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THERMAL AND FTIR INVESTIGATION OF FREEZE-DRIED PROTEIN–EXCIPIENT MIXTURES

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The effect of excipients on the secondary structure of lyophilized proteins was studied through second-derivative Fourier transform infrared (FTIR) spectroscopic analysis. The glass transition temperature (T_g), denaturation temperature (T_d) and moisture content were determined by differential scanning calorimetry (DSC) and thermogravimetry (TG). T_g, T_d and the preservation of protein secondary structure were found to be dependent upon the type and amount of the excipient included in the formulation. Meanwhile, the lyophilized proteins easily adsorbed amounts of moisture during storage to reduce their T_gs and stability.

Keywords: *excipients, freeze-drying, proteins, secondary structure, stabilization*

Introduction

Due to the chemical and physical instabilities of many therapeutic proteins in liquid formulations [1], freeze-drying has commonly been used to prepare dry protein formulations so that they can have satisfactory stability and shelf life [2]. However, many proteins lose their biological activity during the freeze-drying process because of irreversible structural change or resulting aggregation [3–5]. To stabilize the native structures of proteins, protective excipients are generally included as components of the final formulations [6, 7].

Apart from the preservation of protein's native structure during freeze-drying, glass transition temperature (T_g) of the amorphous phase containing the protein has been found to be important in determining the stability of the lyophilized protein formulation during long-term storage [8, 9]. In general, the long-term stability of a protein formulation requires T_g to be well above storage temperature [10, 11]. As an effective glass-forming excipient in a formulation, it is desirable that the excipient has a number of properties, such as non-reactivity, good glass-forming, strong resistance to crystallization, high T_g, etc. [12].

There has been numerous reports on the ability of excipients to stabilize proteins during lyophilization and storage in dried solids [13, 14]. Various excipients possess different physicochemical properties and corresponding molecular interactions with proteins in frozen solutions and freeze-dried solids [15], which thus have different protein-stabilizing effects during freezing, drying and storage processes [16]. Moreover, the stabilizing effects conferred by excipients are found to be a function of the concentration of the excipients, which are generally protein-specific. Unfortunately the selection of the amount of excipients in many previous studies and even commercial formulations has not been necessarily optimized yet [17].

To date, the exact mechanisms of the effect of excipients on the preservation of protein native structure in dried form and on the long-term stability of protein formulations are not quite clear [18]. Two non-exclusive mechanisms, vitrification (glass formation) and direct interactions (water substitution) [19–23], have been proposed to account for the stabilization of proteins conferred by excipients. However, there is no mechanism to account completely for the stabilization of proteins conferred by excipients during lyophilization [24–26].

The main objective of this study was to investigate the influence of the type and amount of excipient on the secondary structure, T_g, denaturation temperature (T_d) and residual moisture content of the freeze-dried protein–excipient mixture. Four model proteins were lysozyme, hyaluronidase, chymotrypsinogen A and thermolysin. The utilized excipients included small molecular additives such as sucrose, trehalose, etc., and large molecular mass species, like dextran. The secondary structure of protein in the dried form was obtained using second-derivative Fourier transform infrared spectroscopy (FTIR). T_g, T_d and moisture content were determined by differential scanning calorimetry (DSC) and thermogravimetry (TG).

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Experimental

Materials

Six times crystallized lysozyme was purchased from Seikagaku Kogyo (Tokyo, Japan). Chymotrypsinogen A, hyaluronidase and thermolysin were purchased from Sigma-Aldrich (St. Louis, MO). All proteins were used without further purification. Sucrose, lactose, trehalose, fucose, mannitol, xylitol, lactitol, and dextran (Molecular mass=$5\cdot10^6$–$4\cdot10^7$) were of the highest grade available.

Protein samples for freeze-drying were prepared with a single excipient at various protein-to-excipient mass ratios. Protein solution was mixed with an equal volume of buffer containing double the final concentration of the excipient. The final concentration and pH of the samples for freeze-drying are shown in Table 1.

Samples were frozen in liquid nitrogen for 1 h and then transferred to the shelf of a benchtop freeze-dryer (Alpha 1-4, Martin Christ, Germany). The shelf temperature of the freeze-dryer was maintained at 20°C for 48–72 h, and the chamber pressure was maintained constant at 0.3 mbar. After freeze-drying, the powders were analyzed immediately and then stored in a vacuum desiccator.

Methods

T_g and T_d were determined using a differential scanning calorimeter with an ultra sensitivity (DSC III, Setaram SA, France). A temperature range from –10 to 120°C was employed, and heating rate was 1 K min^{-1}. All T_g and T_d were reported as the midpoint of the transition.

TG was performed with a model 2960 modulated DSC-TG (TA Instruments, UK) in the following experimental conditions: approximately 10 mg powders, platinum pans, 5 K min^{-1} of heating rate, 20 mL min^{-1} of nitrogen flux. Residual moisture content of freeze-dried powders was determined gravimetrically at 105°C.

The secondary structure of proteins was analyzed with a FTIR system (Excalibur Series FTS-3000MX spectrometer with Merlin software, Bio-Rad Co., Cambridge, MA). For each spectrum, 64 scans were collected with a 4 cm^{-1} resolution, and subsequently a 64-scan background was immediately recorded. The vibrational spectra were corrected for the background and the presence of excipient when appropriate [4, 5]. Each sample was measured in duplicate. The experimentally obtained spectra were firstly smoothed with a nine-point smoothing function to remove any possible noise, and then the normalized second-derivative spectra were obtained.

Results and discussion

Effect of excipient on residual moisture content

Thermogravimetric trace for determining the residual moisture content of samples is schematically shown in Fig. 1. Experimental moisture content of freeze-dried protein-sugar mixtures before storage is summarized in Table 2. When sugar was added, the residual moisture content in freeze-dried protein–excipient mixture was decreased, which was dependent upon the type and amount of both excipient and protein included. The effect of sugar on the decrease in the moisture content can be partially attributed to the increased hydrogen bonding that should occur between sugar and protein as the sugar concentration is increased [27].

The moisture content of a protein-containing powder often controls long-term protein stability, both physically and chemically. High moisture content generally leads to rapid deterioration rate of the protein [28]. In fact a lyophilized protein may easily adsorb sufficient amounts of moisture during storage to

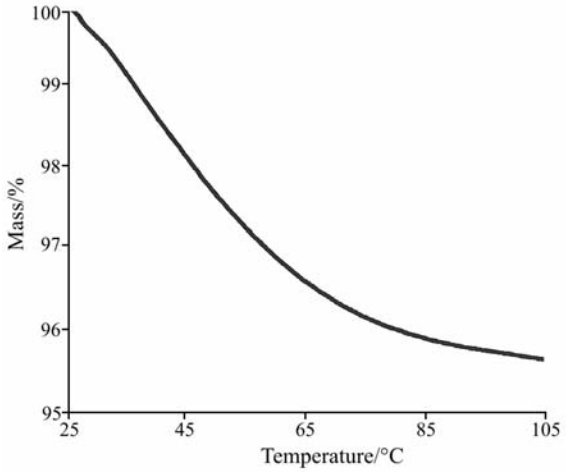

Fig. 1 Thermogravimetric trace of freeze-dried samples for the determination of residual moisture content

Table 1 The concentration and pH of protein samples for freeze-drying

Protein	Buffer	Concentration/mg mL^{-1}
Lysozyme	0.1 M NaAc, pH 4.8	20
Hyaluronidase	5 mM NaAc, pH 5.0	5
Chymotrypsinogen A	10 mM Na citrate, pH 5.25	10
Thermolysin	0.05 M tris-HCl, pH 8.27	6

Table 2 The residual moisture of freeze-dried protein–sugar mixtures before storage

Protein	Excipient	Protein-to-excipient/mass ratio	Residual moisture/g water (100 g solid)$^{-1}$
Lysozyme	none	1:0	4.83
	sucrose	1:3	3.15
	sucrose	1:4	2.99
	trehalose	1:3	3.27
	trehalose	1:4	3.21
Hyaluronidase	none	1:0	3.94
	sucrose	5:2	3.89
	sucrose	1:1	3.45
	lactose	5:2	2.99
	lactose	1:1	2.48
	lactose	1:2	2.05
	lactose	1:4	1.85
Chymotrypsinogen A	none	1:0	4.52
	sucrose	2:3	3.21
	sucrose	2:5	3.14
	sucrose	1:4	2.69
	lactose	2:3	3.98
	lactose	1:3	3.20
	lactose	1:4	2.72
	trehalose	2:5	3.09
	trehalose	1:4	2.56
Thermolysin	none	1:0	4.91
	sucrose	1:1	4.80
	sucrose	2:3	4.70
	trehalose	5:2	3.92
	trehalose	2:3	2.47
	trehalose	1:2	2.27

Table 3 Comparison of the residual moisture of freeze-dried powders of hyaluronidase–sugar

Excipient	Hyaluronidase-to-excipient/ mass ratio	Residual moisture before storage/ g water (100 g solid)$^{-1}$	Residual moisture after storage/ g water (100 g solid)$^{-1}$
None	1:0	3.94	4.61
Sucrose	5:2	3.89	4.17
Lactose	5:2	2.99	5.00

reduce its stability [29]. As shown in Table 3, the moisture content in freeze-dried hyaluronidase-sugar mixtures increased markedly after storage in the desiccator for 1 month. The change of moisture content of a lyophilized protein formulation during storage is mainly due to a variety of factors, such as stopper moisture release and leakage, crystallization of an amorphous excipient, or moisture release from an excipient hydrate [30].

Effect of excipient on T_g

Typical DSC curves of freeze-dried protein–excipient mixtures are shown in Figs 2–4. Experimentally obtained T_gs are listed in Table 4. For proteins of lysozyme, hyaluronidase, chymotrypsinogen A and thermolysin studied in this work, excipients such as lactose (high proportion), trehalose, and dextran (>5kk) led to higher T_g, whereas the addition of sucrose, mannitol, fucose or xylitol resulted in lower T_g. The effect of excipients on T_g generally appeared dependent on the type and amount of excipients.

The adsorption of moisture to a glassy formulation can result in a decrease in the T_g of the formulation. Depression of T_g by water may reach 10°C or more for each percent of moisture retained, especially at low-level moisture contents [31]. The glass transition temperatures of freeze-dried mixtures of lysozyme–xylitol and hyaluronidase–trehalose after storage were lower than those before storage by 10.9 and 12.5°C, respectively, which can be attributed to the increase of moisture content during storage. Apart from decreasing the T_g of a lyophilized protein, the moisture adsorbed during storage may drive crystallization of the stabilizing excipient [32], accelerate the instability of the protein [33, 34], and lead to possible product collapse [35]. Increasing moisture content of lyophilized proteins can decrease the stability of the proteins, which has been demonstrated by use of denaturation temperature described thereinafter.

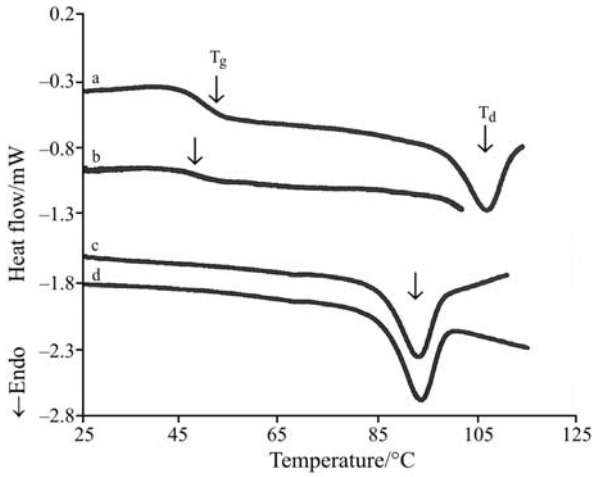

Fig. 2 DSC curves of freeze-dried lysozyme-containing powders: a – lysozyme:lactitol 1:1 before storage, b – lysozyme:lactose 1:1 before storage, c – lysozyme:lactose 1:1 after storage, d – lysozyme:lactitol 1:1 after storage

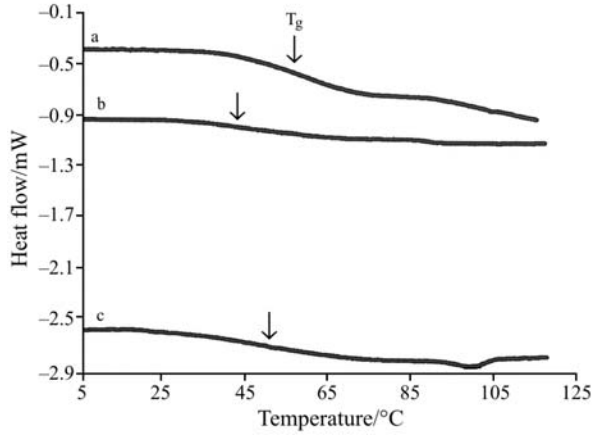

Fig. 3 DSC curves of freeze-dried hyaluronidase-containing and thermolysin-containing powders: a – hyaluronidase alone before storage, b – hyaluronidase:sucrose 5:2 before storage, c – thermolysin:trehalose 5:2 before storage

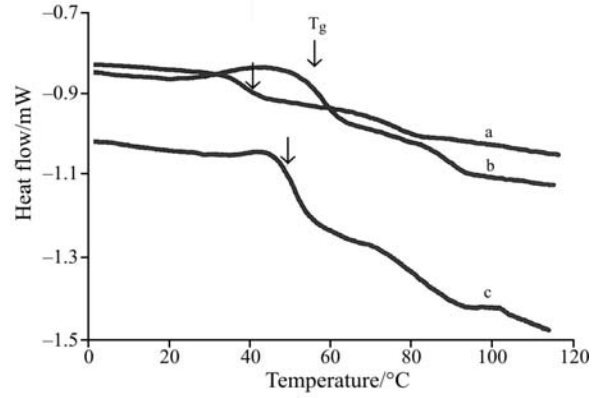

Fig. 4 DSC curves of freeze-dried chymotrypsinogen A-containing powders: a – chymotrypsinogen A:sucrose 1:2 before storage, b – chymotrypsinogen A:lactose 1:2 before storage, c – chymotrypsinogen A:trehalose 1:2 before storage

Effect of excipient on T_d

As shown in Fig. 2, the endotherm in DSC curve corresponds to protein denaturation (melting, or second dehydration), and the temperature at which the denaturation occurred is designated T_d. T_d has generally been utilized to evaluate the thermodynamic stability of proteins, the higher the T_d, the thermodynamically more stable the protein [36]. T_d of lysozyme in solution measured using DSC was 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 [37].

T_ds of lysozyme in freeze-dried lysozyme–excipient mixtures before storage ranged from 84–112°C (Table 5), which were markedly higher than those of lysozyme in solutions. In addition, the effect of excipients on the T_ds appeared to be concentration dependent. The denaturation temperature of lysozyme generally increased with increasing content of sucrose, trehalose, xylitol or lactitol, which was in accordance with the previous studies of spray-dried lysozyme [18]. The apparent difference in the denaturation temperatures of various formulations may be mainly attributable to difference in the moisture content of the final protein products, and the interactions between protein and excipient molecules.

After storage for 1 month, the T_ds significantly decreased (Table 5). In particular, T_d of lysozyme alone in freeze-dried state after storage was 73.8°C, which was quite near to the T_d of lysozyme in solution [37]. The decrease in stability of lysozyme in solid-state can be attributed to the significant increase of moisture content during storage.

Effect of excipient on protein secondary structure in solid-state

Thermal analysis (TA) can study the thermal stability, molecular interaction and compatibility between drug(s) and excipient(s) upon preformulation [38–40]. Moreover, as for biopharmaceuticals, information on the protein structures in freeze-dried solids is useful in designing an appropriate formulation including various excipients that protect proteins through different mechanisms [41]. IR (or FTIR) is probably the most extensively used technique today for studying structural changes in proteins upon freeze-drying [42, 43]. Figure 5 shows the second-derivative FTIR spectra in the amide I spectral region of lysozyme in solid-state. The large peaks at 1654–1656, 1638–1641 and 1675–1705 cm^{-1} denoted α-helix, β-sheet and turn structures, respectively [18]. The reduced intensity of α-helix band in crystals indicated that crystallization resulted in less preservation of α-helical structure than lyophilization. Besides, the intensity of β-sheet band after lyophilization without excipient was significantly

Table 4 Glass transition temperature (T_g) of protein–excipient powders before storage

Protein	Excipient	Protein-to-excipient/mass ratio	T_g/°C
	sucrose	5:2	34.1
	sucrose	1:1	43.2
	mannitol	5:2	12.0
	mannitol	1:1	11.4
	lactose	5:2	45.1
	lactose	1:1	47.3
	xylitol	5:2	–
Lysozyme	xylitol	1:1	30.8
	fucose	5:2	28.5
	fucose	1:1	43.9
	lactitol	5:2	37.3
	lactitol	1:1	49.5
	trehalose	5:2	49.3
	trehalose	1:1	50.5
	trehalose	2:3	68.3
	none	5:0	57.6
	sucrose	5:2	45.3
	xylitol	5:2	57.2
Hyaluronidase	trehalose	1:1	68.9
	lactose	5:2	46.2
	lactose	2:3	51.7
	lactose	1:3	65.2
	sucrose	1:1	49.1
	sucrose	2:5	50.5
	sucrose	1:3	50.5
	lactose	1:1	43.2
	lactose	2:3	49.1
	lactose	1:2	57.9
Chymotrypsinogen A	lactose	1:3	56.3
	trehalose	1:2	50.3
	trehalose	1:4	50.9
	lactitol	1:1	47.6
	lactitol	1:2	39.8
	dextran	1:1	91.2
	dextran	1:2	70.4
	trehalose	5:2	50.2
	trehalose	2:3	60.5
	trehalose	1:2	75.5
Thermolysin	lactose	5:2	46.4
	lactose	1:1	59.6
	lactose	2:3	61.4
	lactose	1:2	61.6

higher than that after crystallization, implying that a higher degree of protein aggregation and/or intermolecular interaction occurred in lyophilization without excipient [4].

Figure 5 also shows that the addition of sucrose, mannitol or dextran caused an increase in the α-helix content and a decrease in the β-sheet content more or less. Various excipients induced different effects in protecting the secondary structure of lysozyme. Co-lyophilization of lysozyme with dextran had small effect in preserving lysozyme's secondary structure, which can be attributed to a poor hydrogen bonding ability of dextran [16]. On the other hand, sucrose showed better effects on maintenance of the secondary structure of lysozyme during freeze-drying than other excipients used in this study (same mass ratio). Moreover, the stabilization of the native α-helical structure of the freeze-dried lysozyme conferred by excipients was found to be a function of the concentration of the excipients. The intensity of the α-helical band increased with increasing sucrose or mannitol content, in the range of mass ratios 0.4–1. These results are similar to those reported for the stabilization of lysozyme during processing by freeze-drying [23].

The second-derivative FTIR spectroscopic analysis of chymotrypsinogen A is shown in Fig. 6. The spectrum was conformationally sensitive in the amide I region, bands at 1638 and 1690 cm^{-1}, which are due to the β-sheet structure. When chymotrypsinogen A was lyophilized or crystallized in the absence of excipient, the resulting IR spectra in solid-state indicated a substantial perturbation of secondary structure, which can

Table 5 Comparison of the denaturation temperature (T_d) of freeze-dried powders of lysozyme–excipient

Excipient	Lysozyme-to-excipient/mass ratio	T_d before storage/°C	T_d after storage/°C
None	1:0	84.5	73.8
Sucrose	5:2	94.1	88.2
	1:1	95.7	94.8
	1:3	96.4	95.1
	1:4	100.5	96.9
Xylitol	5:2	94.6	83.3
	1:1	96.2	93.1
Lactitol	5:2	106.4	88.6
	1:1	108.4	95.0
Trehalose	5:2	96.4	90.3
	1:1	99.0	95.4
	2:3	110.4	96.3
	1:4	111.8	98.2

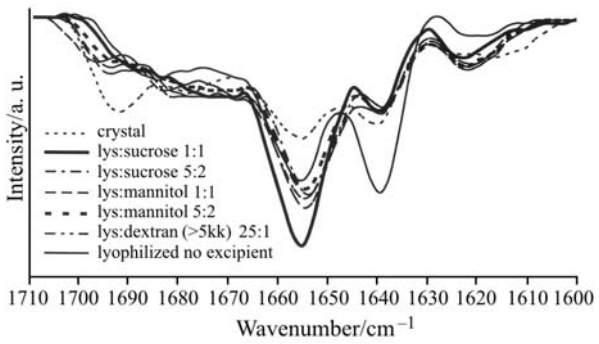

Fig. 5 Second-derivative FTIR of lysozyme in solid form

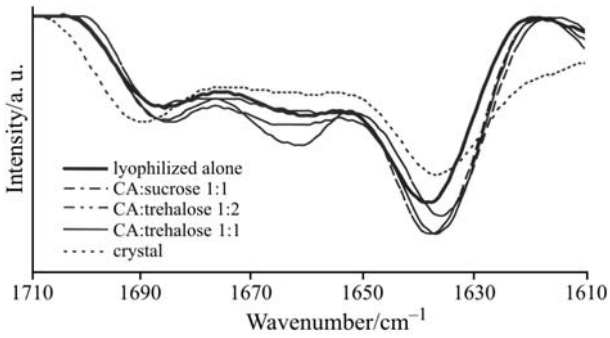

Fig. 6 Second-derivative FTIR of chymotrypsinogen A in solid form

be seen from the lower intensity of the β-sheet band at 1638 cm^{-1}. In the presence of sucrose or trehalose, an increase in intensity at 1638 cm^{-1} is observed. When 66.7 mass/mass% trehalose was used, the degree of structural protection (β-sheet) was almost the same as that noted for 50 mass/mass% trehalose (Fig. 6). Increasing trehalose concentration did not result in further increases in structural protection when trehalose concentration was larger than 50 mass%.

The excipient that is able to induce a large structural preservation may help to prevent aggregation [44]. The extent of changes in overall IR spectrum of a protein upon lyophilization reflects the degree of protein denaturation. Our calorimetric and FTIR spectroscopic experiments showed that trehalose conferred higher T_g and β-sheet structural protection than sucrose (same mass concentration), whereas sucrose was more effective in preserving the α-helical structure of protein during lyophilization than trehalose. Therefore, the benefits of one excipient over the other appear to be equivocal; to optimize both the physical stability of the formulations and the protein stability, more than one excipient might be required [45].

Conclusions

Effect of excipients on T_g of freeze-dried protein–excipient mixture generally appeared dependent on the type and amount of excipient. On the other hand, lyophilized proteins easily adsorbed large amounts of moisture during storage, and increased moisture content of the lyophilized proteins was proven to decrease their glass transition temperatures and stability. In particular, through calorimetric and FTIR spectroscopic analysis, trehalose was demonstrated to confer higher T_g and β-sheet structural protection than sucrose (same mass concentration), whereas sucrose was more effective in preserving the α-helical structure of protein during lyophilization than trehalose. As a consequence of the above findings, it can be suggested that, to optimize both the physical stability of the formulations and the protein stability, more than one excipient might be required and their proportions should be optimized.

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