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Research Paper

Significance of Local Mobility in Aggregation of β-Galactosidase Lyophilized with Trehalose, Sucrose or Stachyose

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Purpose. The purpose of this study is to compare the effects of global mobility, as reflected by glass transition temperature (T_g) and local mobility, as reflected by rotating-frame spin-lattice relaxation time ($T_{1\rho}$) on aggregation during storage of lyophilized β-galactosidase (β-GA).
Materials and Methods. The storage stability of β-GA lyophilized with sucrose, trehalose or stachyose was investigated at 12% relative humidity and various temperatures (40–90°C). β-GA aggregation was monitored by size exclusion chromatography (SEC). Furthermore, the $T_{1\rho}$ of the β-GA carbonyl carbon was measured by ^{13}C solid-state NMR, and T_g was measured by modulated temperature differential scanning calorimetry. Changes in protein structure during freeze drying were measured by solid-state FT-IR.
Results. The aggregation rate of β-GA in lyophilized formulations exhibited a change in slope at around T_g, indicating the effect of molecular mobility on the aggregation rate. Although the T_g rank order of β-GA formulations was sucrose < trehalose < stachyose, the rank order of β-GA aggregation rate at temperatures below and above T_g was also sucrose < trehalose < stachyose, thus suggesting that β-GA aggregation rate is not related to (T-T_g). The local mobility of β-GA, as determined by the $T_{1\rho}$ of the β-GA carbonyl carbon, was more markedly decreased by the addition of sucrose than by the addition of stachyose. The effect of trehalose on $T_{1\rho}$ was intermediate when compared to those for sucrose and stachyose. These findings suggest that β-GA aggregation rate is primarily related to local mobility. Significant differences in the second derivative FT-IR spectra were not observed between the excipients, and the differences in β-GA aggregation rate observed between the excipients could not be attributed to differences in protein secondary structure.
Conclusions. The aggregation rate of β-GA in lyophilized formulations unexpectedly correlated with the local mobility of β-GA, as indicated by $T_{1\rho}$, rather than with (T-T_g). Sucrose exhibited the most intense stabilizing effect due to the most intense ability to inhibit local protein mobility during storage.

KEY WORDS: β-galactosidase; global mobility; local mobility; lyophilized formulation; solid-state stability.

INTRODUCTION

Close correlations between storage stability and molecular mobility have been demonstrated for various lyophilized formulations of peptides and proteins (1,2). Aggregation between protein molecules is a degradation pathway commonly observed in lyophilized protein formulations. The rate of protein aggregation is generally considered to depend on the translational mobility of protein molecules, which is related to structural relaxation (α-relaxation) of the formulation. Correlations between aggregation rates and structural relaxation have been shown in various protein systems in visible ways, such as enhancement of aggregation associated with decreases in glass transition temperature (T_g) (3–6) and changes in the temperature dependence of aggregation rates around T_g (7–10). However, recent studies have suggested that molecular mobility with a length scale shorter than structural relaxation (β-relaxation or local mobility), rather than structural relaxation, is critical to protein aggregation (11,12).

The rate of protein aggregation in lyophilized formulations is also affected by the degree of change in protein conformation produced during the freeze-drying process (1). Greater changes in protein conformation are considered to lead to enhanced aggregation during subsequent storage.

In this study, the significance of local mobility in aggregation of lyophilized β-galactosidase (β-GA), a model protein, is discussed in comparison with the significance of structural relaxation and conformational changes. β-GA underwent significant inactivation during freeze drying with dextran, thus suggesting that significant conformational changes occurred during the process (13). When freeze dried with polyvinylalcohol or methylcellulose, inactivation was not observed during freeze drying (10). However, the time

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courses of aggregation during subsequent storage were describable with the empirical Kohlrausch-Williams-Watts (KWW) equation, thus leading to speculation that there were protein molecules having different conformations resulting from stresses during the freeze-drying process, each aggregating with a different time constant (10,14). The temperature dependence of the aggregation rate measured at the initial stage changed around T_{mc} (T_g determined by NMR relaxation measurement (15)), showing an apparent correlation between aggregation rate and structural relaxation (10). In this study, the significance of local obility in aggregation of lyophilized β-GA was examined in comparison with that of structural relaxation. The aggregation rates of β-GA lyophilized with trehalose, sucrose or stachyose were measured at temperatures near T_g, and correlations were examined between aggregation rate and local mobility (as measured by solid-state NMR), T_g (a primary parameter of structural relaxation), or protein conformational changes (as measured by FT-IR).

MATERIALS AND METHODS

Preparation of Lyophilized β-GA Formulations

β-GA from Aspergillus oryzae (10 U/mg; molecular weight: 105,000; isoelectric point:4.6) was kindly provided by Amano Enzyme Inc. (Nagoya, Japan) and purified by dialysis against 2.5 mM sodium phosphate solution of pH 4.5 (adjusted with HCl). After concentrated by ultrafiltration, trehalose (203-02252, Wako Pure Chemical Ind. Ltd, Osaka, Japan), sucrose (S-9378, Sigma Chemical Co., St. Louis, MO, USA) or stachyose (S-4001, Sigma Chemical Co., St. Louis, MO, USA) solution was added to make a 3.3 mg/ml β-GA solution with various weight fractions of excipient. Two hundred microliters of the solution was frozen in a polypropylene sample tube (2.0 ml) by immersion in liquid nitrogen for 10 min, and then dried at a vacuum level below 5 Pa for 23.5 h in a lyophilizer (Freezevac C-1, Tozai Tsusho Co., Tokyo, Japan). The shelf temperature was between −35 and −30°C for the first 10 h, 20°C for the subsequent 10 h, and 30°C for the last 3.5 h.

Determination of Water Sorption and T_g of Lyophilized β-GA Formulations

Water vapor absorption isotherms were measured gravimetrically at 25°C for lyophilized β-GA formulations containing trehalose, sucrose or stachyose using the automated sorption analyzer (MB-300 G system, VTI Corp., FL, USA). Samples were dried at 25°C under a vacuum level below 0.1 Pa until changes in weight were less than 1 μg per 10 min. Water contents of the samples at partial vapor pressures of 0.10 and 0.20 (corresponding to 10 and 20% relative humidity (RH), respectively) were determined based on equilibrated sample weight (changes in weight of less than 1 μg per 10 min).

The T_g of lyophilized β-GA formulations was determined by modulated temperature differential scanning calorimetry (2920; TA Instruments, DE, USA). Before T_g measurements, samples were stored at 15°C for 24 h in a desiccator with a saturated solution of LiCl H_2O (12% RH). The conditions were as follows: modulation period of 100 s, a modulation amplitude of ±0.5°C, and an underlying heating rate of 1°C/min. Samples were put in a hermetic pan. Temperature calibration was performed using indium.

Determination of $T_{1\rho}$ of β-GA Carbonyl Carbon by ^{13}C Solid-state NMR

The rotating-frame spin-lattice relaxation time ($T_{1\rho}$) of β-GA carbonyl carbon in lyophilized formulations containing various weight fractions of trehalose, sucrose and stachyose was determined at 25°C using a UNITY plus spectrometer operating at a proton resonance frequency of 400 MHz (Varian Inc., CA, USA). Lyophilized samples were pre-equilibrated at 12% RH. Spin-locking field was equivalent to 19 kHz. The rotor size was 7 mm and spinning speed was 4 kHz. Peak height at approximately 180 ppm due to β-GA carbonyl carbon was followed with delay times of 1, 5, 10, 20, 30, 50 and 80 ms. Similar measurement of $T_{1\rho}$ was performed for lyophilized β-GA alone.

Fourier Transform Infra Red (FT-IR) Spectroscopy Measurements

FT-IR spectroscopy was performed using a JASCO FT/IR-6300 spectrometer (JASCO, Tokyo, Japan). A mixture of 100 mg KBr and 1–2 mg lyophilized β-GA formulation was pressed into a pellet under vacuum. A total of 256 scans and a resolution of 4 cm^{-1} were used for each spectrum. The second-derivative spectra were obtained from intact spectra without smoothing using Spectra Manager software version 2 (JASCO, Tokyo, Japan). The area of spectral absorbance was calculated using a baseline drawn between 1,600 and 1,700 cm^{-1}, and normalized for comparison between the formulations containing different excipients.

Determination of β-GA Aggregation Rate in Lyophilized Formulations

Lyophilized β-GA formulations containing trehalose, sucrose or stachyose, pre-equilibrated at 12% RH, were stored with a tight screw-cap at a constant temperature (40–90°C), removed at various times, and stored in liquid nitrogen until assayed. Samples were reconstituted in 1.7 ml of 200 mM phosphate buffer (pH 6.2), and injected into a size exclusion chromatography as described previously (10). The column (Tosoh G3000SW, 30 cm×7.5 mm, Tokyo) was maintained at 30°C, and 200 mM phosphate buffer (pH 6.2) was used as the mobile phase. The detection wavelength was 280 nm. Monomeric β-GA was determined based on the peak height of its chromatogram.

Reconstitution of lyophilized β-GA formulations after storage was also carried out using reconstitution mediums of 200 mM phosphate buffer (pH 6.2) containing 0.5% additives (dextran sulfate (197-08362, Wako Pure Chemical Ind. Ltd, Osaka, Japan), 2-hydroxylpropyl-β-cyclodextrin (C-0926, Sigma Chemical Co.), poly-L-lysine (P-7890, Sigma Chemical Co.), or pluronic (F-68, P-7061, Sigma Chemical Co., St. Louis, MO, USA).

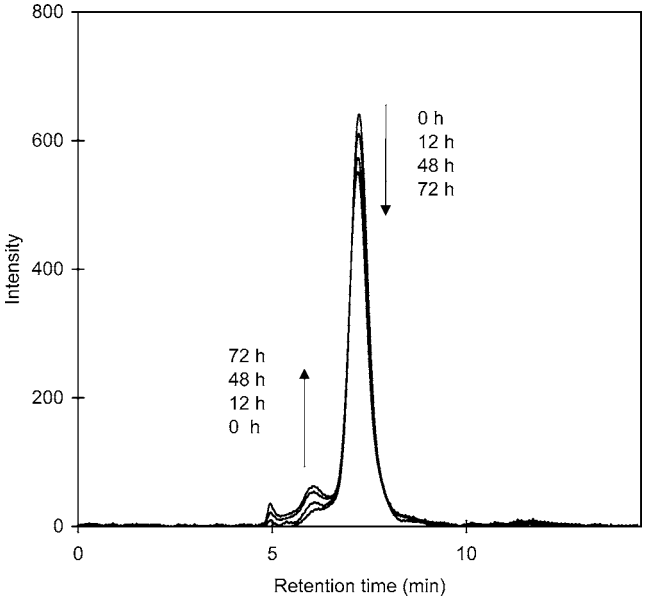


Fig. 1. Size-exclusion chromatograms of β-GA lyophilized with trehalose after various periods of storage at 80°C and 12%RH. The weight fraction of trehalose : 0.5.

RESULTS AND DISCUSSION

Aggregation of β-GA during Storage of Lyophilized Formulations

Figure 1 shows representative size-exclusion chromatograms of β-GA in lyophilized formulations, indicating that monomeric β-GA aggregates to larger sizes during storage. Tables I and II show the effects of reconstitution medium on the amount of monomeric β-GA remaining after storage of the formulation containing trehalose at temperatures below and above T_g, respectively. The amount of monomeric β-GA was not significantly affected by the addition of dextran sulfate or poly-L-lysine. Addition of pluronic also had no significant effect on the amount of monomeric β-GA. For lyophilized interleukin-2, the amount of aggregates after reconstitution was decreased by addition of poly-ions with high charge density, such as dextran sulfate and poly-L-lysine, and increased by addition of surfactants, such as pluronic, into the reconstitution buffer solution (16). This may be explained by assuming that the formation of aggregates from partially unfolded intermediates, as well as the reverse formation of native protein from intermediates, occur during the reconstitution process. In contrast, the lack of the effects of additives observed for β-GA aggregation indicates that neither formation of aggregates nor reverse formation of native protein from partially unfolded intermediates occurs during the reconstitution process. This finding suggests that β-GA aggregation occurs during the storage of lyophilized formulations, even at temperatures below T_g. Because large-scale diffusion of protein molecules is considered to be very limited in glassy solids, β-GA aggregation is considered to occur between protein molecules that are adjacent to each other without large-scale diffusion.

Temperature Dependence of β-GA Aggregation Rate

Figure 2 shows time courses of aggregation of β-GA at 50°C (below T_g) for lyophilized formulations with an excipient fraction of 0.33, and at 80°C (above T_g) for lyophilized formulations with an excipient fraction of 0.5. Similar time courses were obtained for formulations with various excipient fractions and at various temperatures. The wide range of the time courses could be better described by the KWW equation, but the initial stages of aggregation were describable with first-order kinetics. The solid line in Fig. 2 represents the theoretical time course of first-order kinetics.

The time required for 10% degradation (t_{90}) was calculated from the apparent first-order rate constant. Figure 3 shows the temperature-dependence of t_{90} determined for aggregation of β-GA lyophilized with sucrose, trehalose or stachyose at an excipient fractions of 0.33 and 0.5, 12% RH and various temperatures. For the sucrose and trehalose formulations, the temperature dependence of t_{90} exhibited a change in the slope at around T_g, suggesting significant effects of molecular mobility. For the stachyose formulation, the change in the slope was not obvious, because few data were available at temperatures above T_g. The values of t_{90} at T_g largely depended on the excipient; sucrose > trehalose > stachyose. The finding that the t_{90} values at T_g varied significantly between these three formulations suggests that β-GA aggregation rate is not primarily related to ($T-T_g$).

Table I. Effects of Reconstitution Medium on β-GA Aggregation Below T_g for 0.09 Trehalose Formulation

Additives in Reconstitution Medium	Peak Height for Monomeric β-GA (Relative to Solution Prior to Freeze Drying)			
	After Freeze Drying		After 24 h-storage at 70°C	
None	0.97	(0.01)	0.65	(0.01)
Dextran Sulfate	0.96	(0.00)	0.62	(0.01)
2-hydroxylpropyl-β-cyclodextrin	0.95	(0.01)	0.62	(0.00)
Poly-L-lysine	0.94	(0.01)	0.62	(0.00)
Pluronic	0.97	(0.01)	0.66	(0.01)

0.5% additives
Values in brackets represent standard deviation (n=3)

Table II. Effects of Reconstitution Medium on β-GA Aggregation Above T_g for 0.33 Trehalose Formulation

Additives in Reconstitution Medium	Peak Height for Monomeric β-GA (Relative to Solution Prior to Freeze Drying)			
	After Freeze Drying		After 9 h-storage at 90°C	
None	0.99	(0.01)	0.74	(0.03)
Dextran Sulfate	1.00	(0.00)	0.74	(0.01)
2-hydroxylpropyl-β-cyclodextrin	0.98	(0.00)	0.73	(0.01)
Poly-L-lysine	0.98	(0.01)	0.74	(0.01)
Pluronic	1.00	(0.01)	0.76	(0.02)

0.5% additives
Values in brackets represent standard deviation (n=3)

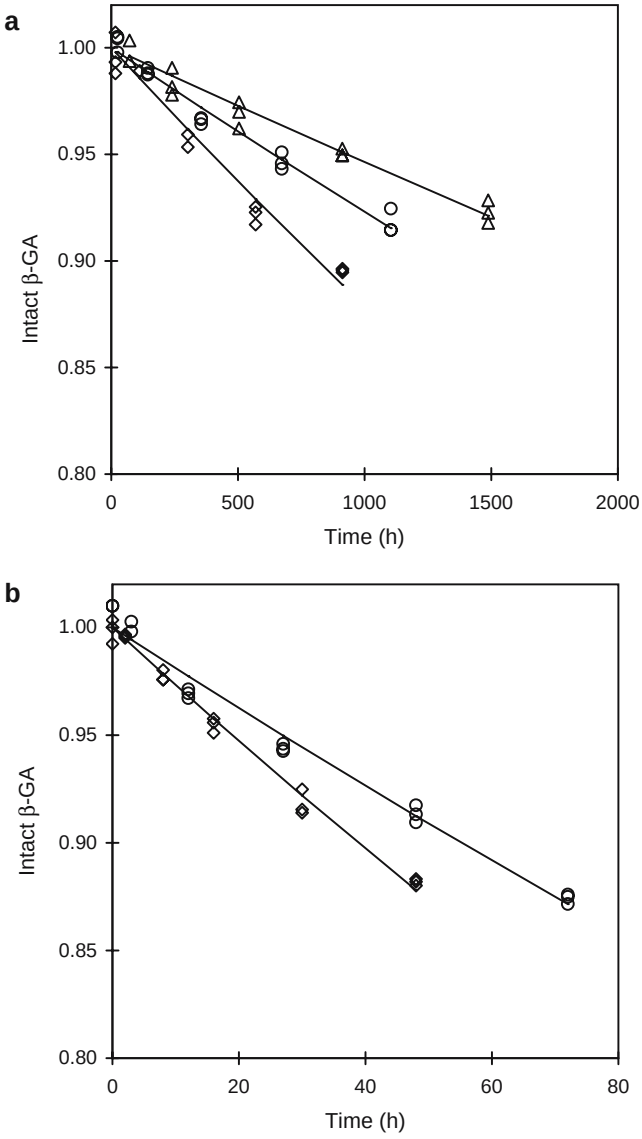


Fig. 2. Time courses of aggregation of β-GA lyophilized with sucrose (△), trehalose (O) or stachyose (◇). (**a**) aggregation at 50°C and excipient fraction of 0.33. (**b**) aggregation at 80°C and excipient fraction of 0.5.

Excipient-Fraction Dependence of β-GA Aggregation Rate

Figure 4 shows the dependence of t_{90} on the weight fraction of excipient at temperatures below T_g (50°C) and above T_g (80°C). As the weight fraction of excipient increased, t_{90} increased for all formulations. The values of t_{90} for the sucrose formulation in the amorphous state could not be determined at fractions above 0.5 at 50°C or above 0.33 at 80°C, because crystallization occurred during storage (crystallization was confirmed by the lack of crystallization peak in DSC thermograms). The t_{90} observed for β-GA aggregation showed a log-linear dependence on the excipient fraction, as reported for other proteins (11,12).

Figure 5 shows the water content and T_g determined for the lyophilized β-GA formulations with various weight fractions of trehalose, sucrose or stachyose. It has often been reported that lyophilized proteins without excipients do not show a distinct change in heat capacity in DSC thermograms. The T_g of lyophilized β-GA alone could not be determined in the dry state, but it could be estimated at 12% RH from small changes in heat capacity (Fig. 6). The T_g value determined at 12% RH depended on the excipient fraction (Fig. 5); T_g decreased with increasing excipient fraction from 0 to 0.3. Only a single T_g was observed in the range of excipient fractions from 0 to 0.3 for all formulations, suggesting that these formulations are a single glassy phase on levels detectable by DSC.

As shown in Figs. 4 and 5, the rank order of t_{90} at a certain excipient fraction was sucrose > trehalose > stachyose, whereas that of T_g of β-GA formulations was sucrose < trehalose < stachyose. The value of t_{90} increased significantly with increasing excipient fraction, even at small excipient fractions, in which T_g decreased significantly with increasing excipient fraction. These findings indicate that β-GA aggregation rate is not primarily related to (T-T_g).

Figure 7 shows the amount of monomeric β-GA remaining after freeze drying with sucrose, trehalose or stachyose. Significant changes were observed at an excipient fraction of 0.09 for all formulations. The stachyose formulation exhibited the largest degree of β-GA aggregation during freeze drying. This finding suggests that freeze-drying processes cause changes in protein conformation at differing degrees between excipients, which in turn leads to the differences in β-GA aggregation rate observed between excipients. FT-IR is known to be useful for detecting changes in protein conformation produced during the freeze-drying process (17). Figure 8 compares the second derivative FT-IR

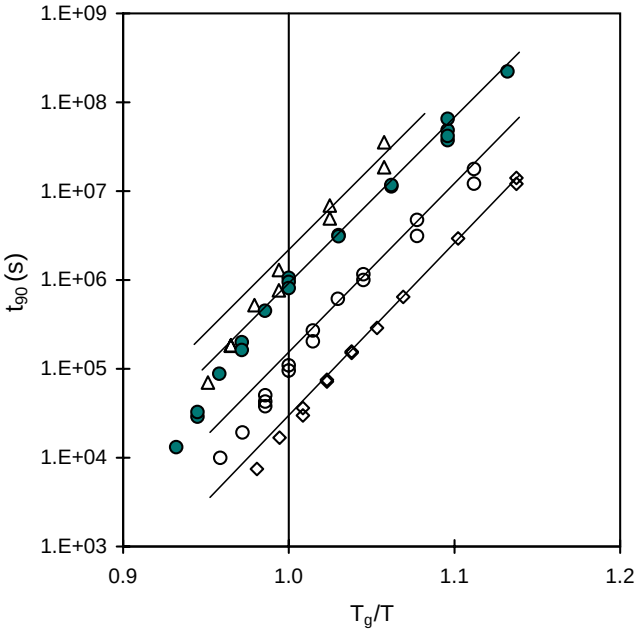


Fig. 3. T/T_g-dependence of t_{90} for aggregation of β-GA lyophilized with sucrose (△), trehalose (O●) or stachyose (◇). The weight fraction of excipient : 0.33 (△O◇) and 0.5 (●).

spectra between the sucrose, trehalose and stachyose formulations. Significant differences in spectra were not observed between the excipients, and the differences in β-GA aggregation rate observed between the excipients could not be attributed to differences in protein secondary structure. It is known that changes in the tertiary structure of protein molecules created during freeze-drying processes can lead to

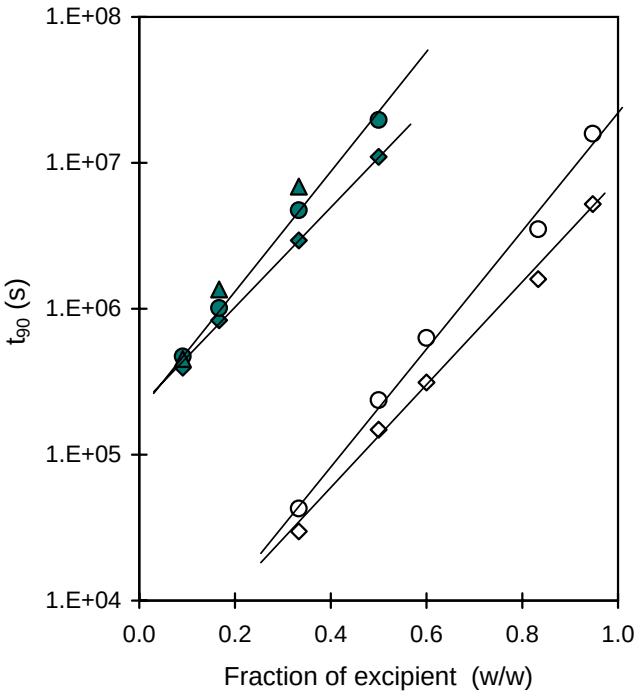
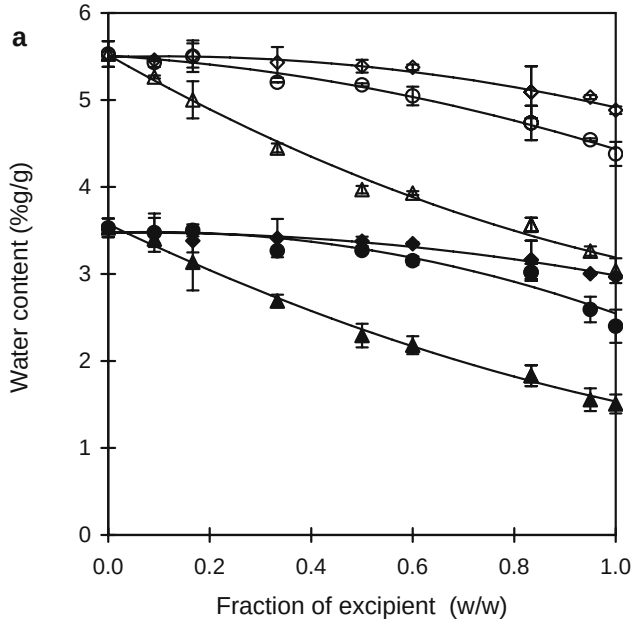


Fig. 4. Dependence of t_{90} on the weight fraction of excipient. The value of t_{90} was determined at 80°C and 12%RH for trehalose (O) and stachyose (◇), and at 50°C and 12%RH for trehalose (●), sucrose (▲) and stachyose (♦).



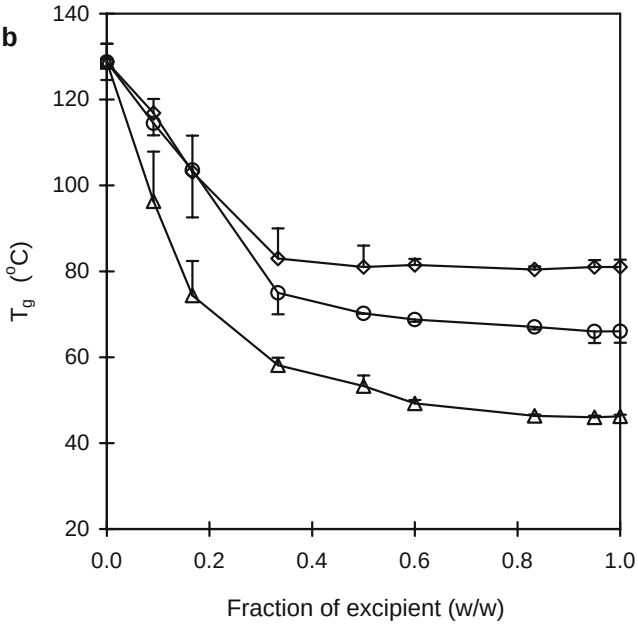


Fig. 5. Water content (**a**) and T_g (**b**) of lyophilized β-GA formulations containing trehalose (O●), sucrose (△▲), or stachyose (◇♦) as a function of the weight fraction of excipient. (**a**) *closed symbols*: 10%RH; *open symbols*: 20%RH. 25°C. (**b**) 12%RH. sd (n=3).

protein aggregation during storage. A possibility that a tertiary structural change is responsible for the differences in β-GA aggregation rate observed between the excipients cannot be excluded.

Significance of Local Mobility, as Determined by $T_{1\rho}$ of β-GA Carbonyl Carbon, and Structural Relaxation in Protein Aggregation

It is generally considered that the rate of protein aggregation, an intermolecular reaction, is mainly determined by structural relaxation that allows for large-scale

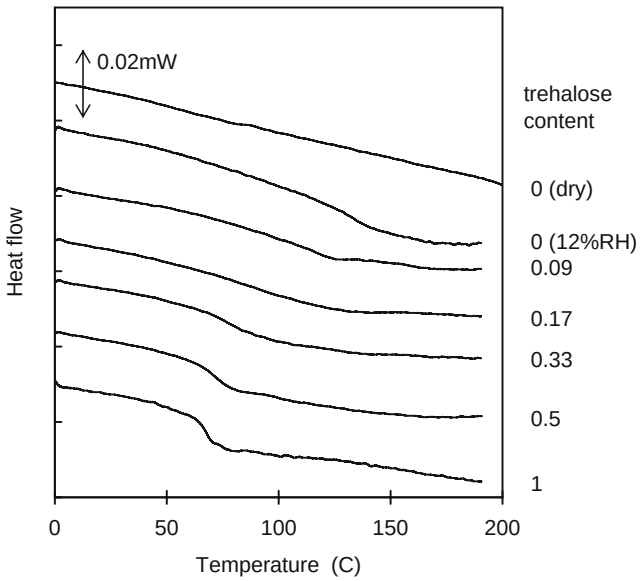


Fig. 6. DSC thermogram for β-GA lyophilized with various weight fractions of trehalose.

diffusion of reactants. From the finding that the t_{90} versus T_g/T plots for the lyophilized β-GA formulations exhibited a change in slope around T_g, β-GA aggregation rate appeared to correlate with structural relaxation. Although β-GA aggregation rate was not related to $(T\text{-}T_g)$, this may be explained by assuming that the fragility and fictive temperature of the formulation vary with the excipient. Because the structural relaxation times of the β-GA formulations were not determined in this study, correlations between β-GA aggregation rate and structural relaxation could not be elucidated.

Meanwhile, the local mobility of β-GA was determined by $T_{1\rho}$ of β-GA carbonyl carbon. Figure 9 shows the time course of rotating-frame spin-lattice relaxation at 25°C and 12% RH for the carbonyl carbon of β-GA lyophilized with sucrose, trehalose or stachyose at an excipient fraction of 0.5.

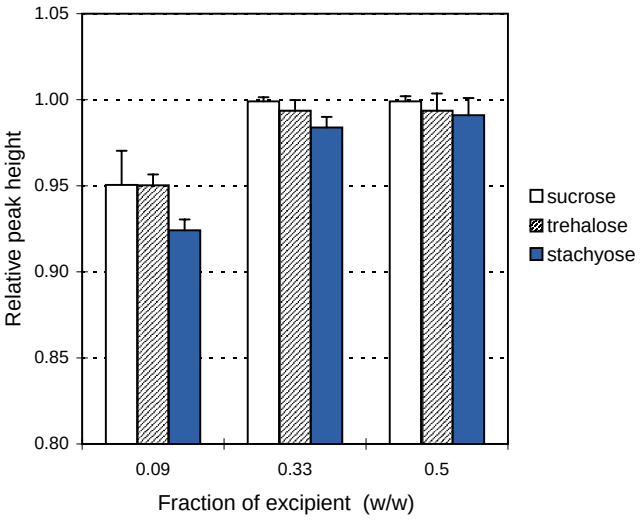
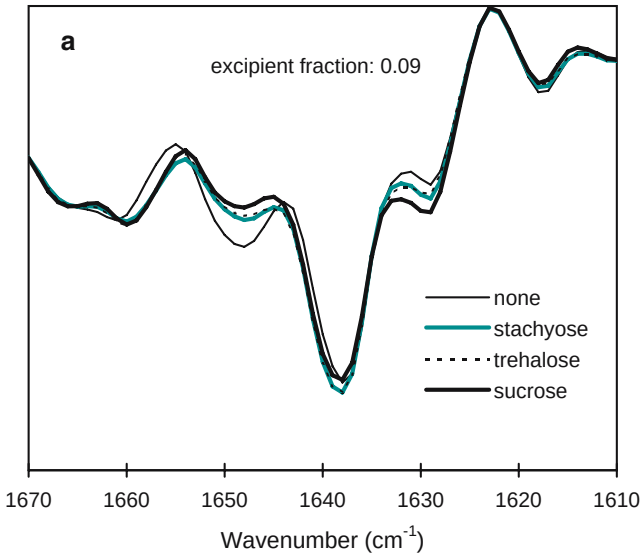


Fig. 7. Ratio of monomeric β-GA remaining after freeze drying with sucrose, trehalose or stachyose. *Bars* represent standard deviation (n=3).

Spin-lattice relaxation was significantly retarded by the addition of excipient. Sucrose resulted in the largest degree of retardation, and there were no significant differences in the degree of retardation between the trehalose and stachyose formulations. The time course of spin-lattice relaxation was describable with a bi-exponential equation including two different $T_{1\rho}$ values. The longer $T_{1\rho}$ value was estimated by curve fitting using a shorter $T_{1\rho}$ of 9 ms and a proportion of 13% for carbonyl carbons with the shorter $T_{1\rho}$. Figure 10 shows the estimates for the longer $T_{1\rho}$ of the dominating proportion, plotted as a function of the excipient fraction. The $T_{1\rho}$ for the sucrose formulation increased significantly with excipient fraction. For the stachyose formulation, in contrast, increases in $T_{1\rho}$ were not significant at an excipient fraction of 0.09, and $T_{1\rho}$ was less than in the sucrose formulation at higher excipient fractions. $T_{1\rho}$ for the trehalose formulation exhibited intermediate behavior when compared to the sucrose and stachyose formulations. The rank order of the ability of excipients to decrease the local



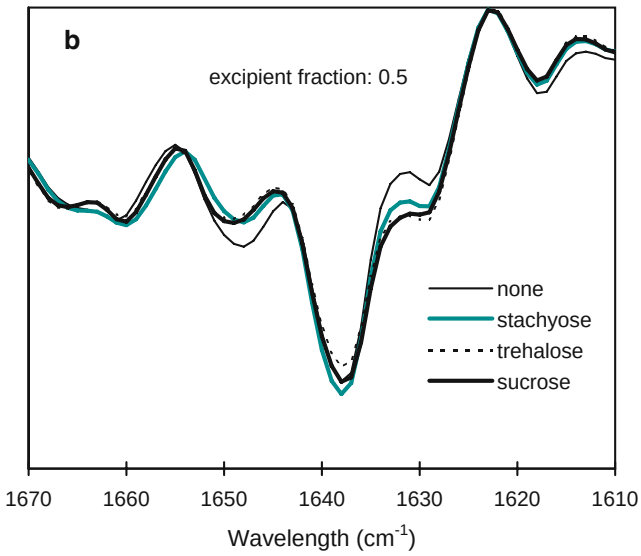


Fig. 8. Second derivative FT-IR spectra for β-GA lyophilized with sucrose, trehalose or stachyose of 0.09 (**a**) and 0.5 fractions (**b**).

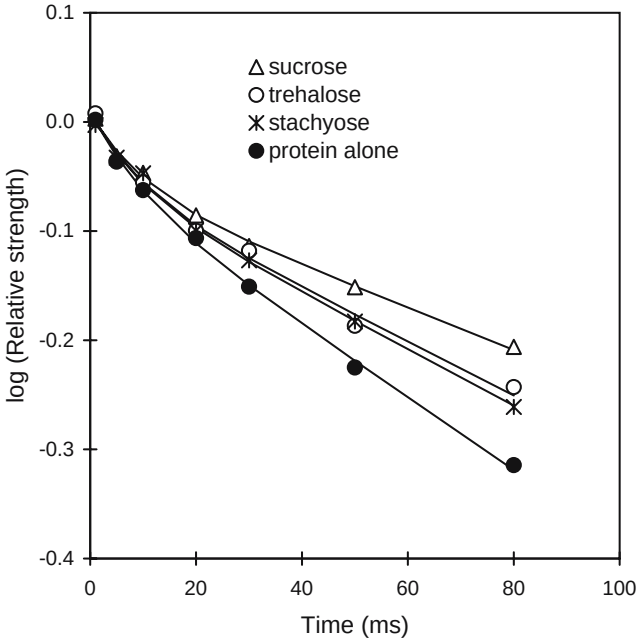


Fig. 9. Time course of spin-lattice relaxation at 25°C and 12%RH for carbonyl carbon of β-GA lyophilized with sucrose, trehalose or stachyose. The weight fraction of excipient : 0.5.

mobility of β-GA appeared to be the same as the rank order of their ability to decrease aggregation rate. This finding suggests that local mobility is a primary factor that affects the stability of lyophilized β-GA formulations; sucrose more potently inhibits local mobility of β-GA, and thus more strongly inhibits β-GA aggregation.

Local mobility is generally considered to follow Arrhenius kinetics. If local mobility is mainly responsible for β-GA aggregation, the temperature dependence of t_{90} should not show a change in slope around T_g. The non-Arrhenius temperature dependence observed for the t_{90} of β-GA aggregation, which is considered to be governed by local mobility, may be explained by assuming that local mobility of protein is coupled with structural relaxation. For bovine serum γ-globulin, the local mobility of protein, as measured by the laboratory-frame spin-lattice relaxation time (T_1) of protein carbonyl carbon, exhibited Arrhenius temperature dependence when lyophilized without excipient (18). When lyophilized with dextran, in contrast, the local mobility of protein exhibited a change in the slope of temperature dependence around the T_{mc} (T_g determined by NMR relaxation measurement), as did local mobility of dextran, as measured by T_1 of dextran methine carbon. These findings suggested that the local mobility of protein was coupled with the structural relaxation of lyophilized solids. The same may be said for the local mobility of protein and structural relaxation of β-GA lyophilized with sucrose, trahalose or stachyose. The local mobility of β-GA may exhibit Arrhenius temperature dependence in the absence of excipient. Upon the addition of excipient, local mobility may become to be coupled with structural relaxation, and the temperature dependence of protein local mobility may become to deviate from Arrhenius behavior.

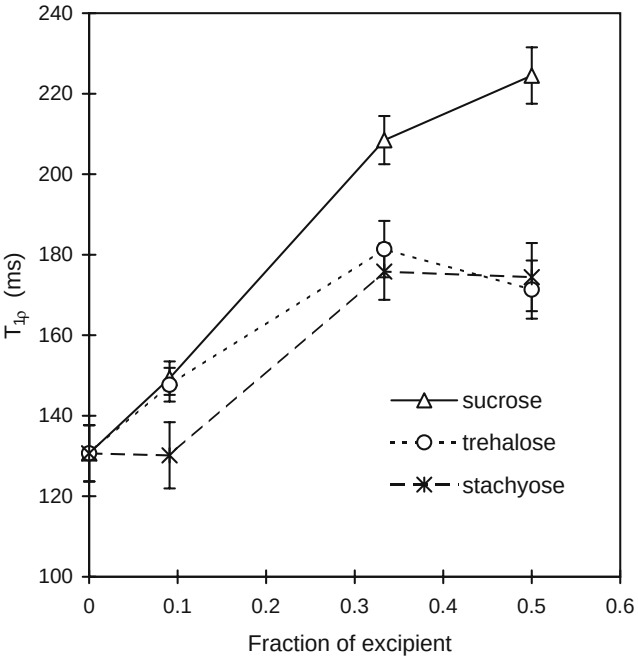


Fig. 10. Effect of weight fraction of excipient on $T_{1\rho}$ of carbonyl carbon at 25°C and 12%RH for β-GA lyophilized with sucrose, trehalose or stachyose

The great increase in t_{90} with increasing excipient fraction observed for β-GA aggregation rate, as indicated by log-linear dependence on the excipient fraction, may be attributed mainly to the effect of excipient inhibiting protein local mobility in addition to the effect of excipient diluting protein molecules.

CONCLUSION

The aggregation rate of β-GA lyophilized with sucrose, trehalose or stachyose unexpectedly correlated with the local mobility of β-GA rather than with ($T-T_g$). An increase in the weight fraction of excipient appeared to increase the effects of excipient decreasing local mobility, resulting in increases in the stability of β-GA. Sucrose exhibited the most intense stabilizing effect due to the most intense ability to inhibit local protein mobility during storage.

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