Effects of Sucrose and Trehalose on the Preservation of the Native Structure of Spray-Dried Lysozyme

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Purpose. To investigate the effects of sucrose, trehalose, sucrose/dextran mixtures, and sucrose/trehalose mixtures on the preservation of the native structure of spray-dried lysozyme in the solid state.
Methods. The intensity of the α-helical band and the melting enthalpies (ΔH_m) of spray-dried lysozyme in the dried form and in aqueous solution were obtained using second derivative FTIR and differential scanning calorimetry (DSC) respectively.
Results. The intensity of the α-helical band and the ΔH_m of spray-dried lysozyme obtained were linearly correlated and both suggest that the stabilization of lysozyme in the dried form was excipient concentration-dependent with a close to maximum stabilization being conferred by sucrose or trehalose at a mass ratio 1–2 (sugar:enzyme). Sucrose appeared to be more effective than trehalose on a weight by weight basis whilst stabilizing effects of dextran/sucrose or trehalose/sucrose mixtures were found to be additive.
Conclusion. Dehydration during spray drying was considered the main stress to the denaturation of lysozyme. A major effect of the sugars in protecting lysozyme against dehydration was attributable to hydrogen bonding between the sugar and protein molecules, which lead to an increase in the change in the negative value of the free energy between native and denatured states.

KEY WORDS: spray-drying; lysozyme; dextran; protein stabilization; denaturation enthalpy.

INTRODUCTION

Increasing interest in developing pulmonary delivery systems for proteins and peptides has lead to the proposal that spray drying may provide one of the principal means of producing inhalable powders of such potentially labile biopharmaceuticals (1–4). However, any commercial inhalable protein formulation must retain its physical and chemical stability within shelf-time requirements and the active component is required to have appropriate aerodynamic properties for pulmonary delivery. Thus, for delivery to the deep lung, particles with an aerodynamic diameter of 1–3 μm are generally considered desirable (5).

The processes involved in spray drying impose potential stresses on the product, including exposure to high temperatures, distribution of air-water interfaces and dehydration. Any of these could denature proteins and thus compromise the biologic activity (2–4,6–7). To stabilize the native structure of proteins, protective excipients are generally included as components of the final formulations. The excipients generally utilized include polyols, sugars, polymers, surfactants, amino acids and proteins. In particular, it has been found that the addition of disaccharides such as sucrose or trehalose to a protein formulation often enables the protein structure to be stabilized effectively (4,6). Such stabilizing effects have been correlated with the concentration of excipient, with a minimum sugar to protein mass ratio being required to preserve the native structure of proteins. In contrast, any excessive excipient might lead to the destabilization of proteins and a decrease in the physical stability of the formulation as indicated by a decrease in the glass transition temperature (Tg) and re-crystallization temperature of small sugars (Tc) (4). The inclusion of surfactants might also promote the stabilization of proteins. For example, in a study involving the spray-drying of recombinant human growth hormone, a protein known to be sensitive to adsorption to air-water interfaces, about 25% of the protein was aggregated in the absence of a surfactant, whereas the addition of polysorbate 20 to the formulation reduced the aggregation by 90% (2). However, the effects of surfactants on the stabilization of the protein native structure in the dried form and the long-term stability of protein formulations are unclear.

To achieve optimum long-term stability of a protein formulation, the Tg should be maximized whilst the perturbation of native structure in the dried state minimized (8–9). The latter requirement is mainly due to denatured proteins in the dry state being more reactive than native proteins and therefore generally susceptible to aggregation and degradation during storage. Thus, the long-term stabilization of proteins in the dried form cannot be guaranteed by demonstrating that the native structure of the proteins in the rehydrated solution immediately after processing is retained (8–9). Trehalose has been identified as one of the most suitable sugars for protecting proteins from denaturation during dehydration and storage, due to the high Tg it confers (10). However, sucrose has been more extensively utilized in the pharmaceutical industry than trehalose, despite the Tg of trehalose being over 40°C higher than that of sucrose (10). The benefits of one excipient over the other appear to be equivocal. For example, sucrose has been found to be more effective in preserving the native structure of lysozyme during lyophilization compared to trehalose, as determined by the measurement of the secondary derivative FTIR of dry lyophilized lysozyme (11). In addition, it has also been reported that sucrose contains a greater capacity to stabilize freeze-dried monoclonal antibody than trehalose when stored at 5°C (12–13). In fact, in nature, seeds commonly contain a large amount of sucrose together with a high proportion of oligo- and polysaccharides, a combination that was found to elevate the Tg and maintain the seed viability for long-term storage (14). Subsequently, Allison and colleagues reported that the addition of dextran to sucrose- or trehalose- stabilized actin, increased Tg, without affecting the capacity of the sugar to stabilize the protein during freeze-drying (15).

Thus, one of the aims of this study was to investigate the effects of trehalose and sucrose on the preservation of the native structure of a model protein—lysozyme during spray

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drying and to compare the stabilizing capacity of the two excipients. A second aim was to determine the effects of trehalose or dextran in combination with sucrose on the protective ability of the latter during processing. Furthermore, attempts were made to investigate the mechanism by which lysozyme was stabilized during spray drying.

MATERIALS AND METHODS

The buffer phosphate salts (ACS reagent grade), sucrose, trehalose, dextran (MW ~70,000), lysozyme (3× crystallized, dialyzed and lyophilized, Lot 57H7045, 77H7032 and 20K0956) and *Micrococcus lysodeikticus* (Lot 39H8615) were purchased from Sigma-Aldrich Co., UK.

Experimental Design

Spray-dried protein formulations were prepared containing a single excipient at the following lysozyme to sugar mass ratios: 10:1, 5:1, 5:2, 1:1, 1:2, 1:4 and 1:10. Alternatively, a combination of either trehalose or dextran with sucrose was used in place of the single excipient. In the latter formulations, the lysozyme to sugar mixture mass ratio was held constant but the sucrose content in the sugar mixtures varied from 0%–100%. In all cases, the lysozyme concentration was maintained at 5 mg/ml in 5 mM potassium phosphate buffer (pH 7.0) and all ratios and compositions were constituted in terms of a mass basis. At least 3 batches of each formulation were prepared.

Spray-Dried Procedures

Lysozyme and excipients were dissolved in buffer and spray dried using a Model 190 Buchi mini spray-dryer. A two fluid nozzle (0.5 mm) system was utilized to atomize the feed solution that was pumped peristaltically through a silicone tube (3 mm). Cooling water (0°C) was circulated through the jacket around the nozzle at a rate of 36 ml/min. The processing parameters comprised a feed rate of 3 ml/min, an atomizing air-flow rate of 700 l/h, and an inlet temperature of 120°C. Outlet temperatures were found to range from 85°C –90°C. The solution volume of each spray-drying batch was between 50 and 100 ml and each process lasted between 17 and 34 min. After all the feed solution had been processed but without cooling down to room temperature, the collected powder was transferred to a 7-ml vial. The vial was immediately sealed by capping and transferred to a freezer (–20°C). In certain spray drying runs, the inlet temperatures were varied between 85 and 180°C (85, 95, 105, 120, 140, 160, 170 and 180°C) and the corresponding outlet temperature ranged from 59 to 134°C (59, 68, 76, 88, 101, 113, 124 and 134°C).

DSC Analysis

The melting enthalpy (ΔH_m) and Tg of spray-dried powders were determined using a model 2920 modulated DSC (TA Instruments, UK), which was calibrated with indium prior to analysis. Approximately 5 mg of dry sample was placed in an aluminum pan that was hermetically sealed, and then equilibrated at –25°C in the sample compartment. Data were collected while the sample was heated at a rate of 10°C/min between –25°C and 200°C. For liquid samples, approximately 30 μl of lysozyme solution was hermetically sealed, and data were collected between 5°C and 95°C at a heating rate of 10°C/min.

Residue Moisture Analysis

The moisture content was determined using a TGA 2050 thermogravimetric analyzer (TA Instruments, UK). Samples (3–7 mg) of powders were placed in an open aluminum pan and loaded into the sample compartment. The sample was equilibrated at 25°C and then the data were collected using a heating rate of 10°C/min between 25°C and 200°C.

Determination of Enzyme Content and Biologic Activity

Lysozyme was assayed as previously reported (16), the biologic activity being measured using an assay based upon the lytic action of lysozyme on *M. lysodeikticus* cells.

Fourier Transform Infrared Spectroscopy

FTIR spectra were recorded on a Perkin–Elmer 1600 series spectrometer and analyzed using PE-GRAMS/32 1600 software. The second derivative FTIR spectra of lysozyme in both solid and solution states were obtained using previously reported methods (17). 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, Kent, UK). The baseline of second derivative amide I spectrum (1600–1710 cm^{-1}) was leveled and zeroed, then the second derivative spectrum of the sample was normalized for area. The intensity of the α-helix band (1654–1658 cm^{-1}) was measured.

RESULTS

Effects of Excipients on the Moisture Content

As the drying protection conferred by an excipient is related primarily to the mass ratio between protein and excipient (8), it was the sugar to lysozyme mass ratio that was used to generate the required compositions. The moisture content of spray-dried powders as a function of lysozyme to sugar mass ratio is shown in Fig. 1. The moisture content of the spray-dried lysozyme formulations was 6.68 ± 1.37%

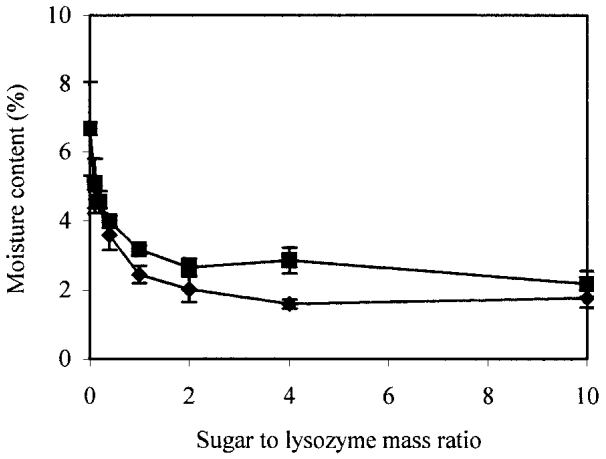

Fig. 1. The effects of sucrose/trehalose on the moisture content (% w/w) of spray-dried lysozyme-sucrose/trehalose powders. Sucrose (♦); trehalose (■) (mean ± SD, n ≥ 3).

(w/w) in the absence of excipient and this was found to decrease with increasing sucrose or trehalose content. However, for both sucrose and trehalose formulations, there were only small decreases in the moisture content with further increases in sugar concentration when the sugar to lysozyme mass ratio exceeded 1. The effect of sugar on the decrease in the moisture content was partially attributed to the increased hydrogen bonding that occurs between sugar and protein as the sugar concentration is increased (4). The moisture content in the sucrose powders appeared slightly but not significantly lower than that in the trehalose formulations ($p > 0.05$, paired student's *t* test). The formulations that were spray-dried from solution containing 10 mg/ml of a mixture of sucrose and trehalose, equivalent to a mass ratio of 2, were found to contain 3.35%–3.95% w/w of moisture content (Data not shown), whereas the moisture content of spray-dried lysozyme in the presence of a mixture of sucrose and dextran at a mass ratio of 1 were between 2.5% w/w–5.0% w/w.

Effects of Sucrose or Trehalose alone on Melting Enthalpies (Denaturation Enthalpies, ΔH_m)

Typical DSC thermograms of spray-dried sucrose-lysozyme, lysozyme alone and lyophilized amorphous sucrose are shown in Fig. 2. The amorphous sucrose was prepared by freeze-drying because it was not possible to produce the sugar in a glassy state using spray drying. DSC thermograms of all spray-dried lysozyme formulations contained an endotherm corresponding to lysozyme melting/denaturation peaks, the temperature at which the denaturation occurred being designated Tm, and a glass transition when the formulations contained sucrose or trehalose at a sugar to enzyme mass ratio of 2:5 or higher. In contrast, the DSC thermogram of amorphous sucrose alone displayed an exothermic peak at about 118°C, which was attributed to the re-crystallization of sucrose. Such an exothermic peak was only apparent in the spray-dried sucrose-lysozyme formulations when the sucrose content was greater than a sugar to lysozyme mass ratio of 4.

Tm has generally been utilized to evaluate the thermodynamic stability of proteins, the higher the Tm, the thermodynamically more stable the protein (18). The Tm of lysozyme in solution measured using DSC has been reported to be 74°C in 50 mM citrate at pH 4.0 and this was increased to 80°C when 1 M sucrose was included (19). Under the conditions utilized in this study (5 mM phosphate buffer, pH 6.2), the Tm determined by DSC was found to be 75.6 ± 0.2°C and was elevated to 78.3°C –78.6°C when 0.6 M sucrose or trehalose was added. The Tms of spray-dried lysozyme formulations, the values ranging from 120–145°C (Table I), were found to be markedly higher than that of the untreated enzyme in solution. In addition, the effects of sugars on the Tm appeared to be concentration dependent. The data in Table I, indicate that Tm generally increased with increasing content of either sucrose or trehalose, finally reaching a plateau at a sugar to lysozyme mass ratio of 2:5–1:1, and the Tms of trehalose formulations generally were 4°C–5°C higher than those of the powders containing comparable concentration of sucrose.

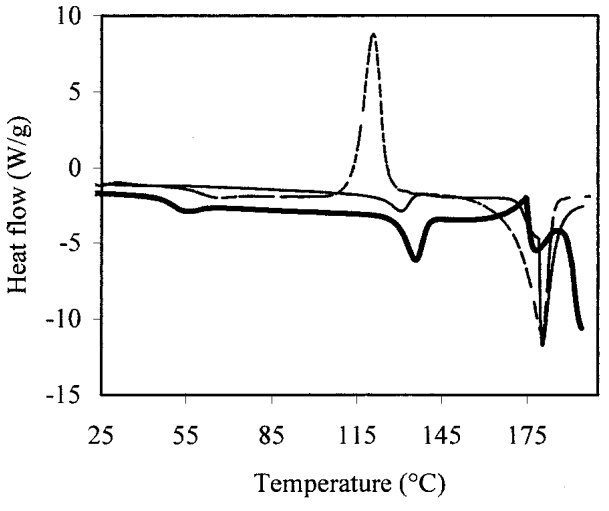

Fig. 2. DSC thermograms of (▬) spray-dried lysozyme:sucrose 1:1; (—) spray-dried lysozyme alone and (- - -) freeze-dried amorphous sucrose.

The Tg values of the spray-dried sucrose- or trehalose-lysozyme formulations appeared generally to increase with increasing sugar content. The lysozyme formulations containing trehalose, the Tgs of which were found to range from 46°C–72°C, appeared to have Tgs 10°C–20°C higher than those containing an equivalent amount of sucrose. The Tgs of sucrose containing formulation were found to be between 34.2°C–58°C, whereas the Tg values of lysozyme formulations containing sucrose in combination with either trehalose or dextran appeared to increase when the proportion of the latter components were increased (data not shown). When the sucrose:lysozyme 1:1 and trehalose:lysozyme 1:1 formulations were equilibrated in a 43% relative humidity desiccator, the moisture contents were found to increase to about 9% and the corresponding Tgs to decrease to 18.8°C ± 1.2°C and 27.4°C ± 1.3°C (n = 3) respectively.

The melting enthalpies of proteins have been suggested to involve both the breaking of internal interactions, which maintain the native structure of protein and the disruption of interactions between protein and excipients (18,20–21). Therefore, the size of the melting enthalpy of a protein is related to the extent of perturbation of the native structure, including both the disruption of secondary and tertiary structures, which occurs in the absence of stabilizing excipients. The melting enthalpies of native lysozyme in the liquid state and spray-dried lysozyme in the absence of excipient were found to be 33.61 ± 0.72 J/g and 12.98 ± 0.98 J/g respectively.

The ΔH_m profile of lysozyme was determined as a function of excipient concentration (Fig. 3). In the range of sugar to enzyme mass ratio 0.1 to 1, a rapid increase in the ΔH_m was

Table I. T_m of Lysozyme-Sugar Powders Spray-Dried from Solutions Containing 5 mg/ml of Enzyme (Mean ± SD, n = 3)

Sugar: lysozyme mass ratio	Sucrose T_m (°C)	Trehalose T_m (°C)
0:1	128.5 ± 10.4	128.5 ± 10.4
1:10	124.6 ± 4.9	128.0 ± 5.2
1:5	131.2 ± 3.5	128.5 ± 5.0
2:5	126.5 ± 3.2	140.1 ± 4.5
1:1	135.3 ± 3.0	139.6 ± 1.3
2:1	135.5 ± 0.5	139.1 ± 2.2
4:1	136.1 ± 2.0	139.6 ± 1.7
10:1	136.6 ± 0.7	141.4 ± 1.4

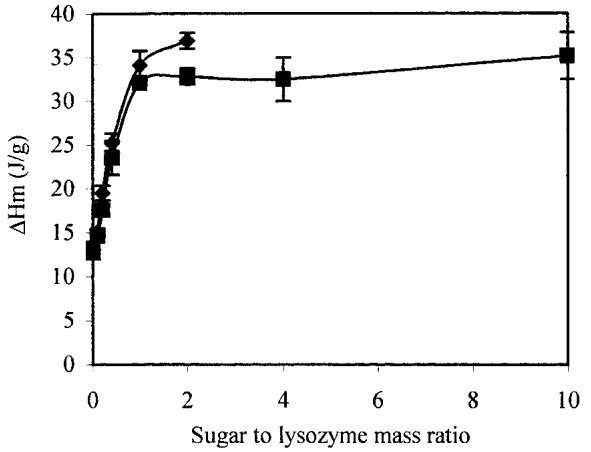

Fig. 3. The relationship between the excipient to lysozyme mass ratios and the denaturation enthalpy (ΔH_m) of lysozyme (mean ± SD, n = 3). Sucrose (◆); trehalose (■). Values are the mean ± SD, n ≥ 3.

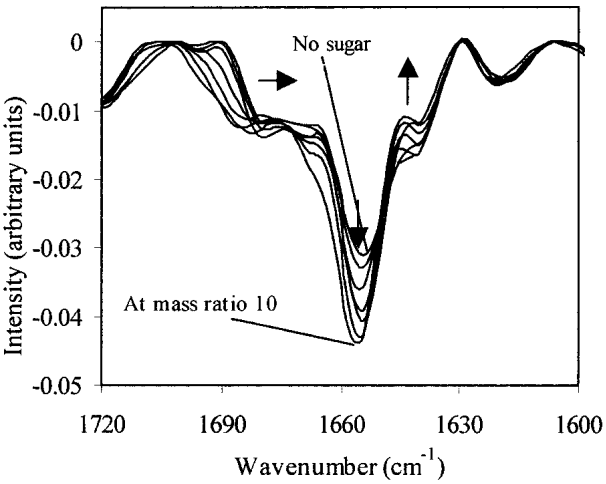

Fig. 4. Second derivative FTIR spectra in the amide I region of spray-dried lysozyme in the presence of sucrose at sugar to enzyme mass ratio 0, 0.2, 0.4, 1, 2, 4 and 10. The arrows show the direction of spectra changes as a function of sucrose amount.

found when either sucrose or trehalose were used (Fig. 3). A further increase in the content of trehalose did not lead to an increase in the ΔH_m, whereas the ΔH_m of formulations with a high sucrose content (mass ratio more than 2) could not be determined due to interference with the event that was attributable to sucrose re-crystallization. The ΔH_m of the sucrose formulation was found to be slightly higher than the corresponding formulations containing an equivalent weight of trehalose when the sugar to lysozyme mass ratio was less than 1. In addition, significant differences in ΔH_m were found between the effects of sucrose and trehalose at mass ratios of both 1 ($p < 0.05$, two-tailed t test, n = 9) and 2 ($p < 0.01$, two-tailed t test, n = 6).

Effect of Sucrose or Trehalose alone on Lysozyme Structures in the Solid State

The biologic activity of lysozyme, spray-dried in the absence of excipient, after rehydration was found to be 86.8 ± 2.45% (mean ± SD, n = 3) of the original activity, whilst there was found to be no detectable change in the presence of any of the sugar excipient(s) (data not shown).

The intensity of the α-helix band in the second derivative FTIR spectra has been utilized previously to indicate the integrity of the secondary structure of lysozyme (11). In the present study, spray-dried lysozyme in the absence of stabilizer also underwent a sizeable perturbation of secondary structure as indicated by the intensity of the α-helix band obtained in the solid state using FTIR. The intensity of the α-helix band in the second derivative spectrum was found to be only 60% of that exhibited by the native structure in the liquid form (a maximum protection of 88% was achieved in the presence of sucrose at a sucrose to enzyme mass ratio 10:1). The stabilizing effects conferred by either sucrose-trehalose or sucrose-dextran mixtures were found to be related to the sucrose content in the mixtures. In the second derivative FTIR spectra (Fig. 4), with increasing sucrose content, the α-helix band at 1654–1656 cm^{-1} shifted slightly to a higher wavenumber and increased in magnitude, whilst β-sheet band at 1638–1641 cm^{-1} as well as β-sheet and turn bands (at 1675–1705 cm^{-1}) decreased or narrowed in size.

The stabilization of the native α-helical structure of the spray-dried lysozyme was found to be a function of the sugar concentration (Fig. 5). The intensity of the α-helical band increased with increasing sucrose content, whilst for trehalose, a similar relationship was found only in the range of mass ratio 0.1–1 and a further increase in the mass ratio from 1–10 did not appear to enhance the preservation of lysozyme structure. In addition, the response between excipient concentration and the increase in the intensity of the α-helical band was found to be greater for sucrose than trehalose. When the concentration of the different sugars was increased to a mass ratio of 1 or more, the capacity of sucrose to preserve the native α-helical structure of spray-dried lysozyme was significantly greater than that of trehalose ($p < 0.01$, paired student's t test, n = 9).

The intensity of the α-helical band in the second derivative FTIR spectra appeared to be independent of the inlet/outlet temperatures utilized. When lysozyme in combination with an equivalent mass of sucrose were spray dried using inlet temperatures varying between 85°C and 180°C, the intensities of the α-helix band of these formulations were found

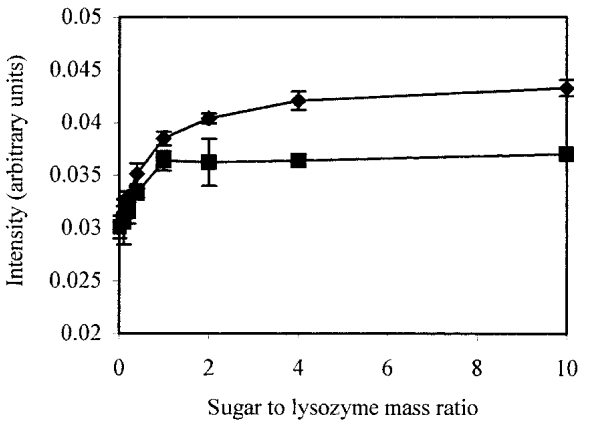

Fig. 5. Relationship between the excipient to lysozyme mass ratios and the intensity of α helix band of spray-dried lysozyme (5 mg/ml) obtained from the amide I region of the second derivative FTIR spectra. Sucrose (◆); trehalose (■) (mean ± SD, n = 3).

to be between 78.2%–82.0% of that exhibited by the untreated lysozyme in solution. There was no significant difference between the intensities of the α-helix band determined for the formulations spray-dried using different inlet temperatures ($p > 0.05$, ANOVA single factor).

To determine whether the differences between ΔH_m and the stabilization of α-helical structure by either sucrose or trehalose were dependent on the batch of protein, three different batches of lysozyme were used to prepare the spray-dried lysozyme formulations containing sugar at a mass ratio of 1. There was no significant difference in either the ΔH_m or the helical content between batches ($p > 0.05$, ANOVA single factor). In addition, the ΔH_m and α-helical structure of lysozyme within the sucrose-based formulations were always larger than those the corresponding trehalose containing product (data not shown).

Effects of Sucrose in Combination with Either Dextran or Trehalose on ΔH_m and α-Helical Content of Lysozyme

Either trehalose or dextran in combination with sucrose appeared to stabilize spray-dried lysozyme in an additive manner, when assessed using the intensity of α-helix in amide I (Fig. 6). Dextran appeared to have no stabilizing ability to preserve the native α-helical structure of lysozyme at a mass ratio of 1, and the capacity of trehalose was significantly lower than that of sucrose at a mass ratio of 2. However, combining either dextran or trehalose with sucrose produced spray-dried enzyme with greater retained structure than when dextran or trehalose was used alone but with less structure than when sucrose was present alone. The effects were directly dependent on the sucrose content.

When the total sugar content was maintained at a sugar to lysozyme mass ratio of 2, then the ΔH_m was also found to be dependent on the amount of sucrose relative to trehalose that was present and increased with increasing sucrose content in the mixture (Fig. 7).

DISCUSSION

In this study, the degree of stabilization of lysozyme induced by spray drying the protein with different concentration of sucrose or trehalose was determined using either DSC or FTIR. A linear correlation was found between the intensity of the resultant α-helix band and ΔH_m for both sucrose and trehalose (Fig. 8). Such results demonstrate that ΔH_m may be a useful parameter to evaluate the stabilization of proteins in the dried form. Furthermore, the stabilization of lysozyme led to some increases in ΔH_m of over 270% (from 12.98 J/g to 36.89J/g), whereas the corresponding increase in the intensity of FTIR was only about half of this (ca. 134%). Thus, DSC may be a more sensitive tool relative to FTIR for determining the extent of structural similarity to the native protein.

The Tm values of the spray-dried lysozyme, which increased with increasing sucrose content, were different to previously reported data (18). The earlier study showed that any increase in sucrose content actually depressed the Tms of freeze-dried lysozyme. Such apparently contradictory results may be mainly attributable to differences in the moisture con-

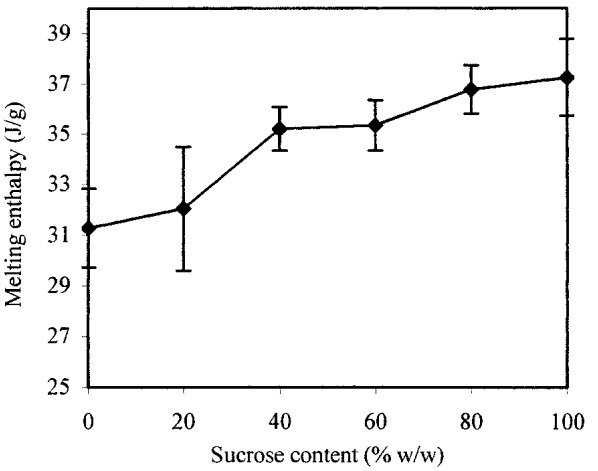

Fig. 7. Relationship between sucrose content (% w/w) in an excipient mixture of trehalose and sucrose (10 mg/ml in total) and the melting enthalpy determined by DSC of spray-dried lysozyme (5 mg/ml) (mean ± SD, n = 3).

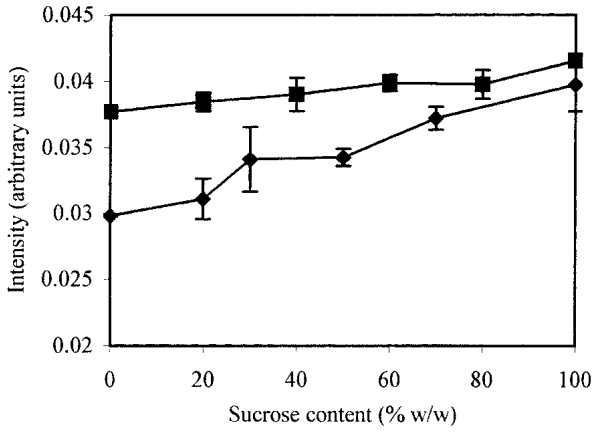

Fig. 6. Relationship between the intensity of α-helix band obtained from the amide I region of the FTIR spectra of spray dried lysozyme (5 mg/ml) and sucrose content, (◆): in formulations containing a mixture of sucrose and dextran (5 mg/ml in total); (■): in formulations containing a mixture of sucrose and trehalose (10 mg/ml in total), (mean ± SD, n = 3).

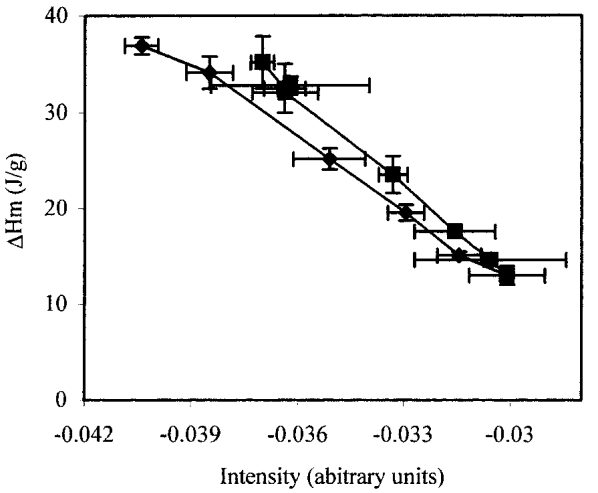

Fig. 8. The relationship between the intensity of α-helix band in the amide I region obtained by second derivative FTIR and the melting enthalpy determined by DSC, (◆): sucrose ($R^2 = 0.995$), (■): trehalose ($R^2 = 0.998$), (mean ± SD, n = 3).

tent of the final protein products that resulted in the two studies. In this study, it is possible that any effect acting to depress Tm, conferred by sugars *per se*, could have been overcompensated by the rise in Tm that could have resulted as a consequence of the decrease in the moisture content.

Both FTIR and DSC results demonstrated that the addition of sucrose or trehalose led to a concentration-dependent stabilization. Sucrose was found to be more effective in protecting the native structure of lysozyme than trehalose on a weight by weight basis during spray drying. These results are similar to those reported for the stabilization of lysozyme during processing by freeze-drying when sucrose was also found to be more effective than trehalose (11).

Lysozyme is a thermophilic protein having high thermodynamic stability as indicated by the high Tm. In addition, the effect of heat stress on the native structure of lysozyme during spray drying appeared to be relatively small in terms of any changes detected in the intensity of the α-helical band. Therefore, it is reasonable to assume that any stress imposed by high temperature is not primarily responsible for the denaturation of lysozyme structure. Furthermore, Lu and colleagues (22) have reported that the exposure of the enzyme to an air-water interface does not disturb the globular structure of lysozyme or lead to any indication of denaturation. It can therefore reasonably be concluded that the main reason for denaturing the native structure during spray drying results from the stresses attributable to dehydration.

The mechanisms by which proteins are stabilized by excipients during the dehydration include the vitrification and water substitution hypotheses. The latter mechanism involves the formation of hydrogen bonds between the sugar and protein molecules, which are believed to be responsible for the inhibition of the unfolding of the proteins (8,23), whilst the vitrification mechanism depends on the immobilization of protein molecules that accompanies glass formation (24). Pikal (25) proposed that the water substitution hypothesis describes a thermodynamic mechanism that is dependent on the free energy of unfolding, whereas the vitrification hypothesis is purely kinetic in origin and is related to the rate of unfolding.

It has been previously reported that the conformational changes of lysozyme upon dehydration start at 22% w/w of moisture content and are completed by 10% w/w (26–27). When the moisture content of a lysozyme-sugar mixture (e.g. sucrose/trehalose:lysozyme 1:1) was between 22%–10% w/w, the Tg would likely be lower than the value of 19°C–27°C in this study for the sucrose and trehalose formulations containing about 9% moisture. During spray drying, although the product temperature could have been lower than the processing outlet temperature (85°C–90°C), it is still likely to have been far higher than the Tg of protein-excipient mixtures. For example, the Tg of spray-dried sucrose:lysozyme 2:5 was found to be 34.2°C, which was 50°C lower than the processing outlet temperatures and about 20°C lower than the temperatures at the collection jar. Therefore, the stabilizing effects conferred by the excipients cannot be attributed to glass formation. It has been reported that the stabilization of poly-L-lysine when air-dried rapidly could be kinetic in origin (28). However, in this experiment, it was not the case because lysozyme in the absence of excipients underwent apparent denaturation despite the drying rate being far faster than those used by Wolkers and colleagues (28).

As the lysozyme-stabilizing effects of combinations of either trehalose or dextran with sucrose were dependent upon the sucrose content, this also contradicts a kinetic mechanism. Both trehalose and dextran have higher glass transition temperatures than sucrose, and both should be more effective than sucrose in protecting the structure of lysozyme, if a kinetic mechanism dominated.

Conversely, as mentioned earlier, the moisture content results suggested hydrogen-bonding interactions between protein and sugar molecules. In addition, the ΔH_m of spray-dried sucrose-lysozyme formulations were found to be larger than that of native lysozyme in the liquid state when the sugar to enzyme mass ratios were at 1 or higher (Fig. 3). The increase in the magnitude of the ΔH_m of the dried formulations could be partially due to protein and sugar interactions. The value of ΔH_m is related to the change in free energy (ΔG), as shown in Eq. 1:

$$\Delta G = -RT \ln K = \Delta H - T\Delta S \quad (1)$$

Thus, any stabilization of lysozyme conferred by sugar during spray drying might result from an increase in the negative value of the free energy change of denaturation. Therefore, the results obtained tend to support the thermodynamic-based water substitution hypothesis.

The lysozyme-stabilizing profiles of sucrose and trehalose were different, with sucrose being apparently more effective (Fig. 5). Such a difference in effect could be due to the competition between protein-excipient and excipient-excipient interactions (4). As the water content decreases, excipient molecules hydrogen bond with protein molecules and gradually approach the surface of the molecules. However, there is also a propensity for the excipient molecules to self-associate and if such interactions are prevalent then the excipient molecules are unable to approach the surface of the protein molecules and form protein-excipient hydrogen bonds. In the sugar to lysozyme mass ratio range from 1–10, the stabilizing effects of trehalose did not lead to an enhanced preservation of lysozyme structure. Such a finding could be attributable to the preferential exclusion effects (4,6,29), which results in clusters of sugar molecules being formed in solution when the solutes are concentrated. The preferential exclusion effects are excipient-specific and concentration-dependent and trehalose has been reported to have a larger such effect than sucrose (6). As a result, trehalose at a high concentration cannot increase the stabilizing capacity, and thus it was found to be significantly less effective than sucrose at the same concentration. Similarly, preferential exclusion effects have previously explained the destabilization of spray-dried trypsinogen at a high sucrose concentration (4).

CONCLUSION

Dehydration during spray drying was the main stress responsible for the denaturation of the native structure of lysozyme and the stabilization conferred by excipients during dehydration is brought about primarily by these excipients substituting for water molecules on the surface of protein. In addition, a finding of these studies is that the melting enthalpy of proteins obtained by DSC could prove to be a useful tool for studying the stabilization of proteins and predicting their shelf life.

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