Pharm Res (2011) 28:540–552
DOI 10.1007/s11095-010-0300-x

RESEARCH PAPER

The Use of Disaccharides in Inhibiting Enzymatic Activity Loss and Secondary Structure Changes in Freeze-Dried β-Galactosidase during Storage

Ville Petteri Heljo • Kirsi Jouppila • Timo Hatanpää • Anne M. Juppo

Received: 16 April 2010 / Accepted: 5 October 2010 / Published online: 22 October 2010
© Springer Science+Business Media, LLC 2010

ABSTRACT

Purpose The purpose of this study is to show how disaccharides differ in their ability to protect lyophilized β-galactosidase from enzymatic activity loss and secondary structure changes during storage.

Methods β-galactosidase was lyophilized with trehalose, sucrose, cellobiose or melibiose at 2:1, 20:1 and 40:1 excipient/protein weight ratios, and stored up to 90 days at 45°C. Protein enzymatic activity was studied using o-nitrophenyl-β-D-galactopyranoside cleavage test, and its secondary structure in lyophilizates analyzed using Fourier transform infrared spectroscopy. The crystallization tendencies, glass transition temperatures and water contents of lyophilizates were evaluated using x-ray powder diffractometry, differential scanning calorimetry and thermogravimetry, respectively.

Results The enzymatic activity of β-galactosidase decreased more slowly in lyophilizates containing trehalose or melibiose at 2:1 excipient/protein weight ratio when compared to those containing sucrose or cellobiose. Similar behavior was observed when analyzing the protein's secondary structure in lyophilizates. In 20:1 and 40:1 excipient/protein weight ratio lyophilizates the decrease of enzymatic activity was less dependent on the excipient, but activity was always amongst the highest in melibiose lyophilizates.

Conclusions Melibiose was shown to be effective in protecting lyophilized β-galactosidase during storage. The protein secondary structure was shown to change at comparable rate in lyophilizates as its enzymatic activity after rehydration.

KEY WORDS β-galactosidase · disaccharides · lyophilization · protein secondary structure · storage stability

INTRODUCTION

The instability of protein-structured pharmaceuticals is one of the main reasons why the number of marketed drug products containing such active compounds is still limited (1). To counter this inherent instability, pharmaceutical protein formulations may be dried in the hopes of making the proteins more resistant to structural changes over time. The most common method used in protein pharmaceutical stabilization during the last decades has been freeze drying. However, even if the protein is not irreversibly damaged by the freezing and dehydration, long-term storage can cause protein degradation, especially at relatively high temperatures. Excipients such as disaccharides are often used to prevent degradation during dehydration and storage, but in order to protect the protein, the excipient must stay in amorphous form and not crystallize (2). Amorphous excipient's ability to protect proteins from conformation changes has been attributed to the high viscosity of the glassy state which keeps the proteins from unfolding (3), as well as to their ability to replace water as a hydrogen bond former

V. P. Heljo (✉) · A. M. Juppo
Faculty of Pharmacy, Industrial Pharmacy
University of Helsinki
Helsinki, Finland
e-mail: petteri.heljo@helsinki.fi

K. Jouppila
Department of Food Technology, Faculty of Agriculture and Forestry
University of Helsinki
Helsinki, Finland

T. Hatanpää
Department of Chemistry, Laboratory of Inorganic Chemistry
University of Helsinki
Helsinki, Finland

when the protein is dehydrated, thus preserving the protein's native aqueous structure (4). Crystallization of an amorphous excipient may cause proteins to lose their active form (5), but even without such a phase transition a mere increase in the molecular mobility of the amorphous phase can lead to lowered stability (6). Therefore, it can be said that the storage stability of lyophilized protein pharmaceuticals depends on a number of different factors, many of which are still poorly understood.

Protein degradation caused by lyophilization and storage has commonly been analyzed from reconstituted lyophilizates with methods such as enzyme activity analysis (5) and chromatographic studies (7,8). However, it is also possible to analyze changes in protein folding directly from the lyophilizates, for example by using spectroscopic methods, which allow fast characterization of lyophilizes without having to rehydrate the dried product. IR-spectroscopy has been shown to be applicable for this purpose, as it is able to detect changes that occur in protein secondary structure during lyophilization (9). These measurements have often been done by monitoring the amide I region around 1700–1600 cm^{-1}, where the absorbance is due to amide bond in-plane C = O stretching, coupled with in-plane C-N stretching and N-H bending (2,10). The amide II region around 1600–1500 cm^{-1}, which is mostly due to N-H vibrations, has also been used in protein secondary structure studies, although less frequently (11). Changes in protein amide bonds are nonetheless visible in both amide I and II regions, and they can both be used to analyze changes in protein secondary structures.

In this study, the storage stability of lyophilized β-galactosidase was evaluated in the presence of different weight ratios of trehalose, sucrose, cellobiose and melibiose, all of which are disaccharides with the same chemical formula ($C_{12}H_{22}O_{11}$) but have different molecular structures (Fig. 1). Of these disaccharides, trehalose and sucrose have been used extensively in lyophilization studies (6,7), whereas cellobiose has been studied only marginally (8), and according to our best knowledge no research articles have been published that describe the use of melibiose as a lyoprotecting excipient for proteins. The aim was to study the effect of different excipients on the retention of β-galactosidase enzymatic activity during storage and to compare them with possible protein secondary structure changes that were measured directly from lyophilizates using FT-IR. β-galactosidase was selected as the model protein, as its enzymatic activity is easy to quantify (12), and the secondary structures of β-galactosidases have been studied thoroughly with IR spectroscopy (11,13).

MATERIALS AND METHODS

Preparation of Lyophilization Solutions

β-D-galactosidase from *Aspergillus oryzae* (M_w~125 kDa, activity NLT 76.5 U/mg) was a kind gift from Amano Enzyme Inc. (Nagoya, Japan), and the protein was purified by using tangential flow filtration. Pall Minimate TFF system (Pall Co., NY, USA) with Omega 30 K MWCO

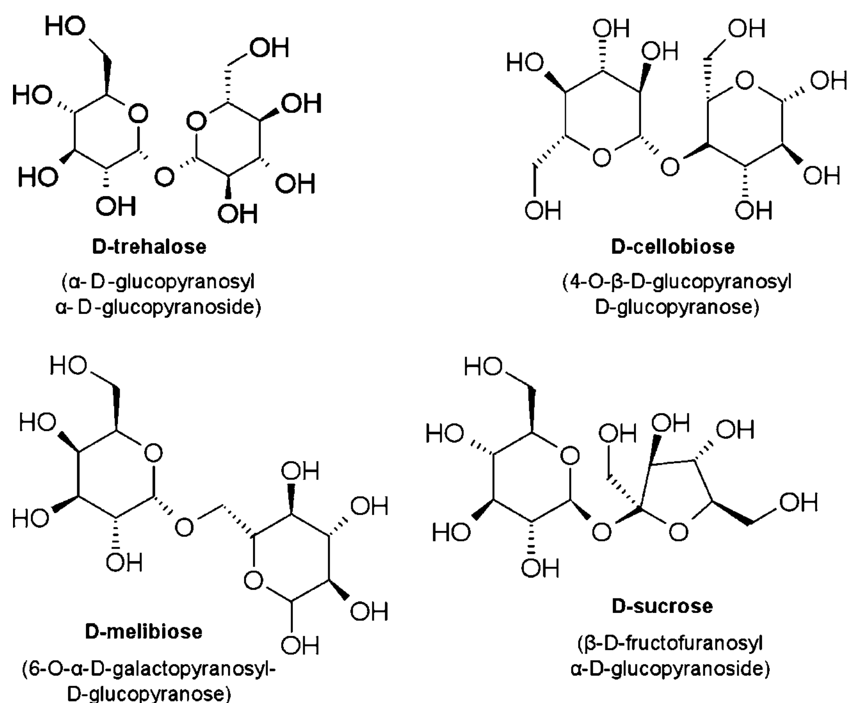

Fig. 1 The molecular structures of the excipients used in the study.

filter was employed with continuous filtration method using purified water as the filtration medium and by collecting 7 diafiltration volumes. After filtration, the initial β-galactosidase activity was measured with enzymatic activity analysis test ($n=3$) as described later. The protein filtrate was then diluted to 0.5 mg/ml concentration with purified water, and D-trehalose (T9531, Sigma-Aldrich, MO, USA), D-sucrose (18219, Sigma-Aldrich, MO, USA), D-cellobiose (C7252, Sigma-Aldrich, MO, USA) or D-melibiose (M5500, Sigma-Aldrich, MO, USA) was added to produce solutions with 1 mg/ml, 10 mg/ml and 20 mg/ml of excipients. The excipient/protein weight ratios of these solutions were, therefore, 2:1, 20:1 and 40:1, respectively. The disaccharides were dried at 60°C/100 mbar for 48 h before use. Protein solutions without excipients (protein concentration 0.5 mg/ml) and excipient solutions without protein (excipient concentration 20 mg/ml) were also produced. One ml of each solution was pipetted into blow-molded 10-ml vials, and the vials were loosely fitted with rubber lyophilization stoppers. The vials were placed randomly on the shelves of Lyostar II freeze dryer (SP Industries Inc., Warminster, USA) and equilibrated at 5°C for 30 min. The vials were then frozen at −45°C for 2 h and at −40°C for 30 min. Primary drying was carried out at −35°C and 0.2 mbar for 21 h, followed by an isobaric heating of 5°C per hour to 35°C, where the samples were held for 2 h. After equilibration to 25°C, the chamber was brought to atmospheric pressure using dry N_2 gas, and the vials were closed by compression before opening the chamber. Aluminum crowns were fitted on the vials after removal from the chamber. No visible collapses were observed in the samples during lyophilization.

Determination of Possible Crystallinity Present in Lyophilizates

The crystallinity of the lyophilizates, or lack thereof, was studied by using Bruker D8 Advance x-ray powder diffractometer (XRPD, Bruker Axs Inc., WI, USA). Samples were analyzed after lyophilization and after 90 days of storage at 45°C to check if crystallization had occurred during this time ($n=1$). The angular range was 5–40° (2θ), and the measurement rate 0.1° per second. Diffractogram peak angles were compared to those acquired from Cambridge Structural Database using ConQuest 1.10 program (CCDC, Cambridge, UK) for crystal identification purposes.

Determination of Lyophilizate Residual Moisture Contents and Glass Transition Temperatures

The residual moisture contents of the lyophilizates were measured with thermogravimetric analysis using TGA 850 (Mettler-Toledo Inc., Switzerland). One to five mg of the lyophilizate was transferred into measurement pans and heated from 25°C to 120°C (20°C/min) and held there for 5 min under 50 ml/min dry N_2 flow. Sample moisture contents were calculated from the weight changes ($n=1$). The glass transition temperatures (T_g) of the lyophilizates were measured with differential scanning calorimetry using DSC823e (Mettler-Toledo Inc., Switzerland). Samples were hermetically sealed in aluminum pans inside a glovebox under low relative humidity (RH ~ 5%) conditions, and the samples to be analyzed were taken from different vials ($n=3$). The T_g of the samples was determined by equilibrating them at 25°C for 3 min and then heating them at 10°C/min at least 30°C over their T_g under 50 ml/min dry N_2 flow. T_g was taken as the midpoint temperature of the glass transition. The T_g' of the aqueous formulations was also measured so that those temperatures would not be exceeded during primary drying. Approximately 5 mg of the solutions were hermetically sealed in aluminum pans, equilibrated at −50°C for 15 min and heated to 10°C at 10°C/min under 50 ml/min dry N_2 flow. The onset of the transition was taken as the T_g' value ($n=1$), so that when the samples were held below this temperature during primary drying, the amorphous phase would remain in glassy state.

Determination of the β-Galactosidase Activity of Rehydrated Solutions

The effect of drying and storage on the enzymatic activity of β-galactosidase was analyzed by rehydrating the lyophilizates with 1 ml of purified water and analyzing the enzyme activity with spectrophotometric o-nitrophenyl-β-D-galactopyranoside (ONPG) cleavage rate test. Briefly, the protein solutions were incubated in the presence of ONPG, a colorless molecule resembling lactose. In its active form, β-galactosidase breaks ONPG into colorless galactose and yellow o-nitrophenol. The concentration of o-nitrophenol in the solution can be quantified by measuring its UV-absorbance at 420 nm. The test described elsewhere (12) was modified to suit the enzyme concentration of this study by using pH 7.3 sodium phosphate buffer and 10 min incubation time. A standard curve of β-galactosidase concentration *versus* ONPG catalysis activity was formed, and the activity of the 0.5 mg/ml β-galactosidase solution after tangential flow filtration and subsequent dilution was assigned as 100% in relative activity. Activities of rehydrated lyophilizates were analyzed from different vials ($n=3$) immediately after lyophilization and after storing them for 7, 30 and 90 days at 45°C. The enzymatic activity present in rehydrated lyophilizates was compared to that of the initial protein filtrate. The standard deviation of the method for

TFF processed solution was ±1.6% ($n=3$), and the drift from day 0 to day 90 was -2.0%.

Analysis of the IR Spectra of Lyophilizates

FT-IR spectroscopy was used to analyze protein secondary structure from the lyophilizates. Approximately 2 mg of the lyophilizate was ground with 250 mg of FT-IR grade KBr (221864, Sigma-Aldrich, MO, USA) and compressed into a pellet under vacuum. The samples were analyzed using Vertex 70 (Bruker Optics Inc., MA, USA) by measuring the spectra 20 times with 4 cm^{-1} resolution between 4000-650 cm^{-1} under dry air flow. Aqueous β-galactosidase samples were measured by placing 100 µl of 10 mg/ml protein solution between two KBr discs and measuring the spectra with the same settings as above. All dry samples to be analyzed were taken from different vials ($n=5$) after lyophilization and after storing them 7, 30 and 90 days at 45°C. The spectra were further treated using OPUS 4.0 program (Bruker Optics Inc., MA, USA) by calculating their second derivatives, after which they were smoothed using Savitzky-Golay function (nine-point smoothing). The resulting spectra were baseline corrected along 1720-1490 cm^{-1} region (amide I and II regions) to minimize the effect of differing baselines on further data analysis. The spectrum of pure water was subtracted from the aqueous protein spectra before further mathematical processing. The protein spectra were interpreted according to literature (11,13–15).

Multivariate Analysis

Multivariate data analysis (partial least squares, PLS) of the spectra was carried out using Simca-P® program (Umetrics AB, Umeå, Sweden). The basic theory of PLS is explained elsewhere (16). Generally, a PLS model is an attempt to relate two data matrices, predictors (X) and response (Y), to each other. The model forms principal components (PCs), which may be regarded as vectors which best describe the variation between the score matrices of X and Y (known as T and U, respectively). As one PC rarely describes data variation adequately, the amount of model PCs can be increased to improve the accuracy at which the model describes the data. Each added PC attempts to explain the variation not explained by the previous components, and adding more PCs generally improves the predictive capability of the model, but only to a certain point. The predictive ability of the model can be described using the cumulative goodness of prediction (Q_{cum}^2) value, where the value 1 indicates that the model predicts the data points perfectly, and as value decreases so does the predictability of the model. In the case of spectroscopy, each spectrum may therefore be expressed by a number of values equal to the number of components in the model. Differences between spectra may be visualized by plotting their PCs on a coordinate system, where each PC is represented by an axis. This kind of plot is known as a score plot. For two identical spectra the PCs would also be identical, which means that they would be superimposed on the score plot. The multivariate model can also be used to identify which sections of the spectra have the greatest impact on different PCs, and when this is drawn on a coordinate system, such plot is known as a loadings plot. In the case of FT-IR spectra, the wave number regions which undergo significant changes have large loading values, which means that loadings plots can be used in conjunction with score plots to identify and interpret spectral changes from very large amounts of data.

PLS modeling has been frequently used in correlating spectral data with changing sample properties, such as the water content of a drug compound (17) and tablet crushing strength (18). In this study, the method was used to relate the FT-IR spectra acquired from protein/excipient lyophilizates to their storage time at 45°C. IR transmittance values between 1720 and 1490 cm^{-1} were plotted as predictors, and the storage time (from 0 to 90 days) was used as the response.

RESULTS

Crystallinity, Residual Moisture Content and T_g of the Lyophilizates

All lyophilizates were found to be amorphous after freeze drying according to their XRPD analyses. After 90 days storage at 45°C all lyophilizates containing protein had remained amorphous, while pure lyophilized sucrose had crystallized during storage (sucrose crystal structure confirmed using ConQuest 1.10 program). It should be noted that crystallinity was not detected in the lyophilizate with 40:1 sucrose/β-galactosidase weight ratio, which indicates that even a small amount of the protein could inhibit the crystallization of sucrose during storage. The thermogravimetric measurements performed on the lyophilizates showed weight loss during the measurements, and the total weight loss was considered as the amount of residual moisture present in the samples. Moisture contents ranging from <1% to nearly 3% (w/w) were measured, as shown in Table I. Increasing the excipient/protein weight ratio of the lyophilizates decreased their residual moisture content. All DSC measurements performed on the lyophilizates showed a single clear endothermic baseline shift, which was interpreted as the glass transition. The glass transition temperatures are also shown in Table I. The transition temperatures generally increased in the order sucrose <

Table 1 Water Contents ($n=1$) and Glass Transtition Temperatures ($n=3$) of Lyophilized Excipient/Protein (E/P) Mixtures and Pure Excipients

	Residual moisture content (%-w/w)				Glass transition temperature			
	Sucrose	Melibiose	Cellobiose	Trehalose	Sucrose	Melibiose	Cellobiose	Trehalose
E/P 2:1	2.6	2.6	2.7	2.7	65°C (± 2°C)	72°C (± 9°C)	65°C (± 2°C)	80°C (± 6°C)
E/P 20:1	1.2	1.3	1.5	2.1	67°C (± 4°C)	81°C (± 2°C)	75°C (± 2°C)	90°C (± 4°C)
E/P 40:1	0.9	1.5	1.2	1.5	69°C (± 3°C)	83°C (± 5°C)	80°C (± 10°C)	94°C (± 6°C)
Pure excipient	0.8	1.2	1.4	1.5	62°C (± 3°C)	82°C (± 5°C)	88°C (± 1°C)	96°C (± 5°C)

Weight loss expressed as%-w/w, standard deviations shown in brackets where appropriate

cellobiose ~ melibiose < trehalose, as the transition temperatures of melibiose and cellobiose often appeared within standard deviation limits from each other. Increasing the excipient/protein weight ratio generally led to a higher T_g, which was caused by the decrease in water content. When measuring the T_g' of the aqueous formulations, the values for trehalose, sucrose, cellobiose and melibiose solutions (20:1 and 40:1 excipient/protein weight ratios) containing β-galactosidase were −31°C, −34°C, −31°C and −32°C, respectively. The concentration of the excipients did not seem to affect T_g' significantly, but in the case of 2:1 excipient/protein weight ratio solutions the transition was too weak to be measured. The measured T_g' values were higher than the shelf temperature of the freeze dryer (−35°C) during primary drying.

Changes in β-Galactosidase Activity during Storage

The relative activities remaining in reconstituted lyophilizates that were stored at 45°C are shown in Fig. 2. The lyophilizates with 2:1 excipient/protein weight ratio had shrunk slightly after 7 days storage at 45°C, but no massive collapses were observed. Freeze drying β-galactosidase without excipients resulted in significantly lower relative

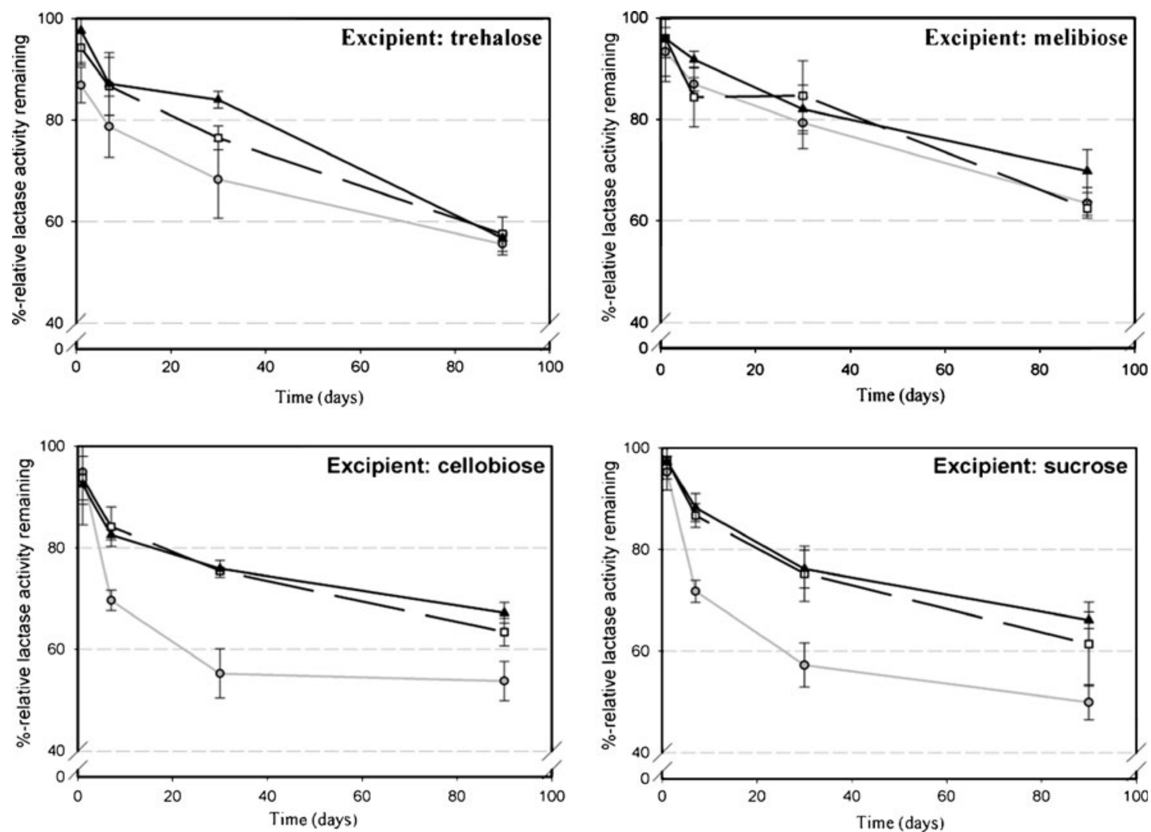

Fig. 2 The relative β-galactosidase activity remaining in reconstituted lyophilized solutions with 2:1 (○), 20:1 (□) or 40:1 (▲) excipient/protein weight ratios stored at 45°C ($n=3$). The average values and standard deviations were obtained from the activity analysis tests, the connecting lines are shown as visual aid.

activity than lyophilizing it in the presence of the excipients (not shown in Fig. 2). The relative activity remaining in the excipient-free β-galactosidase was approximately 13% after lyophilization, and no activity was detected when pure lyophilized β-galactosidase was stored at 45°C for 7 days or longer. The analysis of the formulations containing disaccharides showed that there was no significant difference between their enzyme activities immediately after freeze drying. Melibiose was found to be significantly superior to sucrose and cellobiose at 2:1 excipient/protein weight ratio when stored at 45°C for 7 days or longer ($p \leq 0.05$), and it was also shown to be superior to trehalose when stored at that temperature for 90 days. On the other hand, trehalose was shown to be inferior to other disaccharides at 40:1 excipient/protein weight ratio when stored up to 90 days. There was also a significant difference between the activities of 2:1 and 40:1 excipient/protein weight ratio formulations for cellobiose and sucrose from day 7 onwards and for melibiose at day 90, indicating that from that point forward β-galactosidase activity loss was lower with 2:1 than with 40:1 excipient/protein weight ratio. No clear differences were observed between trehalose formulations of different disaccharide/protein weight ratios at given time points. β-galactosidase activity values at different time points were also fitted into an equilibrium first-order decay model to compare their activity loss rate constants. The results for the 2:1 excipient/protein weight ratio formulations are shown in Table II. Relatively good fit was achieved ($R^2 > 0.98$) for all formulations, and the rate constants of melibiose and trehalose formulations were lower than those of sucrose and cellobiose formulations.

Analysis of Lyophilizate FT-IR Spectra

The analysis of lyophilizate FT-IR spectra revealed clear differences between pure excipient and excipient/protein mixture spectra at 1720–1490 cm^{-1} in some, but not all, of the formulations. The amide I and II region spectra of the 20:1 and 40:1 excipient/protein weight ratio samples did not clearly differ from pure excipient spectra in that region (data not shown), indicating that the amount of protein in the KBr pellets was too small to be detected. However, there were clear differences in the spectra of 2:1 excipient/protein weight ratio formulations and the pure excipients at amide I and II regions, showing that the amount of protein in the pellets was large enough to produce a clear protein spectrum. The second derivative spectra of aqueous β-galactosidase before freeze drying and those of lyophilizates that were freeze dried without excipients or with melibiose or sucrose (2:1 excipient/protein weight ratio) and stored up 90 days in 45°C are shown in Fig. 3. The formulations containing trehalose and cellobiose in the same excipient/protein weight ratio produced similar FT-IR spectra as those containing melibiose or sucrose (data not shown). Before freeze drying, the most intensive peaks of the spectrum are located around 1650 cm^{-1}, 1639 cm^{-1} and 1628 cm^{-1}. The first of these peaks can be attributed to α-helical structures according to literature, while the second and third correspond to β-sheet structures (11,13). After freeze drying, only one β-sheet peak is observed around 1635 cm^{-1}, and the relative intensity of the amide II band is increased. In excipient-free lyophilizates, there is also a peak around 1615 cm^{-1} which can only be seen as a shoulder in the spectra of the formulations containing disaccharides. Absorption at this wavelength has been attributed to intermolecular bonding in β-galactosidase (11), as well as to distorted β-structures in other proteins (14). Furthermore, at least three peaks are visible around 1690-1665 cm^{-1}, which have been attributed to β-turn structures in literature (15). When storing the lyophilizates at 45°C the intensity of the peak around 1650 cm^{-1} increases, a region where α-helix structures of β-galactosidase proteins have been shown to absorb (11). It can be seen from Fig. 3 that this peak was not initially visible in 2:1 sucrose/β-galactosidase weight ratio formulation spectra, but a strong peak can be seen when the lyophilizates are stored for 7 days and longer. Furthermore, the region between 1560 and 1520 cm^{-1} undergoes significant changes during storage in formulations with 2:1 excipient/protein weight ratio. This absorption area has been attributed to α-helical and several different β-structures in different proteins (14), which means that these spectral changes cannot be accurately interpreted.

A three-component PLS model was formed for the second derivative spectral data of the 2:1 excipient/protein weight ratio lyophilizates, which produced a Q_{cum}^2 value of 0.82. The score plot for the data is shown in Fig. 4, where the first, second and third PC values are plotted on x-, y- and z-axes, respectively. It should be noted that the axes are not drawn in scale, because each successive PC describes a

Table II The Results of Fitting β-Galactosidase Activities of 2:1 Excipient/Protein Weight Ratio Lyophilizates during Storage into Equilibrium First-Order Decay Model $\left(y = y_0 + ae^{(-bx)}\right)$

	Sucrose	Melibiose	Cellobiose	Trehalose
R^2	0.983	0.990	0.999	0.990
rate constant (b)	0.124	0.014	0.161	0.029
rate constant error	0.046	0.010	0.010	0.011
Activity remaining (%) at t = ∞	52.7	52.5	54.3	53.2

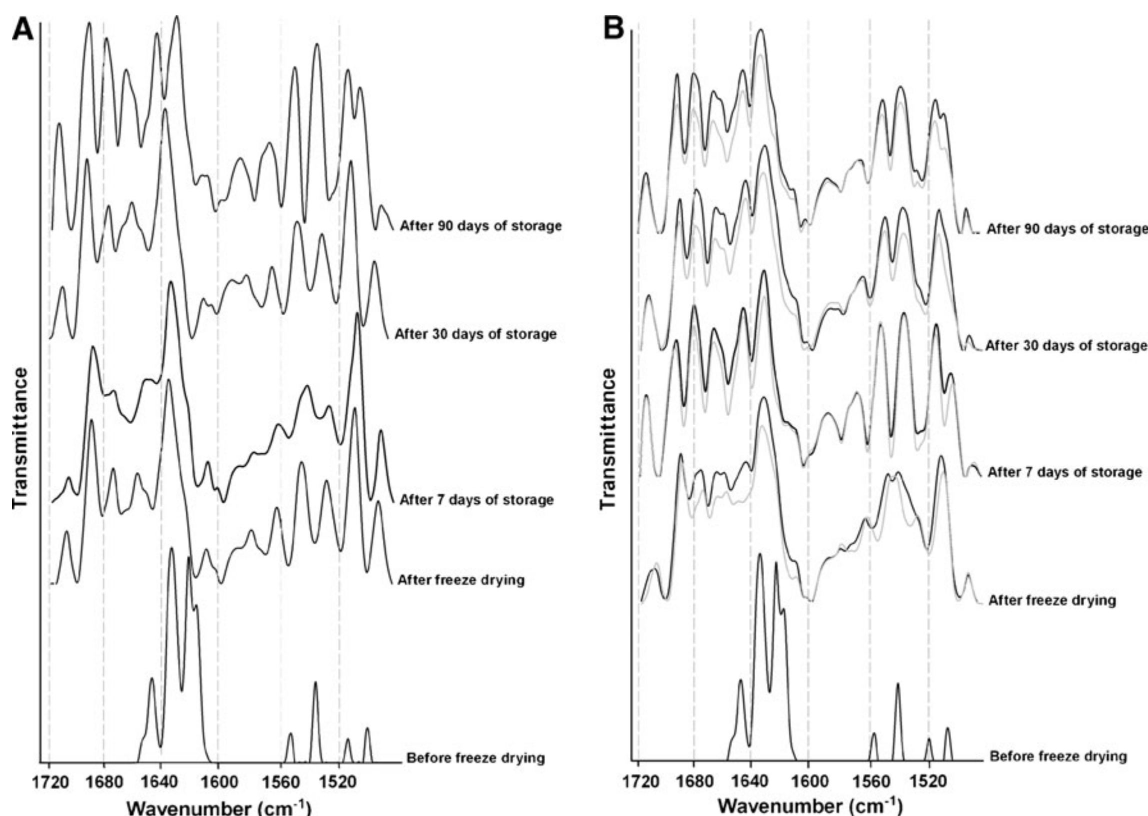

Fig. 3 The normalized 2nd derivative IR spectra (averages, $n=5$) of aqueous β-galactosidase before freeze drying and lyophilizates that were **A** freeze dried without excipients, or **B** with melibiose (*black curve*) or sucrose (*gray curve*) at 2:1 excipient/protein weight ratio and stored up to 90 days at 45°C.

smaller amount of predictor variation when the amount of components is increased. The points describing different samples are generally close to each other at a given time point, indicating that their spectra are fairly similar. However, there is often a clear change in PC values as the storage time increases, indicating that the amide I and II region spectra change as a function of storage time. Storing the lyophilizates at 45°C for 7 days causes mainly

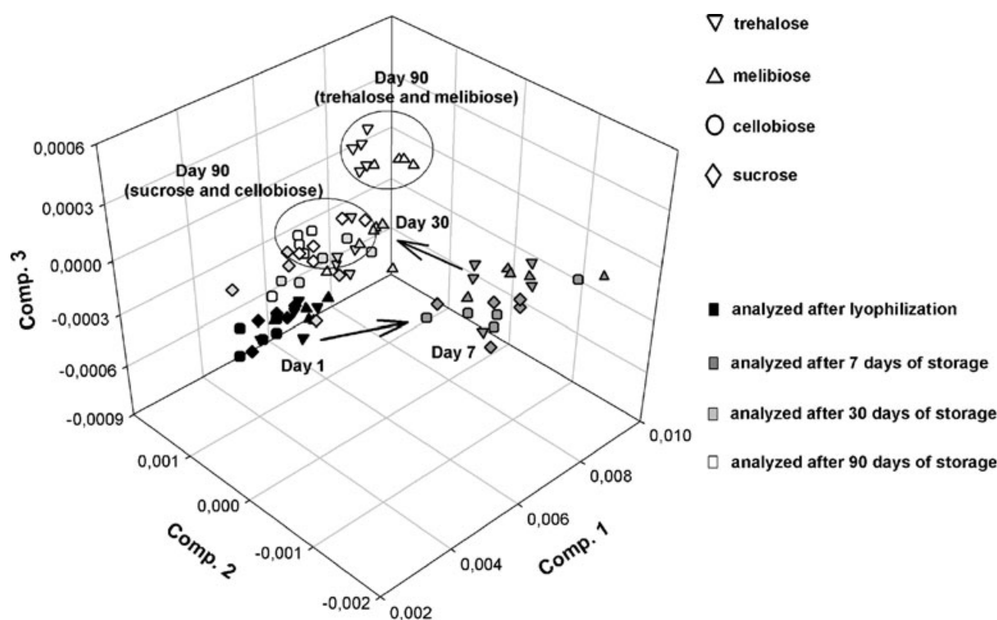

Fig. 4 Three-dimensional PLS score plot of the 2:1 excipient/protein weight ratio lyophilizate FT-IR spectra ($n=5$), where each symbol represents a single lyophilizate spectrum. Geometric symbols (*triangle*, *inverted triangle*, *circle* and *diamond*) represent different excipients and colors (*black*, *dark gray*, *light gray* and *white*) represent storage time before analysis.

an increase in the first and a decrease in the second PC, whereas storing up to 30 days increases the second PC. When stored up to 90 days, there is an increase in the first and third PC values of trehalose and melibiose containing lyophilizates, whereas a smaller shift in second PC is observed in sucrose and cellobiose containing lyophilizates. The PC loading weights are shown in Fig. 5, along with the β-galactosidase IR-absorption region interpretations acquired from literature (11,13–15). The shift in PLS scores when storing the samples for 7 days is partially caused by an increase at the region attributed to the increased absorption around 1650 cm^{-1} (α-helix), as well as changes in the 1560-1520 cm^{-1} region (both α-helix and β-sheet). Storing the lyophilizates up to 30 days shifts the peak originally located around 1635 cm^{-1} towards higher wavenumbers (β-sheet→α-helix). When stored up to 90 days, the spectra of lyophilizates containing trehalose and melibiose are separated from those containing sucrose and cellobiose by increases in the first and third PCs. These increases are mainly due to an increase in absorption intensity around 1690-1665 cm^{-1} (β-turn), 1650 cm^{-1} (α-helix) and in the 1560-1520 cm^{-1} region (both α-helix and β-sheet).

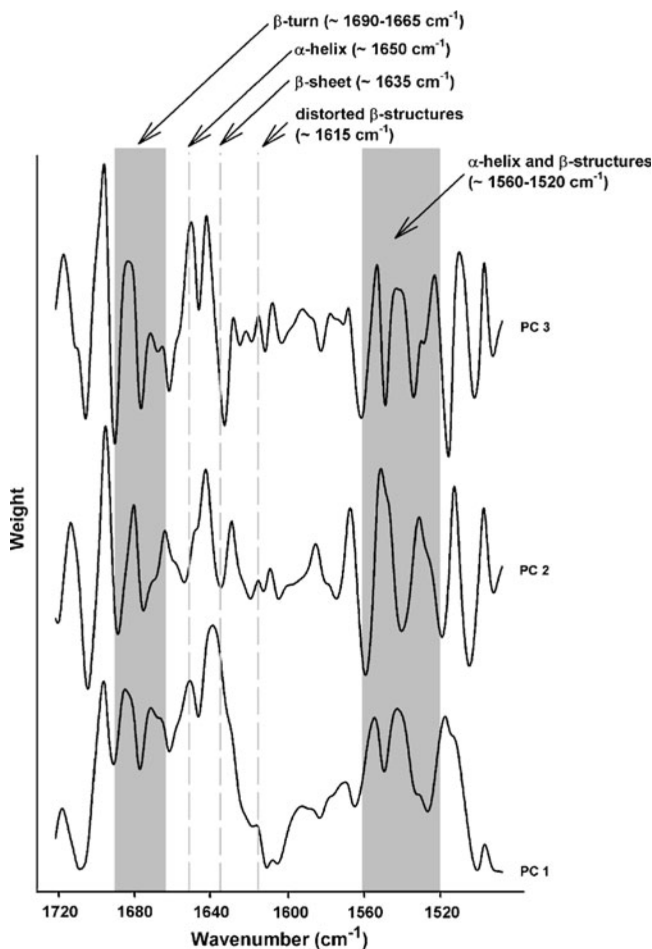

Fig. 5 The loadings plot of the PLS model, where absorption regions have been correlated with protein secondary structure according to literature (11,13–15).

DISCUSSION

Glass Transition Temperatures and Residual Moisture Contents

As increasing the viscosity of the glass matrix surrounding the protein may help to preserve the secondary structure of the protein during storage (3), it could be theoretically possible to improve protein stability by increasing the T_g of the formulations. It was found that β-galactosidase activity loss rate constants were higher for sucrose and cellobiose than for trehalose and melibiose with 2:1 excipient/protein weight ratio, and the initial glass transition temperatures for all formulations generally increased in the order sucrose < cellobiose ~ melibiose < trehalose. When interpreting the equilibrium first-order decay model, it should be noted that the model predicts >0% activity loss at t = ∞, which would not hold true if the formulations were stored indefinitely. However, the empirical model only describes the results in the experimental time scale, and extrapolating such results beyond that region would invariably produce erroneous results. Therefore, the models can only be regarded to represent the storage period of 90 days. The β-galactosidase activity loss rate constants did seem to correlate with initial lyophilizate T_g, but since statistically significant difference between cellobiose and melibiose T_g values could not be established, they cannot be reliably compared. Also, as 2:1 excipient/protein weight ratio lyophilizates shrunk slightly during the first 7 days of storage, it is likely that their glass transition temperatures decreased due to moisture transfer from the stoppers. The vial-to-vial variation in the T_g results may have been caused by the random placement of vials on the shelves of the freeze dryer, which is known to affect the rate of drying (19).

In this study, the initial residual moisture contents were analyzed using thermogravimetry, because the lyophilizates were difficult to analyze using Karl-Fisher titration due to their small size and poor solubility to titration reagents. Similar findings have been published previously by May *et al.* (20). The measurements were carried out because it has been shown that the residual water content of lyophilized formulation has an effect on protein storage stability (21). According to the results of the thermogravimetric analysis, the formulations with lower excipient/protein weight ratio were shown to have relatively higher water content than those with higher weight ratio. In comparison, sucrose

lyophilizates contained slightly less residual moisture than their counterparts, and trehalose lyophilizates slightly more. The residual water content of lyophilizates after freeze drying did not fully correlate with the protein activity loss during storage. For example, sucrose and cellobiose lyophilizates had different residual moisture contents, but their β-galactosidase activities remained fairly similar throughout the experiment. It should be noted that the effect of residual moisture content on protein stability during storage is not a straightforward matter. Even though lowering the water content of the amorphous phase also decreases its molecular mobility, the formulation with lowest water content (and therefore lowest mobility) is not always the most optimal one in protecting a protein from conformation changes. For example, it has been shown that protein aggregation rate during storage can in some cases be slower in formulations with 2–3% (w/w) water content than those with lower water content (21).

To check the reliability of the T_g measurements, pure excipient glass transition temperatures were measured (shown in Table I) and compared to literature values. The glass transition temperatures of pure trehalose, sucrose and melibiose were slightly lower than what is generally given in literature for dry samples by using similar measurement methods, as their T_g values have been reported as 100–120°C (22,23), 70–75°C (24,25) and 85–95°C (22,24), respectively. On the other hand, T_g of pure cellobiose has been given as 77°C (8), which is lower than what was found here. However, in that particular study the sample contained 2–3% (w/w) water, which might have plasticized the amorphous phase and lowered its T_g. To take the water contents of the lyophilizates into account when analyzing their glass transition temperatures, T_g of trehalose and sucrose formulations with 2:1, 20:1 and 40:1 excipient/protein weight ratios were also compared with calculated disaccharide/water mixture T_g values found from literature. For trehalose formulations, the measured glass transition temperatures were all within standard deviation limits from calculated T_g values of disaccharide/water mixtures (26). In the case of sucrose formulations, the other measured T_g values except that of the 2:1 sucrose/protein weight ratio formulation were within standard deviation limits from values calculated by using the Gordon-Taylor equation (27,28). Overall, the glass transition temperatures measured in this study were fairly comparable to those found from literature, even when taking into account their water contents. However, pure sucrose was shown to crystallize during storage, which could have been caused by an increase in the water content of the lyophilizate by moisture transfer from the vial stopper to the lyophilizate. Therefore, the glass transition temperatures of lyophilizates should not be regarded as unchanging, as their water contents may rise during storage. This, in turn, may increase the mobility of the amorphous phase and have a negative effect on protein stability. The residual water contents or glass transition temperatures of the lyophilizates were not measured at the end of the experimental period, so it is not known how these parameters changed during storage.

The thermogravimeter samples were not handed in a dry box before measurement to prevent moisture uptake from the atmosphere, because the apparatus uses open pans to allow evaporation to take place freely. Even if the samples were packed into pans in a dry box, transferring them to the thermogravimeter would have subjected them to atmospheric humidity. This does raise the question whether the measured water content values are systematically too high, but as mentioned previously, the measured glass transition temperatures and water contents correlated fairly well with those found in the literature. This would indicate that the samples did not absorb significant amounts of water during the short period of time when they were subjected to atmospheric conditions.

Protein Secondary Structure Analysis Using FT-IR

Sample pretreatment is an important part of spectroscopic studies, and it is crucial to know if it affects the measurement results in any way. Measuring IR-transmission from pellets compressed from a mixture of KBr and the sample of interest generally yields excellent spectral resolution when compared to ATR measurements, for example. However, when analyzing protein secondary structures from compressed KBr pellets, concern might rise whether the compression affects the interactions between the protein and the excipient or the structure of the protein being analyzed. Studies addressing this issue have been previously published. The compression of KBr, disaccharide and protein into pellets has been shown not to affect disaccharide-protein interactions (29). Similar protein secondary structure spectra may also be measured from compressed pellets and uncompressed samples (hydrocarbon mulls), indicating that the compression does not significantly alter protein secondary structure (30). Therefore, the compression of freeze-dried protein formulations into KBr pellets allows protein secondary structure to be accurately determined without causing significant damage to the sample during pretreatment. Furthermore, the KBr transmission method allows proteins to be analyzed at lower concentrations than when the lyophilizates are rehydrated and their secondary structures determined from aqueous solutions. This is because water can obscure the FT-IR analysis of proteins by absorbing at the same region as the amide I band (31). Based on this, Pretrelski *et al.* (32) concluded that when in hydrated state, protein secondary

structure should be analyzed from highly concentrated solutions, but increasing the concentration may lead to aggregation because the distance between protein molecules decreases. This does not mean that measuring protein secondary structure from aqueous solutions would be impossible or inaccurate, but measuring the spectra directly from lyophilizates will eliminate the need for sample rehydration, a step which might decrease the sensitivity of the analysis method and even alter protein conformation.

In the IR-spectra the amide I and II bands of β-galactosidase were not visible in the formulations with 20:1 and 40:1 excipient/protein weight ratios, as their spectra were indistinguishable from pure excipient spectra. This was most likely the result of the weak amide I absorption of the small amount of protein being overpowered by the O-H stretch absorption of water (present as residual moisture), which also absorbs in this region (31). When the secondary structure spectra of 2:1 excipient/protein weight ratio formulations were measured, it was evident that there were structural changes during storage. This indicates that the changes in protein secondary structure that affected its enzymatic activity occurred at least partially in the solid phase and not only when the lyophilizates were rehydrated. As it is possible that T_g of the 2:1 excipient/protein weight ratio formulations was exceeded during storage, this might have allowed significant protein secondary structure changes to take place. Still, Chang *et al.* (33) found that gross changes in protein structure may occur even when the formulations are stored under their T_g, and these changes can be measurable with FT-IR. It should be noted that in that study the protein secondary structure was analyzed after 24 weeks of storage at 50°C, so it is not known whether the rate of structural alterations was comparable to our studies. Lyophilized β-galactosidase has also been shown to degrade when stored at 50°C, below the T_g of the protein/disaccharide formulations in question (6). However, the excipient concentrations used in that particular study were significantly higher (9–50% w/w) than in our experiments, which must be taken into account when comparing the results. Taking these points into consideration, it can be concluded that when stored in high temperatures (~ 50°C), lyophilized proteins will degrade under experimental time scale even when the glass transition temperature of the formulation is not exceeded.

When assessing the secondary structure changes in β-galactosidase during storage, the intensification of the α-helix peak around 1650 cm^{-1} and the changes in the amide II region were found to be common to all formulations. It is likely that the portion of α-helix structures of all secondary structure types increases at the expense of β-sheet and unordered structures, as has been shown earlier by Muga *et al.* (11). Still, it is difficult to see from the plain FT-IR spectra how different excipients affect β-galactosidase secondary structure changes. In this study, it would have been possible to attempt to quantify the changes in protein secondary structures during storage by calculating the correlation coefficients (9) or the areas of overlap (34) between the aqueous and lyophilized β-galactosidase FT-IR spectra. However, such approach might produce unreliable results for this kind of study, as the amide I and II peaks of the spectra do not simply shift linearly during storage as seen in Fig. 3. Peaks divide and converge in a seemingly random manner, which means that it would not be likely that all spectral changes lead to a decrease in the correlation coefficient or the area of overlap. Furthermore, if protein degradation during storage would affect its spectrum linearly, there should be at least some linear change in the PC values of Fig. 4 as well. Still, it is very likely that all changes in protein secondary structure during storage are unfavorable for its pharmaceutical quality and bioactivity and that these changes reduce the overall similarity between the unprocessed and processed protein. In light of this, we agree with Wang *et al.* (35), who concluded that the correlation coefficient and area of overlap methods should not be used if the results do not agree with visual analysis of the spectra, or if the method seems insensitive for the protein in question. In qualitative analysis, the PLS method can be a potent aid, as even subtle differences in the spectra can be easily identified. Compared to Fig. 3, it is easier to use the PLS model to analyze the changes in protein secondary structure as a function of storage time. It should be noted that the score plot (Fig. 4) shows only the change of arbitrary structure components (PCs) as a function of time, which must be interpreted as absorption changes in amide I and II regions by using the loadings plot (Fig. 5).

The score plot in Fig. 4 shows that initially there is greater variation between different spectra (score points are scattered), but as the lyophilizates are stored, those containing trehalose and melibiose start to become increasingly similar (score points become clustered). The same can be seen with those containing sucrose and cellobiose. It is worthwhile to notice that the spectra of 2:1 excipient/protein weight ratio lyophilizates that contain sucrose or cellobiose remain relatively similar at 30- and 90-day time points, whereas the spectra of those lyophilizates containing trehalose or melibiose change between the storage of 30 and 90 days. This change is very small, as can be seen from the effect of the third PC on the overall spectra. The third PC loading plot in Fig. 5 shows that this small shift is mostly caused by the intensification of the peak attributed to α–helix structures and to some extent β-turn structures. Still, the phenomenon is visible in both FT-IR spectra and enzymatic activity measurements, as the changes in β-galactosidase secondary structure and activity are negligible in sucrose and cellobiose formulations with 2:1 excipient/

protein weight ratio between 30 and 90 days of storage (Fig. 2). It seems that trehalose and melibiose are able to inhibit the change of β-galactosidase from active towards inactive conformation in the early stages more effectively than sucrose and cellobiose with this weight ratio.

The Effect of Disaccharides on β-Galactosidase Storage Stability

β-galactosidase activity loss kinetics in formulations with 2:1 excipient/protein weight ratio resembled that of a biphasic system, as described by Franks (36). In such systems, water is divided unevenly throughout the lyophilizate, leading to accelerated protein degradation in some parts of the sample at the early stages of storage. Water transfer from stoppers to lyophilizates might have also caused such activity loss profiles, as the viscosity of the amorphous phase might have been decreased during storage, resulting in increased degradation rate. However, the effect was not similar in all formulations, and melibiose in particular seemed to be effective in protecting β-galactosidase from activity loss. This might partially be explained by the differences in disaccharide glass transition temperatures. T_g of dry trehalose and melibiose are significantly higher than that of sucrose (22), so more water is required to bring their T_g close to the storage temperature than with sucrose. Even though T(storage)-T_g and protein degradation rate might not always be directly correlated (37), better stability may still be achieved when the storage temperature is decreased further below the T_g of the formulation. This could also be one reason why trehalose and melibiose inhibited β-galactosidase degradation at the early stages of storage more effectively than sucrose with 2:1 excipient/protein weight ratio, where the initial water content was relatively high (> 2.5% w/w). The differences between disaccharides were less pronounced at 20:1 and 40:1 excipient/protein weight ratios, which might have been partially caused by the lower amount of water present in those lyophilizates. Still, this would not fully explain the differences between the excipients, as β-galactosidase activity was lowest with trehalose at 40:1 excipient/protein weight ratio when compared to other disaccharides when stored up to 90 days. This result is corroborated by a previously published study by Yoshioka *et al.* (6), where trehalose (100:1 excipient/protein weight ratio) was found inferior to sucrose in protecting lyophilized β-galactosidase from aggregation when stored at 50°C. Therefore, the excipient glass transition temperatures and water contents may have an effect on β-galactosidase stability during storage, but they do not explain all the results. Furthermore, in our study cellobiose was inferior to melibiose in inhibiting β-galactosidase degradation during storage, even though their glass transition temperatures are very close to each other. The storage stability of lyophilized pharmaceuticals is still a poorly understood field, and more studies on different excipients are required to validate or dismiss the current theories.

Melibiose has not been thoroughly studied as a pharmaceutical excipient, and little is known about its properties in amorphous state. However, taking into account that it inhibited the enzymatic activity loss of β-galactosidase almost equally well with all excipient/protein weight ratios that were tested (significant difference was found only between 2:1 and 40:1 excipient/protein weight ratio formulations after 90 days of storage at 45°C), melibiose appears to be a promising candidate in inhibiting protein inactivation during storage. Further studies are required to screen its usefulness in this area. No comprehensive toxicity studies have been carried out about melibiose, but doses up to 5 g have been administered orally to children without reported side effects (38,39). Furthermore, it has been shown that melibiose is not significantly hydrolyzed to monosaccharides after intravenous administration to rats (40) and that it is mostly secreted to urine without metabolization (41). This suggests that melibiose is relatively inert in mammals. Therefore, its use as a pharmaceutical excipient might be possible, even though more comprehensive toxicity experiments would be necessary to study its safety.

CONCLUSIONS

The changes in enzymatic activity and secondary structure of β-galactosidase during storage at 45°C were dependent on the excipient used. Of all the excipients, melibiose was shown to be most prominent in inhibiting the enzymatic activity loss of β-galactosidase with all excipient/protein weight ratios that were studied. The changes in protein secondary structure as a function of storage time in 2:1 excipient/protein weight ratio samples correlated with the enzymatic activity measurements. It was shown that the IR spectra of the lyophilizates containing sucrose or cellobiose as an excipient changed more during the first 30 days of storage, whereas in the case of trehalose and melibiose the change was more uniform throughout the 90-day storage period. Therefore, a link between the activity loss and β-galactosidase FT-IR spectra during storage was established. The shortcoming of the FT-IR analysis method was that the amide I and II bands of the protein could not be detected in 20:1 and 40:1 excipient/protein weight ratio formulations due to the low protein concentration in the samples. However, in the cases where protein amide I and II bands could be detected, the PLS model was shown to be useful in identifying very small changes in protein secondary structure.

ACKNOWLEDGMENTS

The authors wish to acknowledge Vetcare Oy for providing material funding and Amano Enzymes Inc. for donating a sample of the model protein to be used in the study.

REFERENCES

1. Wang W. Lyophilization and development of solid protein pharmaceuticals. Int J Pharm. 2000;203:1–60.
2. Carpenter JF, Prestrelski SJ, Dong A. Application of infrared spectroscopy to development of stable lyophilized protein formulations. Eur J Pharm Biopharm. 1998;45:231–8.
3. Hagen SJ, Hofrichter J, Eaton WA. Protein reaction kinetics in a room-temperature glass. Science. 1995;269:959–62.
4. Allison SD, Dong A, Carpenter JF. Counteracting effects of thiocyanate and sucrose on chymotrypsinogen secondary structure and aggregation during freezing, drying, and rehydration. Biophys J. 1996;71:2022–32.
5. Izutsu K, Yoshioka S, Terao T. Decreased protein-stabilizing effects of cryoprotectants due to crystallization. Pharm Res. 1993;10:1232–7.
6. Yoshioka S, Miyazaki T, Aso Y, Kawanishi T. Significance of local mobility in aggregation of b-galactosidase lyophilized with trehalose, sucrose or stachyose. Pharm Res. 2007;24:1660–7.
7. Chang L, Shepherd D, Sun J, Quellette D, Grant K, Xiaolin C, *et al.* Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction and/or immobilization in a glassy matrix? J Pharm Sci. 2005;94:1427–44.
8. Costantino HR, Carrasquillo KG, Cordero RA, Mumenthaler M, Hsu CC, Griebenow K. Effect of excipients on the stability and structure of lyophilized recombinant human growth hormone. J Pharm Sci. 1998;87:1412–20.
9. Prestrelski S, Tedeschi N, Arakawa T, Carpenter JF. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophys J. 1993;65:661–71.
10. Surewicz WK, Mantsch HH, Chapman D. Determination of protein secondary structure by Fourier transform infrared spectroscopy: a critical assessment. Biochem. 1993;32:389–94.
11. Muga A, Arrondo JLR, Sancho BTJ, Bernabeu C. Structural and functional strudies on the interaction of sodium dodecyl sulfate with b-galactosidase. Arch Biochem Biophys. 1993;300:451–7.
12. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989.
13. Arrondo JLR, Muga A, Castresana J, Bernabeu C, Goni FM. An infrared spectroscopic study of b-galactosidase structure in aqueous solutions. FEBS Letters. 1989;252:118–20.
14. Cooper EA, Knutson K. Fourier transform infrared spectroscopy investigations of protein structure. In: Herron JN, Jiskoot W, Crommelin D, editors. Physical methods to characterize pharmaceutical proteins. New York: Plenum; 1995. p. 101–43.
15. Dong A, Huang P, Caughey WS. Protein secondary structures in water from second-derivative amide I infrared spectra. Biochem. 1990;29:3303–8.
16. Wold S, Sjostrom M, Eriksson L. PLS-regression: a basic tool of chemometrics. Chemom Intell Lab Syst. 2001;58:109–30.
17. Aaltonen J, Kogermann K, Strachan CJ, Rantanen J. In-line monitoring of solid-state transitions during fluidisation. Chem Eng Sci. 2006;62:408–15.
18. Virtanen S, Antikainen O, Yliruusi J. Determination of the crushing strength of intact tablets using Raman spectroscopy. Int J Pharm. 2008;360:40–6.
19. Pikal MJ, Roy ML, Shah S. Mass and heat transfer in vial freeze-drying of pharmaceuticals: role of the vial. J Pharm Sci. 1984;73:1224–37.
20. May JC, Grim E, Wheeler RM, West J. Determination of residual moisture in freeze-dried viral vaccines: Karl Fischer, gravimetric and thermogravimetric methodologies. J Biol Stand. 1982;10:249–59.
21. Chang L, Shepherd D, Sun J, Tang X, Pikal MJ. Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: implications for the mechanism of protein stabilization in the solid state. J Pharm Sci. 2005;94:1445–55.
22. Roos YH. Frozen state transitions in relation to freeze drying. J Therm Anal. 1997;48:535–44.
23. Hinrichs WLJ, Sanders NN, De Smedt SC, Demeester J, Frijlink HW. Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes. J Control Release. 2005;103:465–79.
24. Orford PD, Parker R, Ring SG. Aspects of the glass transition behavior of mixtures of carbohydrates of low molecular weight. Carbohydr Res. 1990;196:11–8.
25. Taylor LS, Zografi G. Sugar-polymer hydrogen bond interactions in lyophilized amorphous mixtures. J Pharm Sci. 1998;87:1615–21.
26. Simperler A, Kornherr A, Chopra R, Jones W, Motherwell WDS, Zifferer G. The glass transition temperatures of amorphous trehalose-water mixtures and the mobility of water: an experimental and in silico study. Carbohydr Res. 2007;342:1470–9.
27. Hancock BC, Zografi G. The relationship between the glass transition temperature and the water content of amorphous pharmaceutical solids. Pharm Res. 1994;11:471–7.
28. Shamblin SL, Tang X, Chang L, Hancock BC, Pikal MJ. Characterization of the time scales of molecular motion in pharmaceutically important glasses. J Phys Chem B. 1999;103:4113–21.
29. Carpenter JF, Crowe JH. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochem. 1989;28:3916–22.
30. Luthra S, Kalonia DS, Pikal MJ. Effect of hydration on the secondary structure of lyophilized proteins as measured by Fourier transform infrared (FTIR) spectroscopy. J Pharm Sci. 2007;96:2910–21.
31. Akao K-I, Yusei O, Asakawa N, Inoue Y, Sakurai M. Infrared spectroscopic study on the properties of the anhydrous form II of trehalose. Implication for the functional mechanism of trehalose as a biostabilizer. Carbohydr Res. 2001;334:233–41.
32. Prestrelski SJ, Arakawa T, Carpenter JF. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. II. Structural studies using infrared spectroscopy. Arch Biochem Biophys. 1993;303:465–73.
33. Chang BS, Beauvais RM, Dong A, Carpenter JF. Physical factors affecting the storage stability of freeze-dried interleukin-1 receptor antagonist: glass transition and protein conformation. Arch Biochem Biophys. 1996;331:249–58.
34. Kendrick B, Dong A, Allison S, Manning M, Carpenter JF. Quantitation of the area of overlap between second-derivative amide I infrared spectra to determine the structural similarity of a protein in different states. J Pharm Sci. 1996;85:155–8.
35. Wang B, Tchessalov S, Warne NW, Pikal MJ. Impact of sucrose level on storage stability of proteins in freeze-dried solids: I.

correlation of protein-sugar interaction with native structure preservation. J Pharm Sci. 2009;98:3131–44.
36. Franks F. Freeze-drying: From empiricism to predictability. Cryo-Letters. 1990;11:93–110.
37. Pikal MJ, Rigsbee DR, Roy ML, Galreath D, Kovach K, Wang B, *et al*. Solid state chemistry of proteins: II. The correlation of storage stability of freeze-dried human growth hormone (hGH) with structure and dynamics in the glassy solid. J Pharm Sci. 2008;97:5106–21.
38. D'Antiga L, Dhawan A, Davenport M, Mieli-Vergani G, Bjarnason I. Intestinal absorption and permeability in paediatric short-bowel syndrome: a pilot study. J Pediatr Gastroenterol Nutr. 1999;29:588–93.
39. Taylor RM, Bjarnason I, Cheeseman P, Davenport M, Baker AJ, Mieli-Vergani G, *et al*. Intestinal permeability and absorptive capacity in children with portal hypertension. Scand J Gastroenterol. 2002;37:807–11.
40. Clarke F, Solkot R, Corley RC. Metabolism of certain rare sugars. J Biol Chem. 1939;131:135–8.
41. Tomsik P, Sispera L, Rezacova M, Niang M, Stoklasova A, Cerman J, *et al*. Increased melibiose/rhamnose ratio in bile of rats with acute cholestasis. J Gastroenterol Hepatol. 2008;23:1934–40.