms and related substances formed after manufacture and after 6 months storage as a result of insulin degradation is shown in Fig. 3c and 3d. The content of desamido forms after manufacture is generally below 1.5% for all samples. The spray- and freeze-dried samples contained approximately the same level of desamido forms (less than 2%) after storage. This was a little lower than for samples manufactured by other methods of preparation which contained between 2 and 3% of desamido forms.

The content of related substances after processing was between 1.3% (SEDS-2) and 2.8% (SD-1).

After storage, the amount of related substances found in spray-dried samples containing melezitose and sodium taurocholate was 8.5 and 6.0% for SD-1 and SD-2, respectively. This was higher than for freeze-dried samples of the same composition, with 3.7 and 4.8% for FD-1 and FD-2, respectively. The SEDS had an amount of related substances within the same range, except for SEDS-2 which had a content of 13.7%.

The progression in degradation products during storage after subtraction of the initial content is shown in Fig. 5. Degradation products were formed in all samples during storage; however, this effect is most pronounced in SEDS-2 containing the mixture of insulin and melezitose. For this sample the increase in degradation products is above 10%. In most formulations the

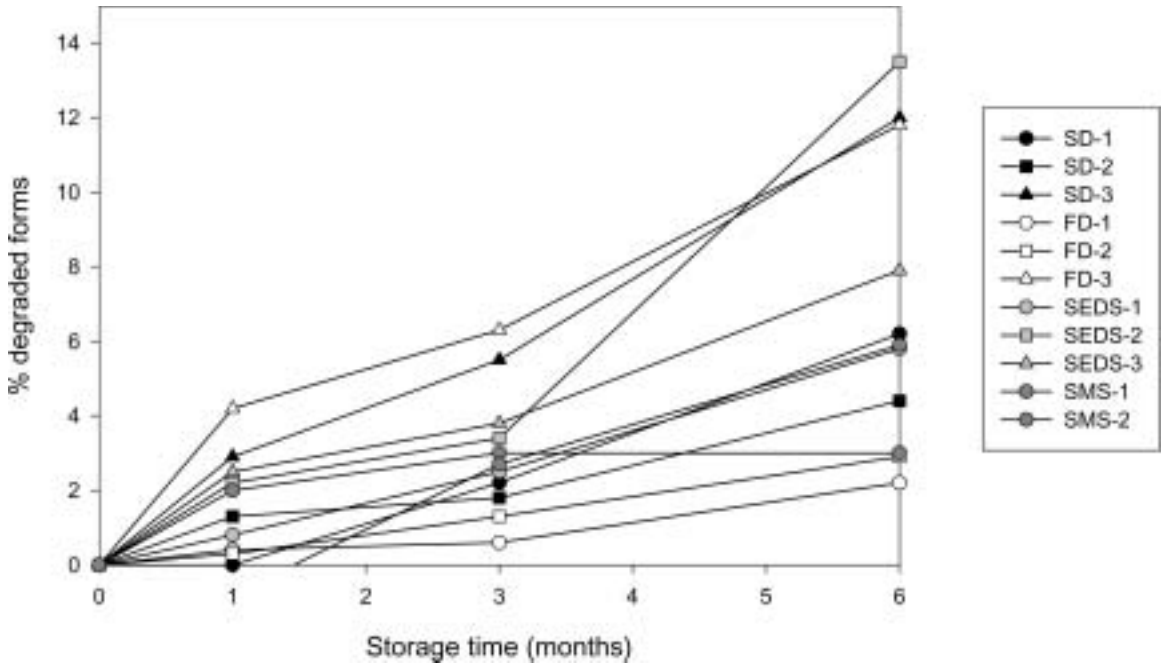

FIGURE 5 Increase in insulin degradation during storage (n = 2).

amount of degradation products formed lies within a range of 4.5–8%. A lower amount of degradation products (<3%) was seen for the freeze-dried samples without starch.

Samples Containing Starch

The level of desamido forms in the spray- and freeze-dried samples containing starch (SD-3 and FD-3) after storage was similar to the other samples manufactured by the same method of preparation and was generally less than 2%. A content of 2–3% was seen for the starch microspheres. In the spray- and freeze-dried samples, the level of related substances was significantly higher than for other formulations prepared by the same method, with contents of 14.4% and 12.5% for spray- and freeze-dried samples, respectively. This is mainly due to the formation of soluble aggregates, which co-elute with other degradation products. The formation of related substances, and thus aggregates, was not so pronounced in the starch microspheres, where the content after 6 months storage was similar to spray-dried formulations without starch.

From the progression in degraded product it can be observed that degradation proceeds throughout the storage period. However, the increase in degradation was less in the starch microspheres compared to the spray- and freeze-dried samples.

DISCUSSION

Insulin powders prepared by spray-drying, lyophilization, SEDS, and microspheres prepared by co-precipitation with starch were compared, and the stabilizing effect of three different excipients, melezitose, sodium taurocholate and starch, was examined. The effect of processing and formulation on the chemical integrity of insulin and on the morphology of the powders will be discussed in the following section.

Effects of Processing

Morphology

Very small particles were produced by spray-drying and SEDS, with the particles produced by spray drying measuring around 2–20 μm and the size of the SEDS particles being less than 5 μm. Freeze-dried samples resulted in brittle flake-like structures with large particle sizes around 50–100 μm, whereas the starch microspheres were spherical with a size in the range 50–200 μm in diameter. Small particles with large surface areas are of interest if they are to be administered in solid form, for example, for pulmonary delivery. On the other hand, the risk of uptake of water and subsequent changes in morphology during storage may be higher for smaller particles due to the larger surface area. This was also observed for spray- and

freeze-dried formulations containing melezitose and sodium taurocholate as will be discussed next.

Chemical Stability

Comparing the chemical stability of insulin with respect to the different methods of preparation indicates that spray- and freeze-dried samples without starch and the SEDS powders generally resulted in products with a low content of soluble aggregates. When starch was included in the spray- and freeze-dried insulin formulations, some polymer formation was seen and thus the chemical stability was observed to be highly related to the excipients used. This will be discussed further in the next section.

The amount of degradation products after processing was comparable in all samples and thus no effect of the method of preparation could be observed here. During storage, however, some differences in the amount of degradation products formed can be observed (Fig. 3). The amount of soluble aggregates and degradation products was generally the lowest in spray- and freeze-dried samples without starch after six months of storage. Thus, even though the SEM micrographs indicated water uptake, this was not observed to have an effect on the chemical stability of the samples. In the samples prepared by SEDS and in the starch microspheres, the level of soluble insulin aggregates and other degradation products after storage was higher than in the spray- and freeze-dried samples, indicating that these methods of preparation resulted in products that were less stable during storage.

Effects of Formulation

Morphology

The SEM-images indicate that spray- and freeze-dried particles containing melezitose and sodium taurocholate experienced significant changes in morphology upon storage. The wrinkled structure observed for the spray-dried samples SD-1 and SD-3 corresponds with the spray-dried particles of pure insulin obtained by Ståhl et al. (Ståhl, 2002). The addition of sodium taurocholate to the spray-dried formulation seems to affect the particle morphology, as the particles (SD-2) were much smoother compared to the raisin-like structures of SD-1 and SD-3. The same effect has also been observed for other spray-dried protein formulations containing surfactants (Maa, 1997; Maa, 1998). For SD-1, SD-2, FD-1, and FD-2 the observed agglomeration of the particles and smoothening of the surface during storage may be explained by uptake of water and fusion of primary particles. The most severe change was seen for the lyophilized samples, shifting from brittle flake-like structures to large molten particles. This may be explained by the very large surface area of the freeze-dried structures after processing, leaving them very susceptible to water uptake. However, water uptake was not observed for the SEDS particles which were even smaller. Indications of water uptake were also observed by solid-state DSC. The observed Tg for melezitose in SD-1, SD-2, FD-1, and FD-2 disappeared after 6 months of storage, which is in accordance with water uptake as suggested by SEM. The Tg appears to have decreased below the temperature range of the DSC method used. Storage of solid protein formulations is generally recommended to take place at least 30°C below Tg, to reduce the molecular mobility and thus minimizing protein reactivity (Hancock, 1995; Yoshioka, 1999). However, it is no guarantee for obtaining sufficient stability (Crowe, 1998; Carpenter, 2002). Also it has been observed that insulin itself is far more stable as amorphous solid compared to the crystalline form (Pikal and Rigsbee, 1997).

A low Tg-value implies that melezitose will crystallize during storage. However, melting transitions were only observed in the presence of sodium taurocholate (SD-2 and FD-2), where melting occurred at 150–160°C. The melting temperature observed corresponds to the melting point of melezitose dihydrate (153°C) (Collins, 1987). Reference samples of melezitose show a melting peak at 160°C, which is due to the dihydrate form. This is in correspondence with XRD data for melezitose (approx. 60% crystallinity). However, since X-ray diffraction showed that the samples were completely amorphous, the crystallization observed in DSC most likely occurred during the analysis and does not correspond to the solid state of the sample. Melting of melezitose dihydrate was not observed in samples with melezitose as the sole excipient (SD-1 and FD-1). Possibly, sodium taurocholate affects melezitose such that it tends to crystallize more easily. The spray- and freeze-dried formulations containing starch as well as starch microspheres and particles prepared by SEDS were not subject to changes in morphology upon storage, which indicates that these formulations are more stable toward morphological

changes upon water uptake. Additionally, the starch microspheres are considerably larger in diameter and thus the surface area available for water uptake was lower.

Chemical Stability

In general, the excipients applied had a significant impact on the chemical stability of insulin. The level of soluble aggregates in spray- and freeze-dried particles containing melezitose was generally low and similar to the amount observed in spray-dried particles of pure insulin (Ståhl, 2002). Thus, melezitose does not induce the formation of soluble aggregates. A pronounced amount of degraded product was observed in spray- and freeze-dried samples containing starch compared to the formulations containing melezitose. As can be seen in Figure 3 a major part of the degraded insulin is aggregated species, and a significant increase was observed during storage. Consequently, starch is less suitable for spray- and freeze-dried formulations compared to the other excipients used. The initial amount of soluble aggregated species in starch microspheres was also quite high, but for these formulations no major increase in aggregation is observed over time. Thus, the stability of insulin toward aggregation is higher in starch microspheres compared to spray- and freeze-dried formulations containing starch, and microspheres may offer a more protective environment for insulin.

For lyophilized proteins it has been observed that the presence of nonreducing disaccharides has a stabilizing effect on the protein. This may be explained by the prevention of dehydration-induced unfolding of the protein as the sugars act as hydrogen donors in place of the removed water (Carpenter, 2002; Wang, 2000). Unfolding of proteins may lead to decrease in chemical stability since the protein may become more sensitive to oxidation and aggregation (Carpenter, 2002). Melezitose is a nonreducing tri-saccharide and may potentially prevent dehydration of insulin. However, the sugar-to-protein mass ratio is quite high (approx. 8:1) in the spray- and freeze-dried formulations tested, which may lead to phase separation and subsequent loss of effect as it has been observed for spray-dried trypsinogen-sucrose mixtures (Tzannis and Prestrelski, 1999). Here the authors found that the optimal mass ratio was 1:1, whereas Carpenter et al. suggest ratios of 3–5:1 for freeze-dried products (Carpenter, 2002).

Carbohydrates of larger size were not observed to have the same protective effect, which is possibly due to steric hindrance interfering with the formation of hydrogen bonds (Prestrelski, 1995; Tanaka, 1991; DePaz, 2002) or phase separation (Randolph, 1997). Thus, the increased degradation of insulin in spray- and freeze-dried formulations containing starch may be due to dehydration of the insulin molecule leading to structural perturbation and subsequently higher susceptibility toward degradation.

Sodium taurocholate is an enhancer, which may improve the uptake of proteins to the body, e.g., in the lungs (Johansson, 2002). Furthermore, the addition of surfactants may reduce interfacial denaturation and aggregation, for example at the ice-water interface during lyophilization (Wang, 2000) or air-water interface during spray-drying. However, sodium taurocholate did not reduce chemical degradation in our study, as the amount of chemical degradation was not significantly lower than for the other formulations included. Thus, sodium taurocholate did not have any stabilizing effect on the formulations.

CONCLUSION

All samples except for the starch microspheres were amorphous after processing. During storage, the spray- and freeze-dried samples containing melezitose and sodium taurocholate experienced significant water uptake, as observed by changes in morphology and disappearance of Tg, but the samples still remained amorphous throughout the storage period. The water uptake did not result in a decreased chemical stability, as the spray- and freeze-dried samples containing melezitose and sodium taurocholate appeared to have the best chemical stability during storage of all samples tested.

The formation of soluble aggregated species during processing and storage was very pronounced in spray- and freeze-dried samples containing starch, and thus this excipient is not preferable in solid formulations manufactured by these methods of preparation when used without other co-additives. The decreased stability may be due to less preservation of the native structure. The effect of starch in the starch microspheres seems to be somewhat different, as the chemical stability in these formulations was comparable to the SEDS samples.

Overall, it has been demonstrated that the generic methods of spray- and freeze-drying resulted in insulin powders with higher chemical stability compared to SEDS powders and the starch microspheres. However, it was also demonstrated that the addition of stabilizing

excipients is of major importance as seen when the addition of starch in spray and freeze dried formulations resulted in inferior products. Thus, optimization of the processing conditions as well as the formulation may result in a slightly modified picture.

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