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Surface characterisation of freeze-dried protein/carbohydrate mixtures

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Abstract

In the present investigation freeze-drying of proteins (BSA or trypsin) together with various carbohydrates, i.e. lactose, sucrose, mannitol, α-cyclodextrin and dextrin, has been studied with particular emphasis on the surface composition of the freeze-dried powders. The proteins were found to be over-represented on the powder surface as compared to the bulk concentration of protein. The mechanism behind the surface accumulation is believed to be that proteins adsorb preferentially over carbohydrates to the ice/liquid interface in the frozen sample. The degree of surface accumulation depended on the carbohydrate used, and was increased in annealed samples compared to reference samples. The activity of trypsin was fairly well preserved (58–90%) in the freeze-dried powders, but depended on the carbohydrate excipient, whilst the surface composition had little effect on the activity. The activity preservation was improved when the protein concentration was raised from 1 to 10% in the solids. The surface composition of powders containing mixtures of mannitol and dextrin as excipients depended on the ratio between the two carbohydrates, with the lowest surface coverage of protein obtained in 50/50 mixtures.

Keywords: BSA; ESCA; Freeze-drying; Surface analysis; Trypsin

1. Introduction

Proteins and peptides are attracting increasing interest as pharmaceuticals. In order to increase the shelf life and to protect the biological activity of these molecules they are often preserved in dry form. Carbohydrates or polyols are often used as bulking agents and protective additives in this context. Freeze-drying is the most common technique for obtaining a dry formulation of proteins (e.g. (Pikal, 1994)), although spray-drying is attracting some interest as well (Broadhead et al., 1994; Mumenthaler et al., 1994; Maa et al., 1998). Generally, the major efforts in studies of freeze-drying of proteins have been devoted to the preservation of the protein activity (Izutsu et al., 1994; Pikal, 1994; Dekeyser et al., 1997), and little emphasis has been placed on the powders as such.

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This is unfortunate, because many properties important in handling of the powder, tableting etc. are determined by or related to the chemical composition of the powder surface. Furthermore, investigating the surface composition of the powders can give some insight in the processes occurring during freezing and drying and thus aid the understanding of how the process influences the powder properties, as well as protein stability and activity.

The elemental composition of the powder surface can be analysed, e.g. by a method using electron spectroscopy for chemical analysis (ESCA), and from this analysis, the composition in terms of molecular species can be estimated (Fäldt et al., 1993). Using this technique, the powder surface has previously been shown to be dominated by protein for spray-dried protein/carbohydrate mixtures (Fäldt and Bergenståhl, 1994; Landström et al., 1999; Millqvist-Fureby et al., 1999). This is because of the protein adsorbing to the air/liquid interface of the spray-droplet, and the composition of this surface being reflected in the surface of the dry powder. For freeze-drying the mechanism is more uncertain, but it is possible that also for this process the adsorption mechanism is valid. If this is the case, the protein would adsorb to the ice crystal surfaces in the frozen material prior to drying, which could be expected to result in an increase in the protein content on the surface of the freeze-dried powder compared to the bulk composition.

When an aqueous solution containing a protein and a carbohydrate is frozen rapidly one would expect the system to be essentially quenched, i.e. having rather even distributions of the two components, and also of water, at least until ice crystals are formed. Once such a system is formed, its storage properties could be expected to depend on whether the storage temperature is above or below Tg′, the glass transition temperature for a maximally freeze-concentrated solution. Below Tg′ the system is in a glassy state and ice crystal formation and growth is limited Slade and Levine, 1991; Roos, 1997). Above Tg′, on the other hand, crystallisation is possible, and one would, therefore, expect ice crystal growth and a parallel freeze concentration, the rate of which depends on the temperature difference between the storage temperature and Tg′ (Slade and Levine, 1991). Under these conditions the solute concentration in the unfrozen phase is high, and thus phase separation is possible.

Interactions of the protein with the surface is likely to have an effect on the surface composition of the final powder, but may also be detrimental for the activity of an enzyme, because such interactions usually lead to conformational changes (Kondo et al., 1993, 1996). If phase separation occurs, individual protein molecules in the protein-rich phase can interact and form aggregates with subsequent loss of activity. In the case of carbohydrates crystallising rather than turning into a glass, phase separation may also occur and moreover, the growing crystals of ice and carbohydrates may exert mechanical forces on the protein, which can result in loss of structure and thus activity. In either case an effect on the surface composition of the freeze-dried powder can be expected.

In the present paper we present studies of the surface composition of freeze-dried protein/carbohydrate mixtures and relate these findings to the properties of the carbohydrates and to the residual activity of a model enzyme (trypsin) after drying. The effects of freezing conditions and annealing on the surface composition and protein stability are also discussed.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) was purchased from Sigma (St Louis, MO, USA). Crystalline porcine trypsin 4500 K was obtained from Novo Nordisk A/S (Bagsværd, Denmark). Mannitol was obtained from Merck (Darmstadt, Germany), lactose from BDH (Poole, UK), and sucrose from Kebo (Stockholm, Sweden). Dextrin and α-cyclodextrin were purchased from Fluka (Buchs, Switzerland). Tween 80 was obtained from ICI Surfactants (Cleveland, UK). N-α-benzoyl-L-arginine ethyl ester (BAEE) was purchased from Sigma (St Louis, USA). Silica particles were ob-

tained from Nissan Chemical Industries (Tokyo, Japan). Other chemicals were of the highest purity available. Doubly distilled water was used throughout.

2.2. Freeze-drying

Solutions containing specified ratios of protein/carbohydrate (10% total solids by weight) were freeze-dried. In order to be able to perform controlled freeze-drying experiments, the sample tubes were fitted in an aluminium block acting as stable heat reservoir (Fig. 1), with a low volatility vacuum oil between the block and the tubes to ensure direct contact and thus efficient heat transfer. The aluminium block and a reference sample are equipped with thermocouples, allowing continuous monitoring of the temperature.

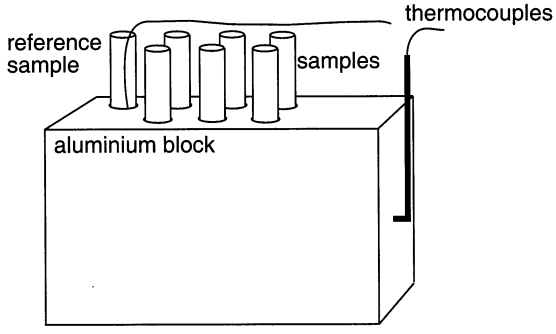


Fig. 1. Schematic of the aluminium block used as a heat reservoir during freezing and freeze-drying. The aluminium block is equipped with a thermocouple in the block and in one of the samples for recording of the respective temperatures during freeze-drying.

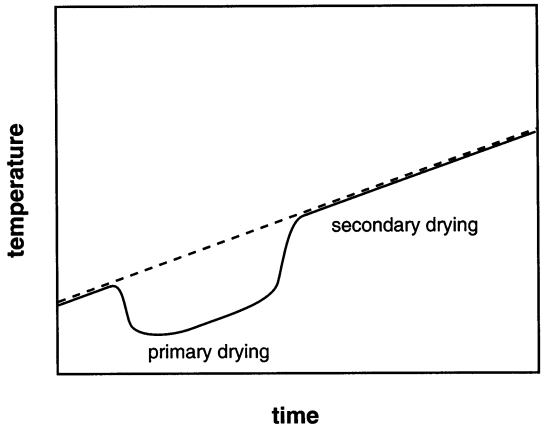


Fig. 2. Schematic illustration of the temperature in the aluminium block (dashed line) and in the reference sample (solid line) during freeze-drying. The primary drying occurs when the sample temperature is below that of the block.

For 'fast freezing' of samples, the block and tubes were pre-cooled to -28°C, thereafter a small volume (300 μl) of the solution to be freeze-dried was added to each tube. The observable freezing during such a procedure was essentially instantaneous. In experiments with 'slow freezing', the samples (300 μl) were added to the sample tubes in the block at room temperature, and the complete assembly was cooled to -28°C over a period of about 12 h. In annealing experiments, the frozen samples were transferred to a cooling bath at -5°C for 48 h. The corresponding reference samples were stored at -28°C for 48 h. The freeze-drying was started from -28°C at $6–8 \cdot 10^{-2}$ mbar, and was continued until the block and sample reached room temperature (typically 48 h). The temperature during primary drying was -34°C, and this phase lasted for about 3 h, Fig. 2 illustrates schematically the temperature in the aluminium block and in the reference sample.

2.3. Seeding experiments

In an attempt to manipulate the number of ice nucleation sites in a controlled manner, small hydrophilic particles (colloid silica particles, diameter 0.1 μm) were added in different concentrations (10^{-12}–10^{-1}% w/w) to a solution containing BSA and lactose (1/99 w/w, 10% total solids by weight). The samples were frozen according to the 'slow freezing' method described above in order to allow time for ice nucleation to occur. Since the silica particles are in the colloidal size range, they did not settle during the duration of the freezing period.

2.4. Electron spectroscopy for chemical analysis

ESCA was used to probe the elemental composition of the powder surfaces with an analysis depth of less than 100 Å. The ESCA measure-

ments were performed with an AXIS HS photoelectron spectrometer (Kratos Analytical, UK). The instrument uses a monochromatic Al Kα X-ray source. The pressure in the vacuum chamber during analysis was less than 10^{-7} torr. In the present investigation, a take-off angle of the photoelectrons perpendicular to the sample holder was used throughout. The area analysed consisted of a circular region of an approximate diameter of 1.3 mm, and three measurements were made for each sample. The standard deviation in the measurements was 1.3% units. The freeze-dried cake was gently crushed before taking samples for ESCA analysis. The surface coverage of trypsin was calculated from the nitrogen content of pure trypsin, as measured by ESCA, and the nitrogen content of the freeze-dried samples.

2.5. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed with a Mettler Toledo DSC821 instrument. Throughout, helium was used as the purge gas at 40 ml/min. The lid of the aluminium pans was pierced just before analysis, allowing desorbed water to leave the pan. Typically, a heating rate of 20°C/min was used. Sample weights were 1–5 mg. The crystallinity of the carbohydrates was estimated as the measured enthalpy of fusion divided by the enthalpy of fusion for completely crystalline sample (taken from the literature), taking the fraction of the carbohydrate in the total solids into account.

2.6. Trypsin activity assay

The enzymatic activity of resolubilised trypsin was determined according to a modified version of a method developed by Schwert and Takenaka (Schwert and Takenaka, 1955). In short, 0.25 mM N-α-benzoyl arginine ethyl ester (BAEE) in 67 mM sodium phosphate buffer pH 8.0 (1 ml) was hydrolysed with trypsin at 25°C (100 μl, 1 μg trypsin), and the progress of the reaction was followed spectrophotometrically at 235 nm, using a Perkin-Elmer UV-VIS Spectrometer. The specific activity was calculated in BAEE-units/mg trypsin, 1 BAEE-unit corresponding to 0.001ΔA/min, and 270 BAEE-unit being equivalent to 1 U (1U = 1 μmol BAEE/min). The activity of untreated trypsin was measured using the same procedure, resulting in a specific activity of 27×10^3 BAEE-units/mg trypsin, and this value was used as a reference value for 100% retained activity. The values reported are the averages of at least two measurements and the standard deviation was typically 1.8% units.

2.7. Scanning electron microscopy

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 Balzers SCD 050 (Balzers Union AG, Liechtenstein) sputter coater. Thereafter, the samples were examined with a Philips SEM 515 (Philips Export BV, Netherlands), operating at 15 kV.

3. Results and discussion

3.1. BSA/carbohydrate mixtures — seeding experiments

It can be speculated that the freezing-rate is determined by the number of, or presence of, ice nuclei in the liquid water. Undercooling could then be explained by slow ice nucleation under certain circumstances. In a solution of protein and carbohydrate, this would allow more time for the protein in the solution to interact with the forming ice crystals, which could be reflected in the powder surface composition. Changing the freezing rate by changing the freezing temperature does not provide an accurate means of regulating the number of ice nucleation sites. Instead we used different concentrations of hydrophilic silica particles, which were assumed to act as ice nucleation sites. As can be seen in Fig. 3, the protein coverage was on average 2.8% with a standard deviation of 1.0% for all samples investigated. Interestingly, there was no change in protein coverage depending on the concentration of silica particles over ten orders of magnitude, indicating either that ice nucleation occurs to a sufficient

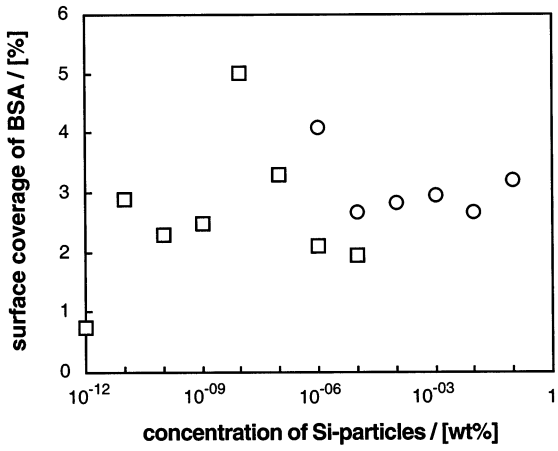


Fig. 3. The protein coverage of freeze-dried powders composed of 1% BSA and 99% lactose, with additions of silica particles. The samples were 'slow-frozen' at −28°C and freeze-dried in two separate batches, both starting from −28°C at $6–8 \cdot 10^{-2}$ mbar. (○) batch 1 and (□) batch 2.

Table 1
Properties of the carbohydrates used in the experiments

Carbohydrate	Tg' (°C)[a]	ΔH_{melt} (J/g)
Mannitol	−40	300[b]
Lactose	−28	150[c] (α-lactose)
		204[c] (β-lactose)
α-Cyclodextrin	−9	
Sucrose	−32	132[d]
Dextrin	−13	

[a] (Slade and Levine, 1991).
[b] (Barone et al., 1990).
[c] (Figura, 1993).
[d] (te Booy et al., 1992).

extent without silica particles, or that the presence of the silica particles had no effect on the ice nucleation, or that the surface coverage of protein is not related to the ice nucleation. Considering the extensive use of silica as a nucleation initiator in organic synthesis, the first of these mechanisms seems most plausible. This also suggests that post-freezing treatments are more important than nucleation-related ones for the resulting surface properties of the freeze-dried powders. This is, therefore, further discussed below. Furthermore, these results also show that the procedures used for freeze-drying and analysis are reproducible, both between samples within a batch and between batches.

3.2. BSA/carbohydrate mixtures – annealing effects

In an attempt to vary the degree of segregation in the frozen material and study the effects of this on the surface composition of the freeze-dried material, we performed a series of investigations with BSA and either mannitol, lactose or α-cyclodextrin, on which we applied reference and annealing treatments (described above) to the frozen solutions before drying. The three carbohydrates were chosen to display a significant difference in their Tg' (Table 1).

The surface coverage of BSA on the freeze-dried powders after annealing and reference treatment was analysed by the ESCA technique, and the results are shown in Table 2. As can be seen, surface accumulation of protein occurred in all annealed samples and also in the mannitol reference sample. Although protein was present on the surface of the other reference samples as well, the amount was very low. These results are consistent with the notion of annealing allowing an increasing ice crystal growth, freeze concentration and possible phase separation, all leading to a higher degree of protein interactions with the ice-crystal

Table 2
Comparison of surface protein content on freeze-dried bovine serum albumin (BSA)/carbohydrate (1/99 w/w) powders obtained from electron spectroscopy for chemical analysis (ESCA) in three different experiments[a]

Excipient	Treatment	BSA coverage (%)
Mannitol	Reference	5
Mannitol	Annealing	5
Lactose	Reference	<2.5
Lactose	Annealing	6
α-cyclodextrin	Reference	<2.5
α-cyclodextrin	Annealing	17

[a] Freeze-dried samples were obtained by rapid freezing to −28°C, followed by annealing at −5°C for 48 h, and subsequent cooling to −28°C prior to drying. The reference samples were stored at −28°C for 48 h instead of the annealing period.

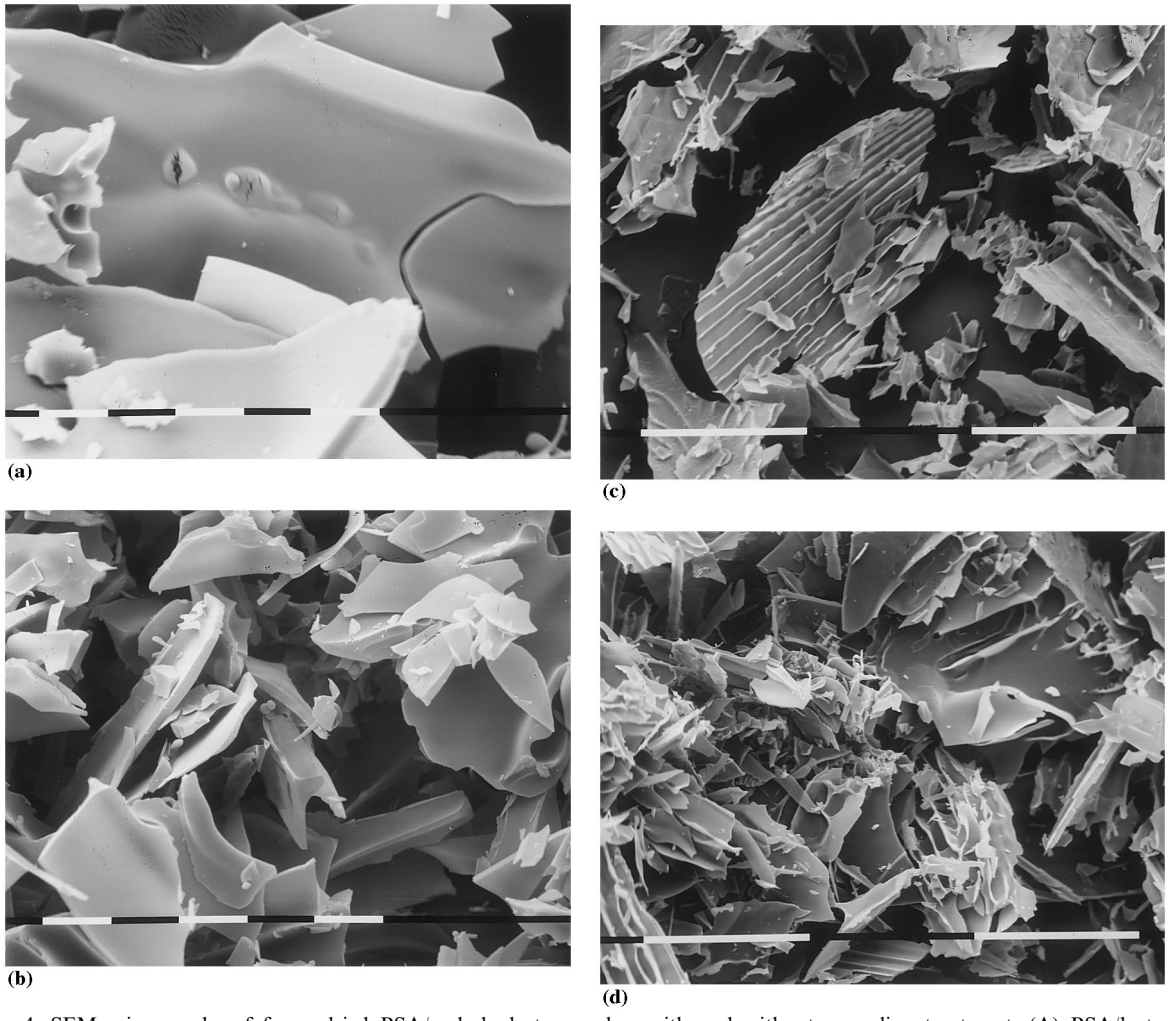


Fig. 4. SEM micrographs of freeze-dried BSA/carbohydrate powders with and without annealing treatment. (A) BSA/lactose, annealed; (B) BSA/lactose, reference; (C) BSA/α-cyclodextrin, annealed; (D) BSA/α-cyclodextrin, reference; (E) BSA/mannitol, annealed; (F) BSA/mannitol, reference. The scale bar corresponds to 10 μm in A, B, E and F, and to 100 μm in C and D.

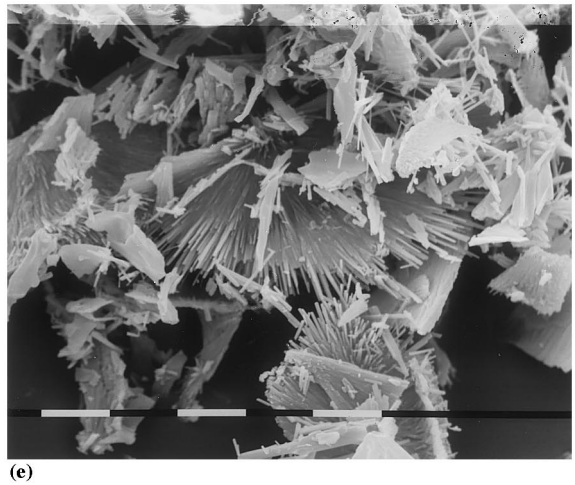
surfaces. Furthermore, during the annealing treatment, a significant portion of the sample is in a rubbery state, rather than a glassy state, which allows the protein and carbohydrate to reorientate. The fact that no significant differences were observed between annealing and reference samples for mannitol may be a result of the low Tg' for this sugar alcohol, resulting in that both reference and annealed samples effectively being stored under annealing conditions.

The morphology of the powders was also studied using SEM. The powders were found to display quite different morphologies, primarily depending on the carbohydrate used (Fig. 4). Although the crystallinity of the two mannitol samples differed only slightly, the textural appearance of the powders is quite different, with the annealed sample showing long rod-like, possibly crystalline, structures. Lactose samples and α-cyclodextrin samples showed more flake-like morphologies, and in particular the α-cyclodextrin samples contained large flakes with channel-like structures, possibly indicating where the ice-crystals were located in the frozen sample.

The samples were also analysed by DSC, showing that lactose and α-cyclodextrin samples were fully amorphous both in the annealed and the reference samples, whilst mannitol samples showed slightly different degrees of crystallinity depending on the freezing conditions, i.e. 68% crystallinity for the annealed sample and 56% for the reference sample

3.3. Trypsin/carbohydrate mixtures

The results presented above for BSA/carbohydrate mixtures showed that the protein accumulates at the powder surface to a certain degree, but using BSA precludes extraction of any information relating to the effects of freezing and freeze-drying on the protein structure, which is of primary importance for its biological function. Therefore, analogous experiments were carried out using trypsin, a protease, for which the activity can easily be assayed. Trypsin is known to unfold readily upon adsorption to an interface, typically resulting in loss of activity (Kondo et al., 1993). It can also be envisaged that mechanical effects, e.g. because of ice crystal growth and any carbohydrate crystallisation during freezing and freeze-drying may affect the protein structure. Essentially all irreversible changes to the native



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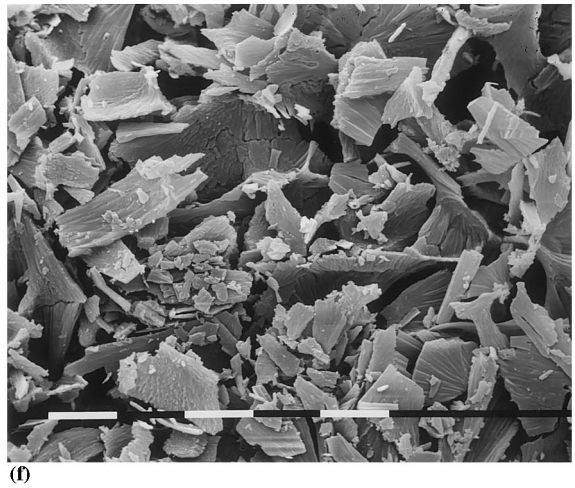

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Fig. 4. (*Continued*)

Table 3
Electron spectroscopy for chemical analysis (ESCA) results and relative activity of freeze-dried trypsin/carbohydrate (1/99 w/w) powders[a]

Sample	Trypsin coverage (%)	Relative activity (%)	Crystallinity (%)
Lactose, reference	<2	81	Amorphous
Lactose, annealed	5.0	81	Amorphous
Lactose/Tween 80, reference	2.2	65	Amorphous
Lactose/Tween 80, annealed	5.2	68	Amorphous
Sucrose, reference	2.0	63	42
Sucrose, annealed	6.1	58	63
Dextrin 15, reference	6.5	74	Amorphous
Dextrin 15, annealed	7.5	74	Amorphous
Mannitol, reference	10.5	73	63
Mannitol, annealed	16.2	77	65
α-cyclodextrin, reference	3.3	92	Amorphous
α-cyclodextrin, annealed	<2	90	Amorphous

[a] Samples were rapidly frozen at −28°C, followed by annealing at −5°C for 48 h, and subsequent cooling to −28°C prior to freeze-drying. Reference samples were stored at −28°C for 48 h in place of annealing treatment.

structure of the protein will result in loss of activity, which thus serves as a sensitive measure of the preservation of the structure.

The surface coverage of trypsin on the freeze-dried powders was analysed and the results are shown in Table 3. From these results it is clear that the surface accumulation of protein is in general on the order of 2–6 times in the reference samples and 5–8 times in the annealed samples, with mannitol being an exception with considerably higher trypsin coverage of the powder surface. Furthermore, the protein coverage is increased after annealing for all carbohydrates except α-cyclodextrin, which shows a low trypsin coverage for both annealed and reference samples. Overall the results agree fairly well with the results obtained for BSA/carbohydrate mixtures, although the highest degree of surface coverage of trypsin was observed with mannitol, whilst the highest surface coverage of BSA was obtained with α-cyclodextrin. Such differences may be a result of different properties of the proteins in terms of their size and tendency to adsorb to interfaces, as well as specific interactions with the different carbohydrates. The increase in trypsin coverage on annealing is consistent with increased ice crystal growth and freeze-concentration, possibly resulting in phase separation. In the case of mannitol, the trypsin coverage is quite high, which may be a result of mannitol crystallising during both annealing and reference conditions, which would lead to phase separation (Sun and Davidson, 1998).

The activity of trypsin was assayed as a sensitive measure on the preservation of the protein native structure. It is a prerequisite for retained enzymatic activity that the protein structure is native after rehydration, however, reversible structure changes induced during freezing and drying cannot be detected by this method. The activity of the rehydrated trypsin is summarised in Table 3. As can be seen, the preservation of the activity was fairly good overall. The activity was best preserved using α-cyclodextrin, followed by lactose. Sucrose turned out to be the least effective protectant, whilst dextrin and mannitol were intermediate. There seems to be no apparent correlation between the protein surface coverage on one hand and the residual activity on the other. Furthermore, it seems that the carbohydrate itself is the important factor in determining the residual activity, rather than the protein interfacial accumulation as such. The importance of the nature of the carbohydrate in the extent of retained activity of trypsin was previously observed with spray-dried trypsin/carbohydrate mixtures, although in that case it seemed that both surface coverage of protein and the crystallinity of the carbohydrate

were important factors in reference to trypsin activity preservation (Millqvist-Fureby et al., 1999).

3.4. Trypsin/mannitol/dextrin mixtures

With the objective to further explore the importance of the crystallinity of the carbohydrate matrix, trypsin was freeze-dried in mixtures of mannitol and dextrin. The trypsin content was 10% on a dry weight basis, whilst the remainder consisted of mannitol and dextrin. As can be seen from Fig. 5, over-representation of trypsin on the surface occurs for all compositions and for both annealed and reference samples. The surface accumulation is found to be highest for the pure carbohydrates in both the reference and annealed samples, and significantly lower in mixtures of the two carbohydrates (Fig. 5). The mechanism behind the increased surface coverage of trypsin is, as discussed above, presumably a combination of adsorption of the protein to the ice-crystal surfaces, and phase separation in the freeze-concentrated phase and crystallisation in the case of mannitol. It is more difficult to explain the lower surface coverage in the mixtures of mannitol and dextrin. A similar result was obtained when using BSA (Fig. 5), and thus it seems that this is a general phenomenon that is not particular to trypsin. It may be that the carbohydrate mixture interacts more favourably with the protein and that less phase separation occurs during freezing.

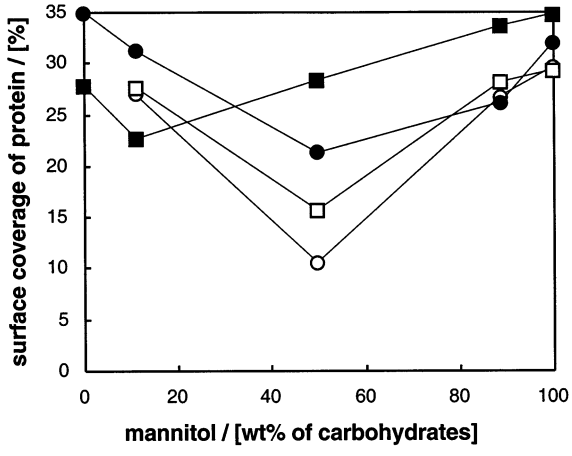

Fig. 5. The surface coverage of trypsin and BSA on freeze-dried powders with varying proportions of mannitol and dextrin, and 10% protein. (●) Trypsin reference samples, (■) trypsin annealed samples, (○) BSA reference samples, and (□) BSA annealed samples.

Table 4
Crystallinity of mannitol in freeze-dried trypsin/mannitol/dextrin powders

Composition (weight%)	Crystallinity (%)	
Trypsin/mannitol/dextrin	Reference	Annealing
10/90/0	68	79
10/80/10	64	64
10/45/45	68	79
10/10/80	Amorphous	Amorphous
10/0/90	Amorphous	Amorphous

The samples were analysed by DSC, typical traces are shown in Fig. 6, and the results are summarised in Table 4. The crystallinity of mannitol was high regardless of mannitol content and annealing, except at the lowest mannitol content. Note that in mixtures of mannitol and dextrin, two endothermic transitions are observed, one occurring below the melting point of mannitol, and one close to the melting point of mannitol. The lower peak was considerably smaller, or even absent, for samples without trypsin, in particular in samples with low mannitol content. This suggests that the amount of protein is important for the crystallisation behaviour of the samples, something which indeed has been found also for other systems (Constantino et al., 1998). In can be noted that the relative sizes of the two endothermic peaks are different in the reference and the annealed samples, indicating that the interactions between the different components are altered during annealing. However, the total enthalpy involved in the transitions is rather similar in all the samples.

The results for the residual activity for these systems indicate that a high content of dextrin improves the preservation of the activity (Fig. 7), however, the activity is recovered to at least 81% in all samples. Furthermore, these results

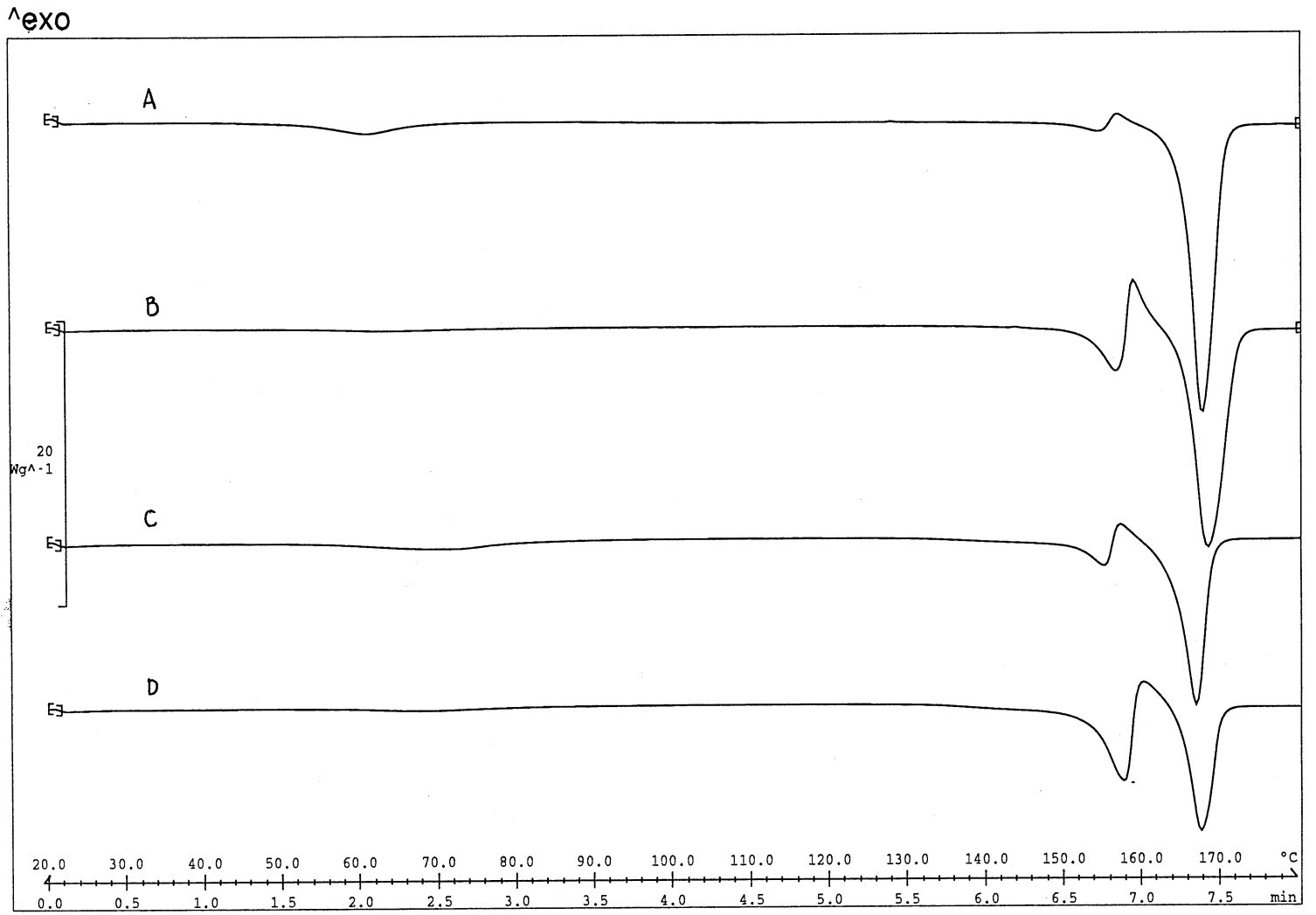

Fig. 6. Typical DSC traces of freeze-dried trypsin/mannitol/dextrin samples. Trypsin/mannitol/dextrin: (A) 10/90/0, reference; (B) 10/90/0 annealing; (C) 10/80/10 reference; and (D) 10/80/10 annealing.

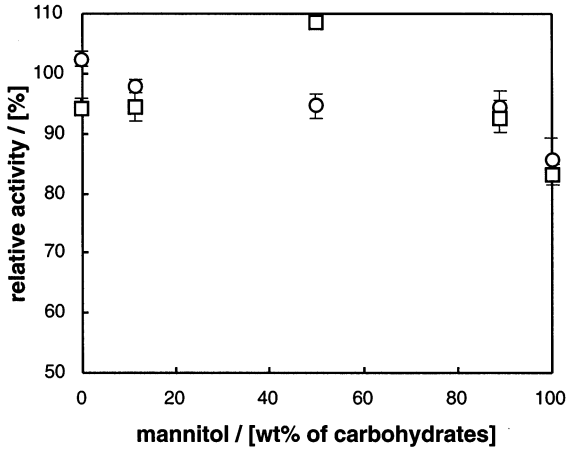

Fig. 7. Residual activity of trypsin after freeze-drying with mixtures of mannitol and dextrin. (○) Reference samples and (□) annealed samples.

suggest that the degree of crystallinity indeed is important for the preservation of the enzyme activity, as noted earlier (Izutsu et al., 1994; Millqvist-Fureby et al., 1999). Annealing has in general only little effect on the activity of trypsin. Comparing these results to the powders with 1% protein (Table 3), the activity recovery is significantly improved in the powders with a high protein content. It can be noted from Table 3 and Fig. 7 that the relative surface accumulation is smaller in the case of the higher protein content (6.5–16 times the bulk concentration for 1% trypsin and 2.7–3.5 times for 10% trypsin). Assuming equal specific surface areas for the powders regardless of protein content, a smaller fraction of the total protein will interact with the ice/liquid interface in the case of 10% trypsin. If these surface interactions are detrimental for the protein, it is expected that the activity recovery will be increased at the higher protein content. A similar trend for improved activity recovery at higher protein contents was noted in spray-drying of trypsin/carbohydrate mixtures (Millqvist-Fureby et al., 1999). However, the positive effect of a higher protein content cannot be expected to be persist at all protein concentrations, eventually extensive direct contacts between protein molecules will occur and thereby the risk for aggregation and accompanying activity reduction is increased (Liu et al., 1991).

4. Conclusions

The present investigation has shown that proteins indeed accumulate at the surface of freeze-dried protein/carbohydrate powders. There are several possible mechanisms behind this phenomenon; it is likely that the proteins adsorb preferentially over carbohydrates at the ice/liquid interface, in the same manner as proteins adsorb at other surfaces. This effect can be supplemented by phase separation in the freeze concentrated matrix, which may further increase the protein surface coverage of the freeze-dried powder. Another possible mechanism is the crystallisation of the carbohydrate, which also may lead to phase separation. The effects of mixed carbohydrates on the surface coverage of proteins are yet unexplained and require additional studies.

The activity of trypsin freeze-dried with pure carbohydrates was fairly well preserved, but the degree of preservation depended on the carbohydrate. However, the activity loss did not show a simple correlation with the crystallinity of the excipient, indeed most of the excipients remained in a glassy state after freeze-drying, and thus there may be important specific interactions between the protein and the excipient. Furthermore, the activity recovery was significantly improved when the protein concentration was increased from 1 to 10% of the solids.

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