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Research paper

Investigation of the stabilisation of freeze-dried lysozyme and the physical properties of the formulations

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Abstract

The long-term stability of a protein formulation requires that the glass transition temperature (T_g) of the formulation should be maximised and the perturbation of the protein native structure in the dried form after processing minimised. In the present study, the stabilisation of lysozyme structure conferred by excipients was monitored using second derivative Fourier transform infrared spectroscopy and the physical properties of protein formulations were investigated using differential scanning calorimetry. The results showed that the preservation of protein native structure during freeze-drying and the T_g of freeze-dried formulations were excipient- and excipient to enzyme mass ratio-dependent. The freeze-dried lysozyme appeared to be less effectively stabilised compared with the spray-dried enzyme when the excipients and the excipient to enzyme mass ratios were the same. In terms of the preservation of the secondary structure of lysozyme, glycerol and sucrose seemed to be more efficient than trehalose, although the T_g of trehalose-containing formulations were found to be higher than the T_g of the equivalent sucrose-based ones. With adding either trehalose or dextran to sucrose-containing formulations, the stabilisation of lysozyme native structure could be as effective as with sucrose alone, whilst the T_g could be enhanced. The results in this study suggested that lysozyme, processed by freeze-drying, is stabilised primarily by the water substitution mechanism.

Keywords: Freeze-drying/lyophilisation; Spray-drying; Protein formulation; Protein unfolding; Protein aggregation; Physical stability

1. Introduction

Proteins and polypeptides are preferably prepared as dry powders by utilising processing methods, such as freeze- and spray-drying, due to both the susceptibility of the active molecule to chemical degradation and physical instability in solution and the difficulty in crystallisation. In addition, the presentation of such biopharmaceuticals in the crystalline state does not necessarily guarantee long-term stability. For example, Pikal and Rigsbee [1] found that insulin in the amorphous state was more stable than in the crystalline form during storage.

However, because of the potential of proteins and polypeptides to be denatured/inactivated during processing and storage, excipients are generally required to increase the acute and long-term stability. In addition, these protectants need to remain in the glassy state and the protein molecules should be homogeneously trapped in the matrix [2]. Moreover, both the maximisation of formulation T_g and the preservation of the protein native structure post-processing have been indicated as important factors for long-term protein stability in a formulation [3–5]. The reason for the maximisation of formulation T_g is due to the fact that the molecular mobility of protein molecules still exists in the glassy state and consequently unwanted changes such as chemical reactivity, crystallisation and structural collapse can occur as a function of temperature and time [6–10]. Although T_g alone is not quantitatively indicative of the dynamics of proteins in the glassy state [11], it has been suggested that a glassy formulation could achieve several years shelf-life when it is stored at 40–50 °C below its T_g [10,12].

The requirement for the processed protein to exist in a folded state similar to its native state in dried forms is due to the fact that exposure of high reactive sites of proteins as

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a consequence of partial or complete unfolding generally leads to higher physical instability and chemical degradation relative to the native states [4,13,14]. The stabilisation of proteins conferred by sugars and polyols during freeze-drying has been explained by several mechanisms. For example, the stabilisation in solution and during freezing has been widely attributed to preferential exclusion [15,16], whilst two possible major mechanisms have been proposed to account for the protection of proteins against dehydration [5,17]. First, replacement of hydrogen bonding between water and protein by that between excipient and protein is considered to be a key factor in the water substitution mechanism by which an excipient stabilises a protein during drying processes [5]. Second, Franks et al. [18] proposed that glass formation is responsible for the stabilisation of proteins during freeze-drying. By trapping protein molecules in a glassy matrix, the molecular mobility of protein-containing system is greatly limited so that the rates of diffusion-controlled reactions, including protein unfolding, protein aggregation and chemical degradation, are reduced relative to the rates which can occur in a rubber state [19]. These two non-exclusive mechanisms are attributable to different origins with the first one having a thermodynamic basis and the other being purely kinetic, but either mechanism alone is not sufficient to account for the stabilising effects of excipients [20].

Since the precise mechanisms by which excipients preserve the native structure of proteins during dehydration is incompletely understood, the optimisation of protein formulations remains a challenge. Indeed, the amounts of excipients employed in many previous studies and even included in commercial formulations have been made on the basis of using iso-osmotic concentrations. Such concentrations are often greatly higher than the minimum amount required to achieve maximum stabilisation and as a result, the physical stability of these products might be compromised, with the resultant products requiring storage in the fridge [21]. Therefore, it is desirable practically to establish the dependence of amount of protein structure retained in the dried form on the amount of excipients employed during freeze-drying with a view to establishing the minimum amount of stabiliser(s) required to achieve an adequate shelf-life. In addition, since a single excipient might not be sufficient to maximise both the T_g and the stabilisation of proteins, mixtures of excipients including high T_g components (e.g. polymers such as dextran) to a protein formulation might be necessary in order to stabilise protein structure adequately. Thus, the potential effect of polymers on the stabilising capacity of small molecular weight sugars requires further investigation.

Therefore, one of the aims of this study was to investigate the quantitative response between the stabilisation of the model protein, lysozyme and the amounts of excipient, including polyethylene glycol (PEG) 200, glycerol, sucrose, trehalose and dextran, present in formulations processed by freeze-drying. In addition, it was planned to study the effects of mixtures of trehalose/dextran and sucrose on stabilising freeze-dried lysozyme and the T_g of the resultant formulations with a view to obtaining a general means of producing protein formulations with both maximal T_g and preserved protein native structure. A final aim of this study was to investigate the effect of drying processes on the stabilisation of lysozyme by comparing the stabilising effects of excipients during freeze-drying with those achieved during spray-drying.

2. Materials and methods

The buffer phosphate salts (ACS reagent grade), PEG 200, glycerol, sucrose, trehalose, dextran ($M_w \sim 70,000$), polyvinylpyrrolidone (PVP) K90 ($M_w \sim 300,000$), PVP K25 (M_w 28,000–34,000), PVP K15 ($M_w \sim 10,000$), polyvinyl alcohol (PVA, M_w 40,000–70,000), and lysozyme (3× crystallised, dialysed and lyophilised, Lot 57H7045) were purchased from Sigma-Aldrich Co., Poole, Dorset, UK.

2.1. Freeze-drying procedure

All solutions were prepared using sodium phosphate buffer (10 mM, pH 6.3). A lysozyme solution (10.0 mg/ml) was mixed with an equal volume of buffer-containing double the final concentration of excipient(s) in 2.0 or 7.0 ml vials. Samples were frozen to −80 °C in a freezer (which was utilised to minimise the potential variation in the freezing of samples), transferred to the lyophilizer (Advantage Benchtop Freeze-dryer XL, VirTis, Gardiner, NY) shelf and processed according to an identical two-step cycle (frozen from room temperature to −80 °C over 1 h, primary drying at −30 °C and 20 Pa for 40 h and secondary drying at 28 °C and 10 Pa for 20 h). The product temperatures were monitored using thermocouples, which were placed in the bottom of the vials. The product temperature during the primary drying of freeze-drying procedure was found to be maintained between −36 and −42 °C.

2.2. Spray-drying procedure

The spray-drying procedure employed was that described previously [22]. Briefly, lysozyme and excipients were dissolved in buffer and spray-dried using a Model 190 Buchi mini spray-dryer. The processing parameters comprised a feed rate of 3 ml/min, an atomising air-flow rate of 700 l/h and an inlet temperature of 120 °C. Outlet temperatures were found to range from 85 to 90 °C. At least three batches of each formulation were prepared.

2.3. Residual moisture analysis

The moisture content was determined using a TGA 2050 thermogravimetric analyser (TA Instruments, Crawley, West Sussex, UK). Samples (2–10 mg) of formulation were placed in open aluminium pans and these were, in turn, loaded into the sample compartment. The sample was equilibrated at 25 °C and then data were collected using a heating rate of 10 °C/min between 25 and 200 °C.

2.4. Differential scanning calorimetry (DSC)

The crystallisation (T_c) and glass transition temperatures (T_g) of freeze- and spray-dried powders and the crystallisation enthalpy (ΔH_c) of sucrose were determined using a model 2920 modulated DSC (TA Instruments, Crawley, West Sussex, UK), in which temperature and heat capacity calibrations were carried out using a protocol provided by the manufacturer. Briefly, the temperature calibration was carried out at heating rates of 10 °C for normal DSC or 5 °C for modulated DSC (MDSC) using water, indium and tin, whilst the heat capacity calibration for MDSC was carried out using sapphire. DSC data were collected as the sample was heated at a rate of 10 °C/min between 25 and 200 °C. MDSC data were collected as the sample was heated between −25 and 200 °C at a rate of 5 °C/min with a modulation period of 40 s and a modulation amplitude of ±0.531 °C. The T_g and the width of transition were obtained using Thermal Advantage™ software and ΔH was calculated on the basis of sucrose weight.

2.5. Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) spectra were measured utilising a Perkin Elmer 1600 series FTIR, and analysed using PE-GRAMS/32 1600 software as described previously [22]. Briefly, a dry protein sample (approximately 0.5 mg protein) was mixed with about 300 mg ground potassium bromide and compressed into a pellet. For each spectrum, 64 scans were collected in absorbance mode with a 4 cm^{-1} resolution, and subsequently a 64-scan background was immediately recorded. The lysozyme solutions were measured at a concentration of ca. 35 mg/ml and using CaF_2 windows with 12 μm Mylar spacer (Graseby Specac, Orpington, Kent, UK). For each spectrum, 256 scans were collected in absorbance mode with a 4 cm^{-1} resolution, and subsequently a 256-scan background was immediately recorded.

The spectra were smoothed with a nine-point Savitsky-Golay function to remove any possible white-noise. The second derivative spectrum was obtained with Savitsky-Golay derivative function software for a five data point window and was smoothed with a seven-point Savitsky-Golay function. The second derivative spectra of experimental samples in the amide I region (1600–1710 cm^{-1}) were analysed. The baseline of the spectrum in the amide I region was levelled and zeroed, then the spectrum of the sample was normalised for area in the region and the intensity of the α-helical band recorded. The intensity of the α-helix band (1654–1658 cm^{-1}) was measured.

2.6. Statistical analysis

All data are expressed as means ± standard deviations (SD). Statistical analysis was performed using either a Kruskal Wallis test or *t*-test (Minitab, Minitab, Inc., State College, PA). In all cases, *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of combining polymer or trehalose with sucrose on the T_g and T_c of freeze-dried excipients

The moisture content of the freeze-dried samples was found to be between 0.5 and 1.1% independent of the constituents of the mixtures. The DSC thermogram of freeze-dried sucrose alone (Fig. 1) showed that an endothermic event occurred at 180 °C, corresponding to the melting peak of sucrose. An exothermic peak at about 118 °C (T_c) was attributed to the crystallisation of sucrose, and a glass transition was detected at about 58 °C (T_g).

A 2% w/w concentration of polymer or trehalose added to sucrose was found to increase the T_c of the resultant combination (Table 1). The increases appeared to correlate with the molecular weight of the added excipient; the larger the molecular weight of the excipient, the higher the resultant T_c. In addition, the DSC thermograms indicated that the crystallisation peaks of some combinations, such as those containing 2% dextran or PVP K90, diminished in size. Such changes could be evaluated by calculating the crystallisation enthalpy (ΔH_c) of sucrose in the different mixtures (Table 1). The ΔH_c of freeze-dried sucrose alone was found to be 94.8 J/g. The addition of 2% w/w trehalose,

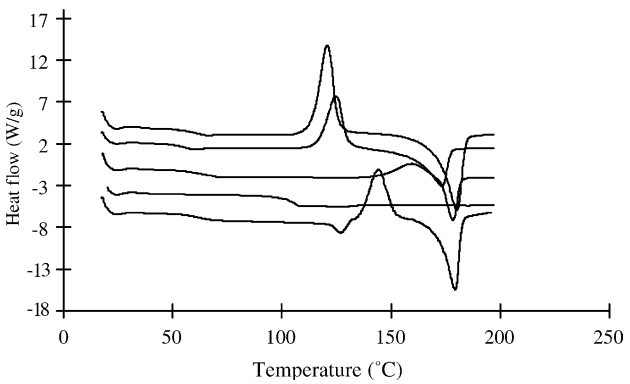

Fig. 1. DSC thermograms of freeze-dried samples obtained at a heating rate of 10 °C/min. From top to bottom, sucrose alone; sucrose +2% trehalose; sucrose:trehalose 9:1; trehalose alone; sucrose:lysozyme 10:1.

Table 1
The glass transition temperature (T_g), crystallisation temperature (T_c) and crystallisation enthalpy (ΔH_c) of freeze-dried sucrose containing 2% w/w of different additives, as measured by DSC using a heating rate of 10 °C (Mean ± SD, $n = 3$)

Excipient	T_g (°C)	T_c (°C)	ΔH_c (J/g)
No excipient	57.8 ± 0.2	118.5 ± 1.0	94.8 ± 4.7
Trehalose	57.9 ± 0.5	125.2 ± 0.7	88.8 ± 2.1
PVP K15	58.8 ± 0.5	127.6 ± 0.9	84.1 ± 12.8
PVP K25	58.9 ± 0.6	129.7 ± 2.4	95.9 ± 2.0
PVA	59.5 ± 0.6	131.3 ± 0.8	87.2 ± 11.7
Dextran	57.7 ± 0.2	140.3 ± 1.0	69.6 ± 7.8
PVP K90	58.2 ± 0.8	146.7 ± 1.0	38.6 ± 19.8

PVP K15, PVP K25 or PVA had no significant effect on the ΔH_c of the resultant formulations ($P > 0.05$, Kruskal Wallis test). However, when 2% w/w of dextran or PVP K90 was included, the ΔH_c of sucrose was decreased considerably to 69.6 and 38.6 J/g, respectively. The effects of adding 2% w/w trehalose or polymers on the T_g, of the sucrose were found to be minimal, the changes in the T_g being insignificant irrespective of the nature of the added excipient (Table 1, $P > 0.05$, Kruskal Wallis test). In addition, the changes in the width of transition also appeared not to be significant (data not shown).

The effects of excipients on the T_cs and T_gs of freeze-dried sucrose were also found to be a function of excipient concentration. As shown in Table 2, the T_cs of sucrose increased with increasing amounts of trehalose up to sucrose to trehalose 9:1. However, when the content of trehalose reached 4:1, the crystallisation of sucrose was completely inhibited. In addition, the ΔH_c of freeze-dried sucrose–trehalose mixtures appeared to be inversely related to the T_c. For example, the ΔH_c was decreased from 94.8 J/g for sucrose alone to 73.5 and 40.0 J/g, respectively, when trehalose was included at ratios of 19:1 and 9:1. The corresponding T_cs increased from 121.4 °C for sucrose alone to 156.2 °C when trehalose was present at 9:1. The effects of excipient content on the T_g of sucrose appeared to be complex. When the trehalose content was increased up to 9:1, there was no significant change in the T_g ($P > 0.05$, Kruskal Wallis test). However, as the trehalose was increased to 4:1 or 1:1, the T_gs of the freeze-dried mixtures were raised to 69.6 and 80.8 °C, respectively, accompanied by an increase in the width of transition (Fig. 1). Broadly, increases in T_g of sucrose conferred by trehalose seemed to be in accordance with the Gordon and Taylor equation [23] (Table 2).

The presence of lysozyme also led to similar concentration-related increases in both the T_c and the T_g of sucrose (Table 3). Generally as the lysozyme content was increased up to about sucrose to lysozyme 10:1, the T_c of the mixture appeared to increase. When the content of lysozyme increased to 10:1, the crystallisation of the sugar was completely inhibited. The ΔH_c of freeze-dried lysozyme–sucrose mixtures was also found to be affected by the addition of lysozyme. Mixtures containing lysozyme at mass ratio of 10:1 were found to have ΔH_c values of 79.7 J/g. The effects of lysozyme on the T_g and the width of transition were found to be insignificant when the amount of the protein was at sugar to enzyme mass ratio 5:1 or less (Table 3) ($P > 0.05$, Kruskal Wallis test). Freeze-dried formulations containing higher than 5:1 of lysozyme were not analysed due to the powders being too bulky. However, results from spray-dried sucrose–lysozyme mixtures showed that the width of transition appeared to increase and the height of the step change in the reversing heat flow decreased with increasing the lysozyme content from 1:1 to 1:4 (data not shown).

3.2. Effects of a single excipient on the secondary structure of freeze-dried lysozyme in the dry state

The activity loss of freeze-dried lysozyme in the absence of excipient was reported to be less than 10%, however, in the presence of any excipient(s), no detectable loss was observed [24]. Therefore, the effect of excipients on the freeze-dried lysozyme was evaluated by changes in the secondary structure, as indicated by second derivative of FTIR. Changes in the secondary structure of lysozyme induced by lyophilisation and spray-drying have been indicated previously by measuring the differences in the intensity of the α-helical band in the second derivative FTIR spectra [22,25]. In this study, it was also decided to utilise such changes in the intensity of the α-helical band to determine quantitatively the changes in the secondary

Table 2
The glass transition temperature (T_g), crystallisation temperature (T_c) and crystallisation enthalpy (ΔH_c) of freeze-dried sucrose containing different concentrations of trehalose, as measured by DSC using a heating rate of 10 °C (mean ± SD, $n = 3$)

Sucrose:trehalose mass ratio	Measured T_g (°C)	Expected T_g (°C)[a]	T_c (°C)	ΔH_c (J/g)
Sucrose alone	60.8 ± 1.5		121.4 ± 3.3	94.8 ± 4.7
49:1	57.9 ± 0.5	61.6	125.2 ± 0.7	88.8 ± 2.1
19:1	60.0 ± 1.6	62.7	143.8 ± 6.4	73.5 ± 20.5
9:1	62.0 ± 1.2	64.6	156.2 ± 4.1	40.0 ± 17.8
4:1	69.6 ± 5.0	68.5	No T_c	0
1:1	80.8 ± 3.3	80.6	No T_c	0
Trehalose alone	103.0 ± 1.6		No T_c	0

[a] Expected T_g were calculated based upon Gordon–Taylor equation.

Table 3
The glass transition temperature (T_g), crystallisation temperature (T_c) and crystallisation enthalpy (ΔH_c) of freeze-dried sucrose containing different amounts of lysozyme, as measured by DSC using a heating rate of 10 °C (mean ± SD, $n = 3$)

Sucrose:lysozyme mass ratio	T_g (°C)	T_c (°C)	ΔH_c
Sucrose alone	60.8 ± 1.5	121.4 ± 3.3	94.8 ± 4.7
200:1	59.0 ± 1.6	119.3 ± 0.7	86.5 ± 5.2
100:1	61.1 ± 0.6	130.9 ± 0.8	92.1 ± 5.8
50:1	61.2 ± 1.4	135.3 ± 1.6	87.9 ± 7.8
25:1	61.9 ± 1.3	137.8 ± 0.7	93.0 ± 4.5
10:1	62.1 ± 0.9	145.7 ± 0.8	79.7 ± 3.3
5:1	63.4 ± 1.3	No T_c	0

structure of the model protein in the dried form upon lyophilisation. The higher the intensity, the greater preservation of the native structure, although the utilisation of a single band for quantitative analysis is only valid when the overall features of those spectra are similar.

The second derivative FTIR spectrum of freeze-dried lysozyme showed bands at 1624 and 1641 cm^{-1}, assigned to β-sheet structure, a minimum at 1654–1658 cm^{-1}, assigned to α-helical structure, and bands between 1665 and 1710 cm^{-1}, assigned to β-sheet and turn structures [22,25] (Fig. 2). Like spray-dried lysozyme [22], the freeze-dried enzyme in the absence of stabiliser also underwent a sizeable perturbation of secondary structure as indicated by the low intensity of the α-helical band obtained in the solid state using FTIR. The intensity of the α-helical band of freeze-dried lysozyme alone was only about 55% of that of the native enzyme whilst the bands between 1665 and 1700 cm^{-1} broadened to 1710 cm^{-1} and the band at approximately 1686 cm^{-1} became more apparent.

In the second derivative FTIR spectra, the intensity of the α-helical band increased with increasing excipient to lysozyme mass ratio as shown for glycerol in Fig. 2, whilst the band between 1665 and 1710 cm^{-1} was narrowed and decreased in size. The capacity of excipients to preserve the native α-helical content of lysozyme was found to be generally a function of excipient to enzyme mass ratio (Fig. 3). In the range of excipient to enzyme mass ratio 0–2, a rapid increase in the α-helical intensity was found to exist when glycerol was employed, whilst the increases induced by the presence of sucrose and trehalose in the lysozyme formulations were found to occur up to 4. However, increasing the concentration of any of these excipients up to 10 did not further affect the native α-helical content of lysozyme ($P > 0.05$, Kruskal Wallis test). Moreover, dextran appeared to confer minimal stabilising effects on the native α-helical content with the exception of the formulation containing dextran at a mass ratio of 10 (Fig. 3). In addition, the relationship between the concentration and the increase of the native α-helical content was similar for both the sucrose and trehalose formulations, although sucrose was found to be always slightly more effective than trehalose at each concentration (Fig. 3) and overall the difference was significant ($P < 0.05$, paired Student's t-test). When the concentration of trehalose was increased beyond a mass ratio 7, the native α-helical content of freeze-dried lysozyme appeared to diminish marginally, in contrast to the formulation containing sucrose at the same mass ratio (Fig. 3).

The intensity of the α-helical band appeared to be dependent upon the sucrose to enzyme mass ratios rather than the bulk concentration of the sugar. An increase in the concentration of sucrose from 4 to 20 mg/ml (corresponding to lysozyme concentrations between 2 and 10 mg/ml) could not be shown to significantly influence the intensity of α-helical band of lysozyme ($P > 0.05$, Kruskal Wallis test) when the mass ratio of sucrose:enzyme was maintained at 2 (data not shown).

The extent of the native α-helical content preserved within freeze-dried lysozyme was different when processed

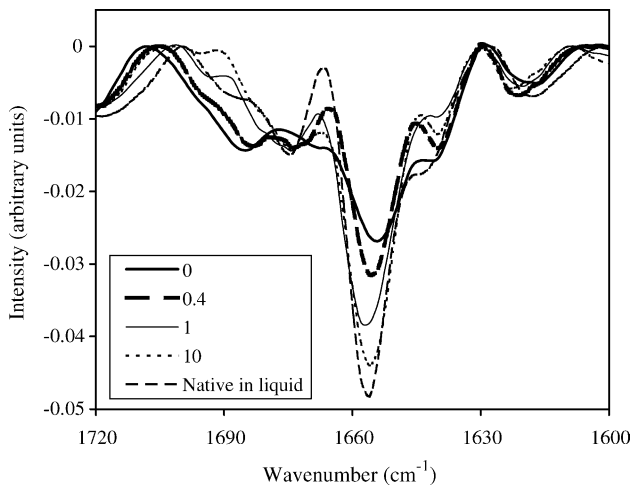

Fig. 2. The second derivative FTIR spectra of freeze-dried lysozyme (5 mg/ml) in the presence of glycerol at different excipient to enzyme mass ratios.

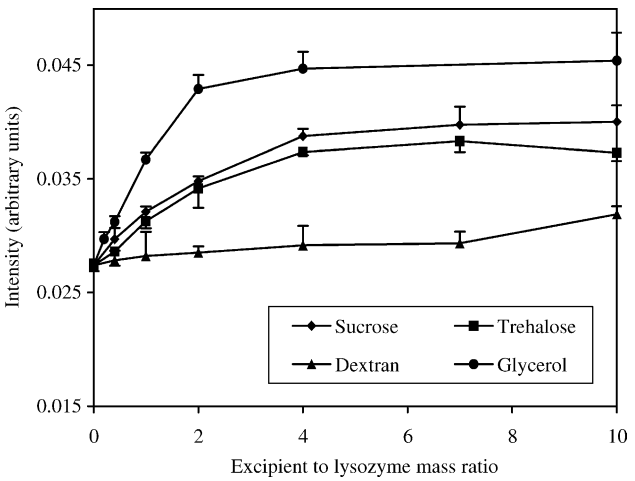

Fig. 3. Relationship between excipient concentration and the intensity of the α-helix band of freeze-dried lysozyme (5 mg/ml) obtained from the amide I region of the second derivative FTIR spectra (mean ± SD, $n = 3$).

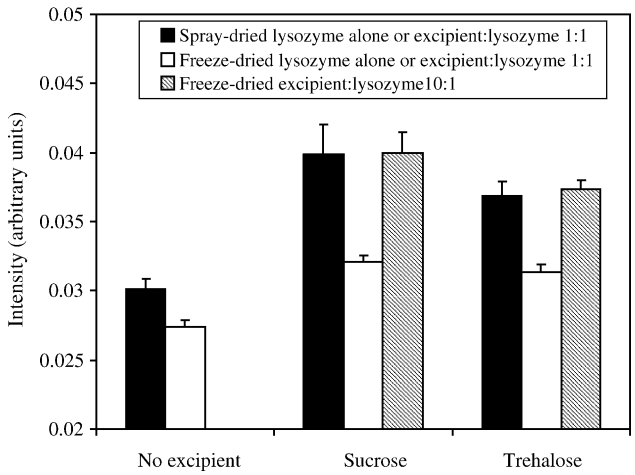

Fig. 4. Comparison of the α-helical intensity of spray- and freeze-dried lysozyme with or without excipient (mean ± SD, $n = 3$).

in the presence or absence of excipients and also dependent upon the method of drying (Fig. 4). The intensity of the α-helical band of the enzyme was found to be significantly lower after freeze-drying than that obtained after spray-drying when excipients was present at an mass ratio 1:1 ($P < 0.01$, one-tailed Student's t-test, $n = 6$). In addition, in order to achieve a similar preservation of the native α-helical content, a higher mass ratio of excipient to enzyme was required for processing by freeze-relative to spray-drying (Fig. 4). Furthermore, the minimum excipient to enzyme mass ratios (1:1–2:1) required to be present during spray-drying [22] to preserve the lysozyme native structure close to maximum in the dried form were found to be about only a half of those (2:1–4:1) necessary to stabilise the native structure during freeze-drying.

The effects of PEG 200 on the secondary structure of freeze-dried lysozyme (Fig. 5) were different from the other excipients employed. The spectral features of the PEG 200 containing formulations appeared to be substantially changed and consequently the intensity of α-helical band could no longer be utilised as an indicator of the integrity of the native structure. When the concentration of the excipient was present at mass ratios between 0.2 and 1, the second derivative FTIR spectra of the freeze-dried products appeared to be similar to that of lysozyme processed alone, with the intensity of the α-helical band being almost identical. However, a new band at about 1700 cm^{-1}, attributable to inter-molecular β-sheet [26], occurred in the spectra of the formulations containing PEG 200 at a mass ratio of 0.6 and 1 with the latter formulation having a higher intensity (Fig. 5a). When the PEG 200 content was further increased to a mass ratio of 2, the α-helical band at 1656 cm^{-1} decreased sizeably whilst the band at 1700 cm^{-1} apparently increased and a new peak emerged at 1624 cm^{-1}, which was also assigned to inter-molecular β-sheet structure [26] (Fig. 5b). The further addition of PEG 200 to obtain a mass ratio of 4 led to a further increase in the intensity of the band at 1624. Whilst yet a further increase in

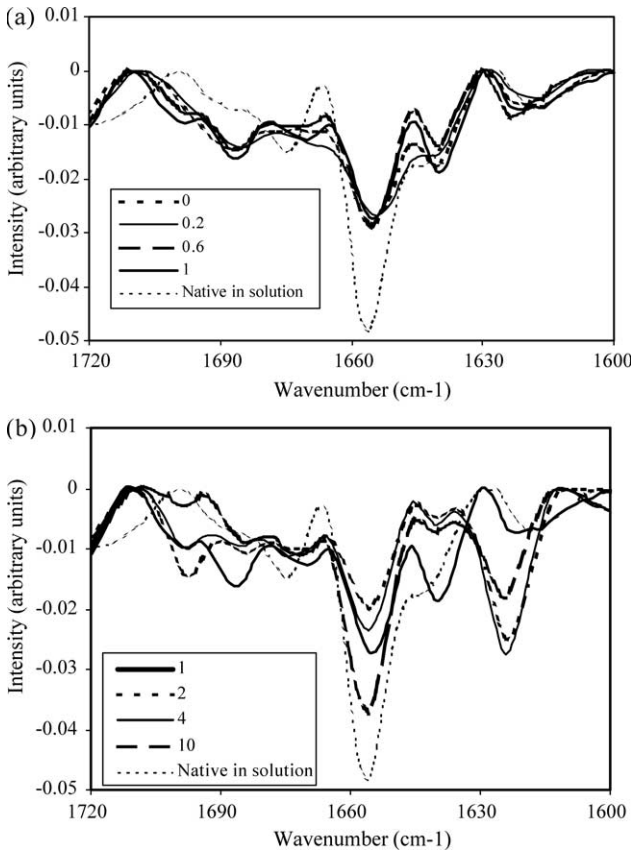

Fig. 5. The second derivative FTIR spectra of freeze-dried lysozyme (5 mg/ml) in the presence of PEG 200 at different excipient to enzyme mass ratios.

PEG 200 content to a mass ratio of 10 resulted in an increase in the native α-helical content as shown by the more pronounced band at 1656 cm^{-1} whilst the bands at both 1624 and 1700 cm^{-1} decreased in size (Fig. 5b).

3.3. Effects of a mixture of sucrose with either trehalose or dextran on secondary structure of freeze-dried lysozyme in the dry state

Sucrose was found to be more effective in stabilising the native α-helical content of lysozyme during lyophilisation than trehalose (Fig. 3), when each excipient was employed at a mass ratio of 10. Samples containing mixtures of trehalose and sucrose at a mass ratio of 10 but with varying compositions were also tested (Fig. 6). With an increasing sucrose content up to 30% w/w, the intensity of α-helical band appeared to increase, whereas a further increase in the sucrose content of the mixture did not lead to further significant changes in the native α-helical content ($P > 0.05$, Kruskal Wallis test).

The effects of combinations of dextran and sucrose on the intensity of α-helical band were found to relate to the sucrose content in the mixture when the total ratio of excipient:enzyme was maintained constant (Fig. 7a and b). When an excipient:protein mass ratio was maintained at 4,

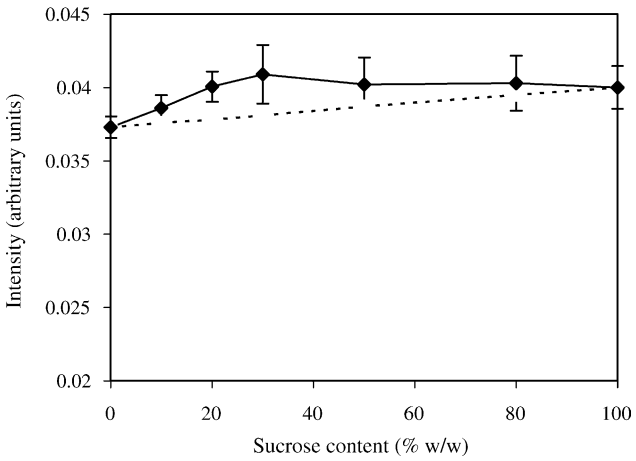

Fig. 6. Relationship between sucrose content (% w/w) in a formulation containing enzyme and a mixture of trehalose and sucrose and the intensity of α-helical band of freeze-dried lysozyme at amide I regions. Total additive content was maintained at a mass ratio of 10 (mean ± SD, $n = 3$). —, measured value; - - -, predicted value assuming an additive effect of the two excipients.

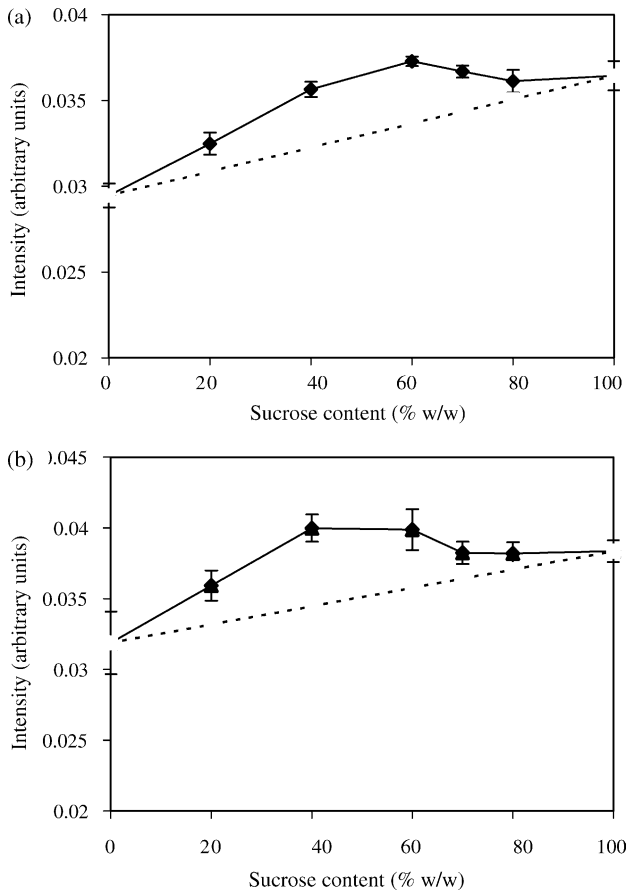

Fig. 7. Relationship between sucrose content (% w/w) in mixture of dextran and sucrose and the intensity of α-helical band of freeze-dried lysozyme at amide I. (a) At a mass ratio of 4 (excipient:protein); (b) at a mass ratio of 10 (excipient:protein). Values are mean ± SD, $n = 3$. —, measured value; - - -, predicted value assuming an additive effect of the two excipients.

the intensity of α-helical band increased with increasing sucrose content up to 60%. For formulations containing a sucrose content at 60% or higher, there was little further change in native α-helical content (Fig. 7a). In addition, as the excipient content was increased to a mass ratio of 10, the substitution of sucrose with up to 60% w/w dextran preserved the α-helical structure to a greater extent relative to sucrose alone (Fig. 7b).

The effects of combinations of dextran/trehalose and sucrose on the stabilising capacity of sucrose during freeze-drying were found to be different from those during spray-drying. The stabilisation of lysozyme conferred by the mixtures of either trehalose or dextran with sucrose during spray-drying appeared to obey a linear relationship between sucrose content in the mixtures and native α-helical content indicating an additive relationship [22]. In contrast, such mixtures were found to promote synergistic effects on the stabilisation of the native structure of lysozyme during freeze-drying (Fig. 7a and b).

4. Discussion

The successful stabilisation of proteins during processing does not ensure satisfactory storage stability unless the T_g of the formulation is higher than the storage temperature [5]. In the present study, the combination of either trehalose or dextran with sucrose as lyoprotectants seemed to result in an equivalent protective capacity to sucrose alone and better than either trehalose or dextran alone. Such results were broadly in agreement with the stabilising effects of dextran and sucrose/trehalose mixtures on actin [27]. In addition, as trehalose gradually replaced sucrose the T_g of the resultant mixtures was raised relative to sucrose alone. Trehalose was found to be more effective in raising the T_g of sucrose in accordance with the Gordon and Taylor equation [23] than lysozyme on a w/w basis. Mixing sucrose with lysozyme appeared to produce a non-ideal system, similar to a PVP–sucrose mixture as previously reported by Shamblin et al. [28]. These earlier workers found that increasing the content of PVP in a PVP–sucrose mixture up to 30% did not lead to a raised T_g relative to sucrose alone and proposed that non-ideal mixing was responsible. Non-ideal mixing could result from sugar–sugar interactions being preferable to sugar–lysozyme interactions and consequently this could lead to the generation of potential sugar–sugar clusters and even phase separation. However, upon including another excipient such as trehalose (Fig. 6) or dextran (Fig. 7), the strength of the sucrose–sucrose interactions could be decreased such that the interactions between lysozyme and sucrose were promoted, leading to an increase in the stabilisation of lysozyme. Although trehalose and dextran might be able to raise the T_g of sucrose-containing formulations, they might also affect the glassy properties, such as the width of transition. The potential effects of changes in the latter properties on the dynamics of

the stabilised protein were beyond the scope of the present study but warrant more studies in future.

The results of the present study tend to support the hypothesis that hydrogen-bonding is primarily responsible for the stabilisation of protein structure, as proposed by Allison et al. [25], and also indicated that vitrification might not be a prerequisite for reducing/inhibiting lysozyme unfolding during freeze-drying process. The stabilisation of the secondary structure of the freeze-dried lysozyme was found to be independent of the formation of glass during the drying process but related to the presence of hydroxyl groups in the excipients. Potential steric hindrance due to the molecular sizes, has been reported to dictate the hydrogen bonding capacity of the excipients [5]. Amongst the excipients utilised, dextran and PEG 200 are less capable of hydrogen bonding with protein molecules and hence there were minimal stabilising effects of the two excipients on the native structure of lysozyme despite dextran being ready to form a glass. In contrast, the excipients with high hydrogen bonding ability such as glycerol, sucrose and trehalose conferred stabilisation upon lysozyme. In particular, although glycerol was unable to exist as a glassy state under the experimental conditions, it appeared to stabilise lysozyme more efficiently than either sucrose or trehalose during freeze-drying.

The stabilisation of proteins also appeared to be dependent upon the accessible surface areas of both the protein and excipient molecules, which correlates with the potential interactions, which can exist between protein, and excipient molecules. The stabilising effects of excipients on the native structure of freeze-dried lysozyme in the present study were found to be excipient-enzyme mass ratio-dependent rather than being dependent upon the bulk concentration of the excipients, which indicated that the stress mainly responsible for the perturbation of the lysozyme structure might be dehydration [5,29]. From the relationship between stabilisation of lysozyme structure conferred by excipients and excipient-enzyme mass ratios, it could be seen that a minimum excipient-enzyme mass ratio was required to achieve close to maximum stabilisation of protein secondary structures with further increases in the mass ratios only leading to a small gain in protective effect. The magnitude of minimum mass ratios appeared to be excipient- and protein-dependent. Results indicated that the smaller the molecular weight of excipients and/or the larger the molecular weight of the proteins, the smaller the mass ratios required. For example, the minimum mass ratios for glycerol, the molecular weight of which is approximately a quarter of those of sucrose and trehalose, to achieve a close maximum stabilisation of freeze-dried lysozyme and catalase [24] were found to be only a half or less than a half of those for the latter excipients. The minimum mass ratio required for the stabilisation of lysozyme appeared to be 2 times higher than those for the stabilisation of catalase, which has a molecular weight approximately 18 times that of the former enzyme. The minimum mass ratios for glycerol, sucrose and trehalose to achieve a close maximum stabilisation of catalase were found to be approximately 0.6, 2, and 2, respectively [24]. The different minimum mass ratios required for different excipients and proteins might be attributable to the different accessible surface area of protein molecules on a weight basis and the hydrogen-bonding capacity of excipients. For large molecular weight proteins, the accessible surface area on a weight basis is lower than that for low molecular weight proteins such that a lesser amount of excipients and a lower minimum mass ratio, is required. On the other hand, low molecular weight excipients such as glycerol are sterically more efficient at gaining access to the surface of protein molecules, and therefore confer a more effective stabilisation of protein structure on a weight basis relative to high molecular weight excipients. Therefore, the excipient to mass ratios to achieve a close to maximum stabilisation of proteins might be estimatable based upon the molecular weights and accessible surface areas of the proteins and the excipients.

However, it should be noted that the formation of hydrogen bonding interactions between protein and excipient molecules might be affected also by the drying process. Indeed, the minimum mass ratios for both sucrose and trehalose to achieve a close maximum stabilisation of spray-dried lysozyme were found to be only a half of that required for the freeze-dried enzyme. Such a difference could result from the competition between protein–excipient and excipient–excipient interactions. As the water content decreases, hydroxyl groups from excipient molecules might be expected to gradually approach and hydrogen bond with protein molecules. During freeze-drying, the mobility of excipient molecules, which are trapped in glassy matrices, is more greatly restricted relative to that during spray-drying. Due to the competition between protein–excipient and excipient–excipient interactions, trehalose is more likely to self-associate relative to sucrose as described previously [22]. As a result, sucrose was found to be more effective in stabilising the enzyme than trehalose when the sugar to enzyme mass ratio was the same. Such results were broadly similar to those of spray-dried lysozyme reported previously [22].

Although the formation of hydrogen bonding between excipients and proteins may be mainly responsible for the preservation of the native structure of proteins, the formation of glass in the present study might play a role in restricting the formation of protein aggregates, which has been previously proposed as an explanation for the possible denaturation of protein structure as a consequence of intermolecular hydrophobic and electrostatic interactions occurring during dehydration [30]. The formation of aggregates can be indicated by the presence of intermolecular β-sheet structure in the second derivative FTIR spectra [26]. Using these criteria, it appeared that, in the present study, lysozyme underwent apparent aggregation in the presence of PEG 200. The formation of such aggregates might be attributable to both the unfolding of lysozyme due

to the lack of hydrogen bonding ability of PEG 200 and the high mobility of molecules within the protein system due to lack of glass formation. The formation of aggregates during freeze-drying might be greatly inhibited by either the inhibition of unfolding or the formation of glass. For example, the presence of glycerol was found to minimise the generation of aggregates since glycerol could thermodynamically stabilise the native state, whilst the inhibition of aggregation conferred by dextran might be due to the limited mobility of the enzyme molecules within the dextran–lysozyme mixture.

5. Conclusion

The stabilisation of lysozyme conferred by sucrose and trehalose was found to be drying method dependent with the spray-dried enzyme being more effectively stabilised at an equivalent sugar to protein mass ratio than the freeze-dried protein. In addition, the stabilising capacity of excipient appeared to be related to the hydrogen bonding ability of the excipient, rather than glass formation. However, the latter could inhibit the formation of inter-molecular aggregates, should the enzyme be partially denatured, as a consequence of the restriction of molecule mobility. The physical stability (in terms of T_g and T_c) of freeze-dried lysozyme formulations was found to be a function of excipient–protein mass ratio and was also composition-dependent with trehalose-containing formulations being superior to the equivalent sucrose-based ones. The inclusion of trehalose to a sucrose-containing freeze-dried formulation could raise the T_g of the resultant formulation and also lead to a synergistic stabilisation of lysozyme, in contrast to an additive manner as occurred in spray-dried formulations [22].

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