Protective Mechanism of Stabilizing Excipients against Dehydration in the Freeze-Drying of Proteins

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Purpose. To investigate the influence of type and amount of excipient on the preservation of the native structure and the biologic activity of freeze-dried lysozyme and catalase.

Methods. The secondary structure of protein in the dried form and in aqueous solution was obtained using second derivative infrared spectroscopy and circular dichroism spectra respectively whilst the activity was determined using bioassay.

Results. Small molecular excipients (glycerol, sorbitol, 1,6-anhydroglucose, sucrose, and trehalose) were found to stabilize the activity and/or the native structure of freeze-dried lysozyme and catalase, despite the processing temperatures being above Tg′ of excipient-protein mixtures. The preservation of catalase activity required excipient to be present at a lower excipient to enzyme mass ratio than that necessary to preserve native structure in the dried form. Combining dextran with sucrose synergistically protected the native structure of catalase but preserved the activity in an additive manner.

Conclusion. The results indicate that the stabilization of catalase and lysozyme by excipients during dehydration was mainly due to water substitution rather than the formation of glass; the latter appearing not to be a prerequisite during freeze-drying.

KEY WORDS: freeze-drying; lysozyme; catalase; protein stability; protein conformation.

INTRODUCTION

Freeze-drying, typically termed lyophilization, is widely used commercially to prepare dry protein and peptide formulations so that they can retain satisfactory pharmacological activity during long-term storage. These techniques are necessary as proteins and peptides are generally susceptible to chemical degradation and physical instability in aqueous solutions (1,2). However, the process of freeze-drying itself can impose stresses on protein and peptide molecules that can subsequently lead to significant changes in conformation in the absence of stabilizing excipients (1,3). Consequently, to minimize the perturbation of protein native structure during lyophilization, it is often vital that non-specific stabilizers such as sugars are incorporated within the protein formulation and that the processing conditions are optimized (4). In general, excipients such as sugars protect proteins from denaturation as a function of concentration during freeze-drying (5). In addition, the amount of excipient present in the resultant formulations can markedly affect the long-term stability of the proteins because the latter is dictated by both the preservation of the protein native structure during processing and the physical properties of the resultant product, including for example factors such as the glass transition temperature (6–7). In other words, insufficient excipient content may not render a full stabilization of protein structure whereas the inclusion of excess excipient might decrease the physical stability of the formulations. The selection of the amount of excipients in many previous studies and even commercial formulations has not been necessarily optimized, but is often done by using iso-osmotic concentrations (8). However, the amount of excipient(s) required to achieve stabilization during processing and provide stability during in-storage of a lyophilized antibody has been reported to be far less than an iso-osmotic concentration (8). Practically, it may be desirable to establish the dependence of the stabilization of protein native structure in the dried form and the rehydrated solution as a function of the excipient concentration used during freeze-drying so that the minimum amount of stabilizer(s) to achieve the shelf-life requirement can be established. In addition, strategies such as the inclusion of higher Tg components (e.g. polymers such as dextran) to a protein formulation have also been recently used with a view to improving the physical stability of the formulation (6). However, the potential effect of polymers on the stabilizing capacity of small molecular weight excipients needs to be further investigated.

The mechanisms by which excipients effect the stabilization of proteins during dehydration are controversial, although the preferential exclusion mechanism (9) has been widely accepted to explain the preservation of the native structure when protein solutions are frozen (7,10). Two non-exclusive mechanisms have been proposed to account for the stabilization of proteins conferred by excipients during the dehydration stage of the freeze-drying procedure; these involve vitrification (glass formation) and water substitution. The latter mechanism involves the formation of hydrogen bonds between the sugar and protein molecules that are believed to be responsible for the inhibition of the unfolding of the proteins (1,11), whereas the vitrification mechanism depends on the immobilization of protein molecules accompanied by glass formation (12). Pikal (13) 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. However, there is no mechanism to account completely for the stabilization proteins during dehydration (7,10,13).

The aim of this study is to investigate the influence of the type and amount of excipient on the stabilization of both the biologic activity and the native structure of two model proteins, lysozyme and catalase. The excipients that are being utilized include small molecular additives such as glycerol, sorbitol, 1,6-anhydro-β-D-glucose, sucrose, trehalose, and a large molecular weight species, namely dextran. The properties of the small molecular weight excipients vary in terms of glass transition temperature and hydroxyl groups on a weight basis. For example, glycerol has the lowest Tg but the most number of hydroxyl groups, 1,6-anhydro-β-D-glucose both a

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low Tg and a small number of hydroxyl groups whilst dextran contains the highest Tg but the least number of hydroxyl groups. Further aims were to study the effects of dextran on the capacity of sucrose to stabilize the same proteins during dehydration and to attempt to elucidate the mechanism by which proteins are stabilized during dehydration.

MATERIALS AND METHODS

The buffer phosphate salts (ACS reagent grade), glycerol, sorbitol, 1,6-anhydro-β-D-glucose, sucrose, trehalose, dextran (Mw ~70,000), catalase (2× crystallized, suspension in water containing 0.1% w/w thymol, Lot 97H7028), lysozyme (3× crystallized, dialyzed, and lyophilized, Lot 57H7045) and *Micrococcus lysodeikticus* (Lot 39H8615) were purchased from Sigma–Aldrich Co., UK. Before use, the catalase suspension was centrifuged at 2,000 *g* for 5 min and the supernatant, containing thymol (used as preservative) was removed. The residue was washed using a suspension volume of phosphate buffer (5 mM, pH 7.0) and recentrifuged. The fresh residue of catalase crystals was added to potassium phosphate buffer (5 mM, pH 7.0) and incubated at 32°C for 2 h to dissolve. The catalase solution was filtered using a polyvinylidene difluoride (PVDF) syringe filter (0.2 μm, Whiteman, UK) and the concentration was determined using UV absorbance at 405 nm ($E^{1\%}_{405}$ = 13.5, obtained from the product batch information).

Freeze-Drying Procedures

Lysozyme solutions were prepared using sodium phosphate buffer (10 mM, pH 6.3), whereas catalase solutions were prepared using potassium phosphate buffer (5 mM, pH 7.0). A lysozyme or catalase 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 in a freezer (–80°C) and then transferred to the freeze-dryer (Advantage Benchtop Freeze-dryer XL, VirTis, UK) shelf and processed according to an identical two steps 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). In certain freeze-drying runs, samples were prepared by using a single step procedure, in which both primary drying and secondary drying were maintained at 25°C and 20 Pa for 6 h, or using the above two-step cycle but varying the time of secondary drying in order to achieve samples with different moisture content.

Residue Moisture Analysis

The moisture content was determined using a TGA 2050 thermogravimetric analyzer (TA Instruments, UK). Samples (2–10 mg) of formulation were placed in an open aluminum pan and 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.

DSC Analysis

The Tg′ (glass transition temperature of the freeze concentrates) and Tg (glass transition temperature) were determined using a model 2920 modulated DSC (TA Instruments, UK) that was calibrated with indium prior to analysis. For the measurement of Tg′, approximately 40 μl of sample solution was placed in an aluminum pan that was hermetically sealed and then equilibrated at –60°C in the sample compartment. Data were collected during heating at a rate of 2°C/min between –60 and 5°C. For the measurement of Tg, approximately 3–5 mg of solid sample was hermetically sealed in an aluminum pan, and data were collected between -30°C and 200°C at a heating rate of 10°C/min. All Tg and Tg′ were reported as the midpoint of the transition.

Assays of Enzyme Activity

Lysozyme was assayed as previously reported (14). The activity of catalase was measured by a published method (15), in which the first-order degradation of hydrogen peroxide was monitored by measuring a decrease in absorbance at 240 nm as a function of time. Briefly, 100 μl of catalase solution, the concentration of which was between 0.4–1.2 μg/ml, was transferred to 2.9 ml of substrate (H_2O_2 solution) contained in a silica cuvette. The A_{240} of the substrate was always initially between 0.520–0.550 and the time required for A_{240} to decrease from 0.450–0.400 was recorded.

Circular Dichroism (CD) Spectroscopy

CD spectra of catalase (approximately 0.13 mg/ml) were measured using a Jasco J-600 spectropolarimeter. Samples were placed in 0.5-mm path length quartz cells and the CD spectra measured across the wavelength range of 185–260 nm at 0.2 nm intervals. Spectra were background corrected and converted to residue ellipticity (deg cm^2/mmol). The secondary structure estimation was based upon the Principle Component Regression method (16).

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra were measured utilizing a Perkin Elmer 1600 series FTIR, and analyzed using PE-GRAMS/32 1600 software as described previously (17). 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 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 (1,600–1,710 cm^{-1}) were analyzed. The baseline of the spectrum in the amide I region was leveled and zeroed, then the spectrum of the sample was normalized for area in the region and the intensity of the α-helical band recorded.

RESULTS

Effects of Additives on the Secondary Structure and Relative Activity of Freeze-Dried Proteins after Rehydration

The product temperatures during freeze-drying were monitored using thermocouples that were placed in the bot-

tom of vials. The product temperature during the main drying of a two-step procedure was found to be between –36°C and –42°C. Whereas the product temperature during the primary drying of a single-step cycle appeared to be a function of time, with temperature varying from –24 to –32°C in the first 1–2 h and increasing gradually to 25°C over the following 1–2 h. Although the endpoint of the primary drying was not clear, it was likely to be higher than –10 to –15°C since there appeared to be ~20%–40% of ice remaining in samples within this temperature range.

All freeze-dried formulations except some of those with glycerol showed good cake structures with no sign of collapse. The moisture content of the formulations prepared using a two-step procedure was found to be between 0.4%–1.3% w/w, whereas that of the formulations using a-one-step cycle was between 2.0%–3.8%. The recovered activity of the freeze-dried proteins was determined immediately after rehydration. The activity of freeze-dried lysozyme recovered in the absence of excipient was found to be 92.6 ± 5.7% (n = 12), whilst in the presence of any excipient(s) it was higher and no detectable loss was observed (data not shown). Furthermore, there was no significant difference ($p > 0.05$, ANOVA single factor) between the recovered activity of samples of freeze-dried lysozyme with a moisture content ranging between 0.9 and 8.3% w/w (Fig. 1A).

Catalase was found to be much more unstable relative to lysozyme upon freeze-drying. In fact, freeze-dried catalase in the absence of excipient lost between 35%–50% activity and the loss of activity slightly increased with decreasing moisture content from 9.1% to 6.1%, however further decreasing moisture content appeared not to decrease the activity significantly ($p > 0.05$, ANOVA single factor, Fig. 1B).

The biologic activity retained was found to be a function of excipient concentration and to increase with increasing sucrose, trehalose or dextran content (Fig. 2A). There were only small increases in the activity with further increases in the excipient content when the excipient to catalase mass ratio exceeded 1 (Fig. 2A) and such results were similar to previously published data (5). There was no significant difference between sucrose and trehalose in the protection of catalase on a w/w basis from the results of the bioassay ($p > 0.05$, paired student's *t* test) studies. The stabilizing effect conferred by glycerol appeared to be different from the above excipients, the activity recovered was found to increase with increasing the mass ratio up to 0.8, at that point the activity of catalase was fully preserved (Fig. 2A). However, a further increase in the concentration of glycerol was found to lead to a loss of the solid cake structure and a drastic decrease in the enzyme activity due to the formation of insoluble aggregates. When the glycerol to catalase mass ratio added was at 4 or 10,

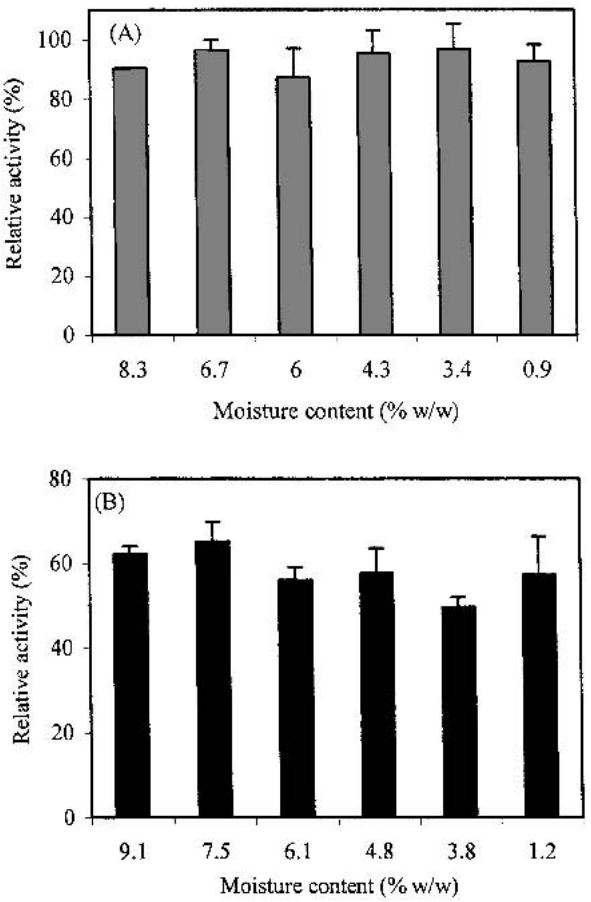

Fig. 1. The effects of moisture content on the recovered activity of freeze-dried enzymes (5 mg/ml) relative to non-freeze-dried enzyme for (A): lysozyme; (B): catalase in the absence of stabilizer (Mean ± SD, n = 3).

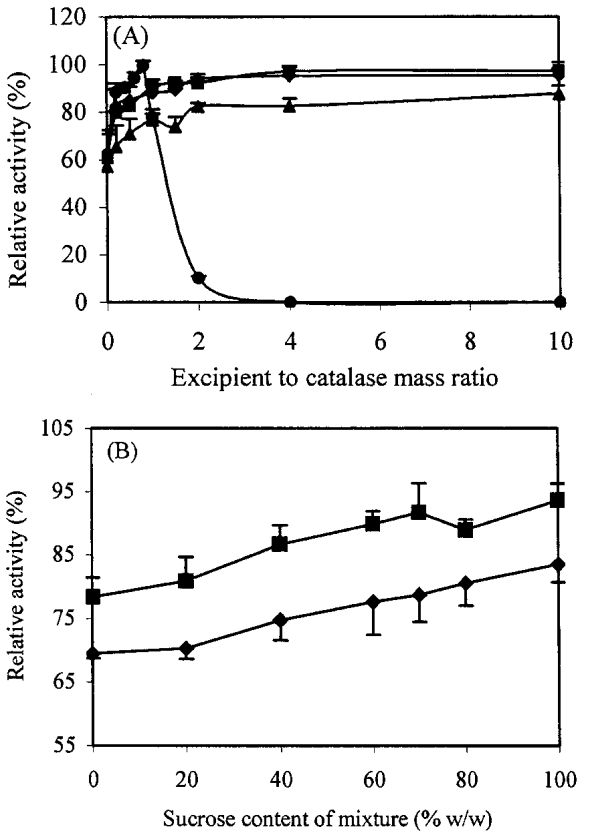

Fig. 2. (A): Relationship between excipient concentration and the activity of freeze-dried catalase relative to untreated catalase (Mean ± SD, n = 3). glycerol (●); sucrose (♦); trehalose (■); dextran (▲). (B): Relationship between sucrose content (% w/w) in the mixture of dextran and sucrose and the recovery activity of freeze-dried catalase. The total excipients at a sugar to enzyme mass ratio of 1.5 (♦); the total excipients sugar to enzyme mass ratio of 10 (■).

catalase solubility appeared minimal and no activity was recovered. In the presence of a combination of sucrose and dextran, the recovered activity of freeze-dried catalase after rehydration was found to correlate linearly with the sucrose content of the mixture (Fig. 2B).

The activity retained by freeze-dried catalase was found to be excipient-dependent and glycerol conferred the least stabilizing capacity amongst the excipients utilized when the excipient to catalase mass ratio was maintained at 1 (Table I). The effects of excipients on the preservation of enzyme activity appeared to be almost independent of the processing procedure (Table I).

Effects of a Single Additive on the Secondary Structure of Freeze-Dried Protein in the Dry State

In the second derivative FTIR spectra of freeze-dried lysozyme and catalase, the bands at 1,624 and 1,641 cm^{-1} were assigned to β-sheet structure, a minimum at 1654–58 cm^{-1} assigned to α-helical structure and bands between 1,665–1,710 cm^{-1} assigned to β-sheet and turn structures (18) (Figs. 3A and 3B). For both lyophilized lysozyme and catalase in the absence of stabilizer, the intensity of the α-helical band from the FTIR spectra was sizably decreased relative to that in the presence of any excipient. In addition it was found that a moisture content between 0.9%–8.3% w/w for lysozyme alone and between 1.2%–9.1% w/w for catalase alone did not significantly influence the intensity of the α-helical bands (data not shown). With lysozyme or catalase formulations containing dextran, trehalose or sucrose at a mass ratio of 10, the intensity of the α-helical band increased, whereas the bands attributable to the β-sheet and turn structures narrowed and decreased in size. Dextran appeared to be less effective than trehalose or sucrose at stabilizing the α-helical structure of either of the two enzymes (Figs. 3A and 3B). The intensity of the α-helical band presented at the amide I region of the second derivative FTIR spectra of both lysozyme and catalase showed excellent repeatability. The range of the relative standard deviation (RSD) of triplicate measurement of the same sample was between 0.3 and 2.64% and the mean RSD was 1.61 ± 0.64% (n = 10). A comparison of the spectra of freeze-dried enzymes with those of the corresponding native protein in aqueous solution indicated that the greater the intensity of the α-helical band in the spectrum of a sample, the more similar the spectra of the sample was to that of and the native protein, as shown in Figs. 3A and 3B. It has been reported that the greater the similarity between the spectrum of a sample and that of the native protein then the greater the amount of the native structure remaining (25). Therefore, in this study, the intensity of the α-helical band was taken as a measure of the amount of native enzyme structure preserved.

The capacity of the excipients to preserve the α-helical structure of catalase as indicated by the intensity in the second derivative FTIR spectra was also found to be a function of concentration (Fig. 4). Sucrose, trehalose and dextran at a mass ratio of 2 were found to produce close to the maximal stabilization of the catalase structure. Further increasing the concentration of excipient from a mass ratio of 2 to 10 did not significantly affect the intensity of the α-helical band of catalase ($p > 0.05$, ANOVA single factor). For the stabilization of lysozyme, the minimal mass ratio for sucrose and trehalose was found to be 4 (data not shown). The results (Fig. 4) showed that there was no significant difference between sucrose and trehalose in the protection of catalase on a w/w basis ($p > 0.05$, student's *t* test). The effects of glycerol on the structure of catalase were different from the above excipients. Although the intensity of the α-helical band appeared to increase with increasing the glycerol content, a new band between 1,695-1,700 cm^{-1} attributable to intermolecular β-sheet was apparent when the glycerol to catalase mass ratio was ≥ 1 (Fig. 3C).

The denaturation of catalase induced by the freeze-drying process in the presence of excipient(s) appeared to be partially or completely reversible upon rehydration, although the reversibility was excipient-dependent. For example, the intensity of the α-helical band of freeze-dried catalase with a sucrose or glycerol to catalase mass ratio of 0.2 in the dry form was found to increase by only 13.4% and 7.8% respectively, relative to lyophilized catalase alone, whereas after rehydration, the activity was found to increase by 36.0% and 42.1% respectively. In addition, glycerol was found to confer a full preservation of activity when the concentration was held at a

Table I. The Activity Recovered and α-Helical Content Obtained from the Amide I Region of the Second Derivative FTIR Spectra of Catalase in Combination with an Equivalent Weight of Excipient (5 mg/ml) Freeze-Dried by a Single Step or Two-Step Procedure and the Glass Transition Temperature (Tg′) of Freeze Concentrated Excipients and Catalase-Excipient (1:1, w/w)

	Freeze-dried by a single step[a] (Mean ± SD, n = 3)		Freeze-dried by two steps[b] (Mean ± SD, n = 3)		With catalase glass transition (Tg′) (°C)	Excipient alone glass transition (Tg′) (°C)
Excipient	Activity recovered (%)	α-helical content (arbitrary units)	Activity recovered (%)	α-helical content (arbitrary units)		
Glycerol	66.9 ± 9.8	0.0174 ± 0.0004	78.2 ± 3.1	0.0175 ± 0.0003	–54.6[c]	–99.9[c]
Sorbitol	91.4 ± 1.3	0.0176 ± 0.0002	90.8 ± 2.4	0.0209 ± 0.0003	–35.2	–48.8
1,6-Anhydro-glucose	92.5 ± 0.6	0.0178 ± 0.0009	92.2 ± 2.8	0.0249 ± 0.0006	ND	–49.8
Sucrose	96.3 ± 2.5	0.0212 ± 0.001	88.1 ± 3.2	0.0206 ± 0.0003	–28.4	–34.5
Trehalose	86.3 ± 0.9	0.0216 ± 0.0006	90.6 ± 3.1	0.0214 ± 0.0008	–27.8	–31.1
Dextran	89.9 ± 4.6	0.0199 ± 0.0008	76.5 ± 4.1	0.0178 ± 0.0005	–16.2	–12.1
No excipient	54.8 ± 7.8	0.0154 ± 0.0004	57.2 ± 9.1	0.0156 ± 0.0003	ND	NA

ND = Not determined; NA = Not applicable.

[a] Both primary and secondary drying at 25°C.

[b] Primary drying at –30°C and secondary drying at 30°C.

[c] The values were obtained by extrapolation using the Gordon–Taylor equation (19).

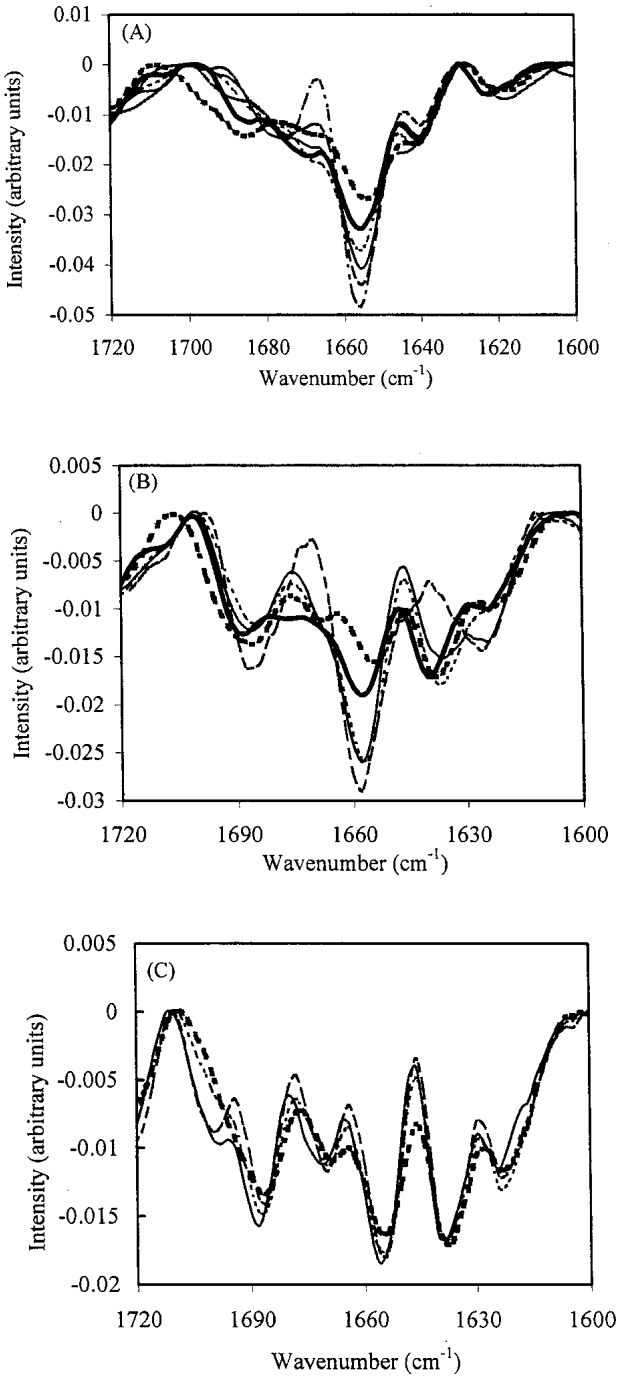

Fig. 3. The second derivative FTIR spectra of freeze-dried enzyme (5 mg/ml) (A) lysozyme to excipient mass ratio 10. (- - -) no excipient; (—) dextran; (– – –) trehalose; (—) sucrose; (— —) glycerol; (- — -) native lysozyme in aqueous solution. (B) catalase to excipient mass ratio 10. (- -) no excipient; (—) dextran; (– – –) trehalose; (——) sucrose(— —) native catalase in aqueous solution. (C) catalase to glycerol mass ratio (––) 0.2; (– – –) 0.6; (— —) 1; (——) 2.

mass ratio of 0.8, whilst its ability to stabilize the native structure in the dry form was found less effective than that of either sucrose or trehalose.

The Tg′ of the excipients was determined by DSC and found to range from –99.9°C to 12.1°C, whereas those of excipients in combination with an equivalent weight of catalase were between –54.6°C and –16.2°C (Table I). The Tg′ of glycerol was obtained by extrapolation by using a correlation between the Tg′ of the mixtures of glycerol and dextran and the content of glycerol based upon the Gordon–Taylor equation (19). The Tg′ values of sorbitol, sucrose, trehalose, and dextran were in agreement with those values reported in a previous study (20). In addition, the Tgs of freeze-dried sucrose- and trehalose-containing formulations were between 60°C–70°C and between 89°C–97°C respectively when the moisture content was between 0.4%–1.3% w/w. However, Tg is very sensitive to the moisture content, for example, the Tgs of catalase in combination with sucrose or trehalose at a sugar to enzyme mass ratio of 1:1 were found to decrease to 19.5°C and 27.9°C respectively at about 9% w/w of moisture content.

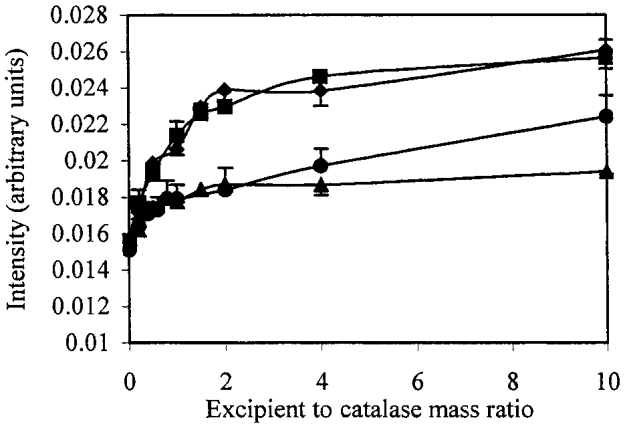

Fig. 4. Relationship between excipient concentration and the intensity of α helical band of freeze-dried catalase (5 mg/ml) obtained from the amide I region of the second derivative FTIR spectra glycerol (●); Sucrose (♦); trehalose (■); dextran (▲). (Mean ± SD, n = 3).

With the exception of dextran, the intensity of the α-helical band of catalase in the presence of an excipient was generally found to increase with increasing Tg′ of catalase-excipient mixtures when the formulations were lyophilized by a single step procedure (Table I). However, the capacity of an excipient to stabilize catalase during freeze-drying using a two steps cycle was independent of the Tg′ of the excipient. The second derivative FTIR spectra showed that 1,6-anhydro-glucose was more effective than any of the other excipients used using the two-step cycle (Table I). However, when a single-step cycle was used, 1,6-anhydro-glucose proved to be amongst the least effective excipients, and was comparable with sorbitol and glycerol at preserving structure.

Effects of Mixtures of Sucrose with Dextran on Secondary Structure of Freeze-Dried Proteins in the Dry State

The mixture of dextran with sucrose appeared to promote synergistic effects on the stabilization of the native structure of catalase (Fig. 5.). When the mixture concentration was at a mass ratio of 1.5 (Fig. 5A), the inclusion of dextran up to 40% w/w preserved the α-helical structure to almost the same extent as sucrose alone at a mass ratio of 1.5 as determined from the second derivative FTIR spectra. In addition, when the total concentration of excipients in the mixture was maintained at a mass ratio of 10 (Fig. 5B), up to 40% w/w dextran showed a similar capacity to preserve the α-helical structure relative to sucrose alone.

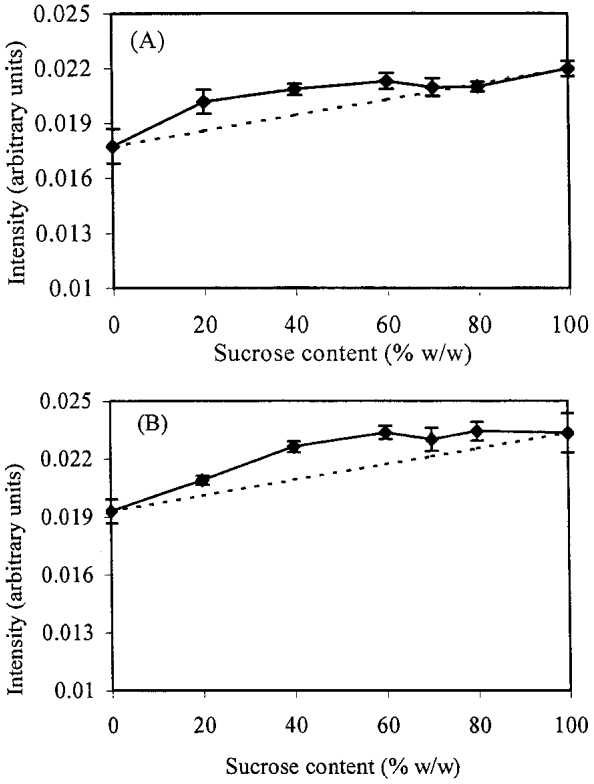

Fig. 5. Relationship between sucrose content (% w/w) in the mixture of dextran and sucrose and the intensity of the α helical band of freeze-dried catalase (5 mg/ml) obtained from the amide I region of second derivative FTIR spectra (Mean ± SD, n = 3). (A) Freeze-dried catalase in the presence of mixtures of dextran and sucrose (at 7.5 mg/ml total excipients); (B) Freeze-dried catalase in the presence of mixtures of dextran and sucrose (50 mg/ml total excipients). Measured value (——); predicted value according to the weighted addition of two excipients (– – –). (Mean ± SD, n = 3).

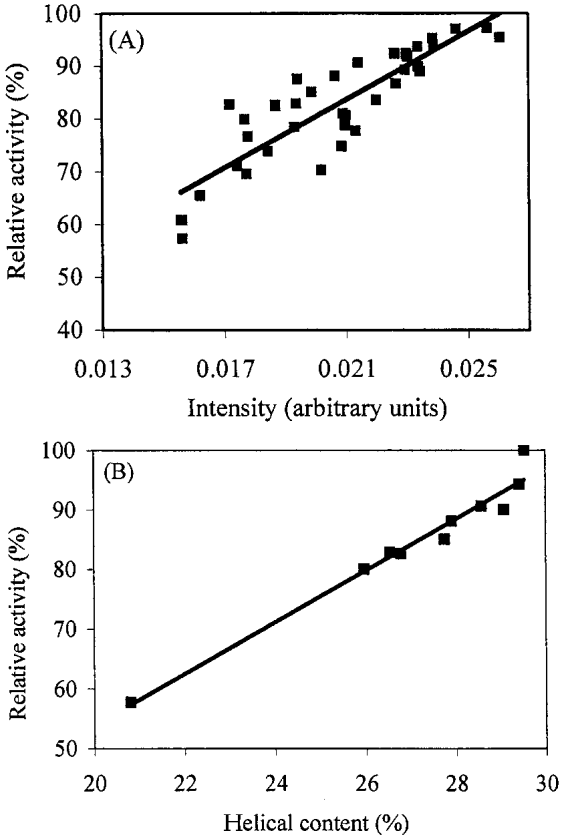

Fig. 6. (A) Correlation between the relative activity and the intensity of α-helical band obtained from the amide I region of the second derivative FTIR spectra of freeze-dried catalase in the presence/absence of sucrose, trehalose or dextran (R^2 = 0.764, n = 36); (B) Correlation between the relative activity and α-helical content estimated by the Principle Component Regression method of freeze-dried catalase after rehydration (R^2 = 0.960, n = 10).

Correlation of Biologic Activity with Secondary Structure of Freeze-Dried Protein in the Dried State and in Aqueous Solution after Rehydration

Although glycerol, 1,6-anhydro-glucose and sorbitol were found to be less effective in preserving the native structure during one-step freeze-drying, the retained biologic activity was comparable to that obtained after stabilization with sucrose or trehalose. In general, freeze-dried catalase stabilized by small excipients such as glycerol, and sorbitol had a better reversibility in terms of recovered activity than sucrose, trehalose, or dextran. Overall there was a weak correlation (Fig. 6A, R^2 = 0.764) between the recovered activity and the intensity of the α-helical band in the dried form. For example, the combination of sucrose and dextran appeared to promote a synergistic effect on the secondary structure in the dried form (Fig. 5) but not on the activity retained (Fig. 2B). Nonetheless, for freeze-dried catalase stabilized by each individual excipient except glycerol, the correlation of the FTIR data with activity was found to be fairly good ($R^2 > 0.900$, data not shown).

Circular dichroism spectroscopy was used to examine the protein secondary structure after rehydration. The α-helical content of freeze-dried catalase was estimated by using previous methods (16). The reduction of the α-helical content without excipient(s) determined by CD spectrum was apparent after rehydration compared to the native catalase spectrum. Figure 6B shows that there was a linear correlation (R^2 = 0.960) between the α-helical content and the activity recovered after freeze-dried samples were rehydrated.

DISCUSSION

Changes in the secondary structure of proteins induced by lyophilization have been monitored previously by measuring the differences in second derivative IR spectra (3,21). When the conformation of α-helical containing proteins is disturbed, the intensity of the α-helical band would be expected to decrease whilst that of the β-sheet and turn bands might increase (11). Therefore, the changes in the intensity of the FTIR spectrum corresponding to the α-helix were utilized to determine the changes in the secondary structure of model proteins on lyophilization. The native secondary structure of catalase determined in the dry state appeared to have a weaker correlation with the recovered activity after rehydration than that between the degree of retained native structure in solution as determined by CD and activity. However, it has been proposed that the storage stability of protein formulations requires the native structure to be preserved in the dried form (7). Partly denatured protein, which can be refolded

upon rehydration, has higher reactivity and can undergo aggregation and/or chemical degradation, which as a function of time might lead to the native structure not being re-attained after rehydration and hence this would lead to a concomitant loss of biologic activity. Thus, simply achieving a full preservation of protein activity over the short term rather than ensuring the stabilization of the native structure, may not predict the shelf life of the protein formulations. The stabilization of catalase structure in the dried form required a larger amount of excipients for maximum effect than that required for preserving activity. For example, a minimum sugar to enzyme mass ratio of 2 for sucrose and trehalose was required for the stabilization of the secondary structure (Fig. 4) in comparison to a mass ratio of 1 for the two disaccharides to afford a similar effect on the activity of catalase (Fig. 2A).

Vitrification and water substitution mechanisms have both been proposed as explanations for the protective effects of carbohydrates during dehydration (1,11–13). In this experiment, the Tg′ of the glycerol and lysozyme formulations were well below –50°C when the glycerol to lysozyme mass ratio exceeded 1. Therefore, the stabilizing effects of glycerol on the native structure of lysozyme were not attributable to the formation of a glass. In the case of the stabilization of catalase, sucrose and trehalose were found to confer a comparable capacity to preserve the secondary structure at different drying temperatures. Any changes in the secondary structure of lysozyme and catalase resulting from dehydration as indicated by FTIR data were found to be complete when the water content of the freeze-dried powders decreased to 8.3% w/w and 9.1% w/w respectively. Further removal of water from the enzymes did not lead to decreases in the α-helical content (data not shown). Such a result concurs with previous reports that indicated the conformational changes of lysozyme upon dehydration start at 22% w/w of moisture content and are complete by 10% w/w (22,23). It was unlikely that a glass was formed in a protein formulation with the moisture content being 9% w/w or more when the shelf temperature was held at 25°C, although further dehydration had resulted in the formation of glass in the final product. Indeed, at about 9% w/w of moisture content, the Tg of catalase in combination with an equivalent amount of sucrose was less than 20°C. On the basis of these results, it is proposed that glass formation was not a prerequisite for the stabilization of the enzymes against dehydration during freeze-drying.

These results tend to support the water substitution hypothesis, which describes that the effects of sugars in protecting proteins against dehydration are mainly attributable to hydrogen bonding between sugar and protein molecules (11). The capacity of a carbohydrate to protect lysozyme/catalase during freeze-drying had nothing to do with the glass transition temperatures of the additives but correlated with the ability of excipient to form hydrogen-bonds with proteins, similar to the results reported by Tanaka and colleagues (5). For example, dextran, which has a poor hydrogen bonding ability conferred less stabilization relative to other excipients except glycerol. In addition, the stabilizing effects on the native structure and the activity of enzyme were found to be concentration-dependent. A minimum excipient-enzyme mass ratio was required to achieve close to maximal stabilization and further increases in the mass ratio only lead to a small gain in protective ability. The minimum mass ratio was previously attributed to a monolayer of excipient forming on the protein surface by excipients (5,24). In the current study, there appeared to be a difference between the excipient to protein mass ratio required to achieve maximum stabilization of the native structure for the different proteins used. Such a finding could result from the difference between surface to volume ratios of proteins due to the hydration of proteins correlating more closely with the amount of protein surface than its volume or mass (22). As a result, catalase, as a larger molecular weight protein, contains a less accessible surface area than lysozyme if the weights are the same. Consequently, the mass ratio of excipient to protein required to replace water during dehydration will be larger for lysozyme than catalase.

The stabilization of the secondary structure of catalase appeared to correlate with the Tg′s of the formulation or excipient (Table I). Low Tg′ excipients including sorbitol and 1,6-anhydroglucose were found to confer a limited stabilization when freeze-drying was effected using a one-step procedure, whereas glycerol rendered a limited stabilization when catalase was freeze-dried using both one- and two-step procedures. Such stabilization might be a consequence of the molecular mobility within the excipient-protein mixtures. For glycerol-, sorbitol- and 1,6-anhydroglucose-catalase mixtures, the product temperature is far higher than the Tg′ of the excipient-protein mixture during the main drying and this might lead to microcollapse of the cake and/or phase separation in the system. In addition, a high mobility might also allow an increase in intermolecular contacts of catalase, leading to possible aggregation and this did appear to occur, on the basis of the FTIR measurement in the case of the glycerol-enzyme mixture. Nonetheless, although the product temperature of sucrose- and trehalose-catalase mixtures using a single-step procedure was also higher than the Tg′, the stabilizing effects in the single-step process and the glassy matrix of the final products was found to be comparable to that using a two-step cycle. Therefore, freeze-drying may be able to be effected at a higher temperature than the Tg′ of protein formulations without inhibiting the stabilizing effects of excipients and the formation of a glassy matrix in the final product, providing the potential phase separation between protein and stabilizers can be inhibited.

CONCLUSION

Small molecular weight excipients (e.g. glycerol) were found using appropriate freeze-drying procedures to stabilize the native structure and activity of lysozyme and catalase as a function of concentration. The stabilizing effects conferred by increasing concentrations of excipients used were protein-specific, being different for lysozyme and catalase. Combining dextran with sucrose synergistically improved the protection of the native structure of catalase in the dry form, but preserved the retained activity of catalase only in an additive manner. The results suggested that the stabilization of catalase was effected by both water substitution and restriction of the mobility within the formulation, if not the formation of glass, although water substitution may have been sufficient to maintain the integrity of lysozyme structure. The requirements of glass formation and a processing temperature below Tg′ appear not to be prerequisites during freeze-drying process, although the final product should remain as a glassy state.

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