Inactivation and Aggregation of β-Galactosidase in Lyophilized Formulation Described by Kohlrausch-Williams-Watts Stretched Exponential Function

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Received May 27, 2003; accepted June 30, 2003

Purpose. To examine whether the empirical Kohlrausch-Williams-Watts (KWW) equation is applicable not only to protein aggregation but also to protein denaturation in lyophilized formulations. Lyophilized β-galactosidase (β-GA) formulations containing polyvinylalcohol and methylcellulose were used as model formulations. The possibility of predicting storage stability based on the temperature dependence of the estimated parameters of inactivation/aggregation—time constant (τ) and its distribution (β) is discussed.

Methods. Protein aggregation in lyophilized β-GA formulations at 10–70°C and 6–43% relative humidity was determined as a function of time by size exclusion chromatography. Enzyme activity was also determined using 2-nitrophenyl-β-D-galactopyranoside as a substrate.

Results. Inactivation and aggregation of β-GA were describable with the empirical KWW equation, regardless of whether the temperature was above or below the NMR relaxation-based critical mobility temperature (T_{mc}) or whether protein molecules with different degrees of deformation resulting from stresses during lyophilization exist in the formulation. The estimated β parameter for protein aggregation decreased rapidly as temperature increased beyond T_{mc} because the mobility of polymer molecules increased in the initial stages of glass transition. The time required for 10% enzyme to aggregate (t_{90}) calculated from the τ and β parameters exhibited a change in temperature dependence gradient near T_{mc}. In contrast, t_{90} for protein inactivation exhibited temperature dependence patterns varying with the excipients.

Conclusions. The t_{90} calculated from the estimated τ and β parameters was found to be a useful parameter for evaluating the stability of lyophilized β-GA formulations. The prediction of t_{90} by extrapolation was possible in the temperature range in which β did not rapidly vary with temperature.

KEY WORDS: inactivation; aggregation; β-galactosidase; molecular mobility; glass transition.

INTRODUCTION

The empirical Kohlrausch-Williams-Watts (KWW) equation [Eq. (1)] has been used to describe molecular relaxation processes within amorphous pharmaceuticals (1–4):

$$\phi(t) = \exp\left[-\left(\frac{t}{\tau_{KWW}}\right)^{\beta_{KWW}}\right] \quad (1)$$

where $\phi(t)$ is a relaxation time function and t is time. If it is assumed that nonexponential relaxation behaviors arise from multiple relaxation processes with a distribution of relaxation times, τ_{KWW} represents an average relaxation time, and β_{KWW} a measure of the distribution of relaxation times.

Application of Eq. (1) to protein degradation has been proposed, assuming that the protein exists in a number of configurations, each configuration degrading in first-order fashion with a different rate constant (5). Protein aggregation in lyophilized γ-globulin formulations containing dextran and methylcellulose (MC) was describable with the empirical KWW equation, assuming that there were protein molecules with different degrees of deformation resulting from stresses during the freeze-drying process, each aggregating with a different aggregation time constant (6). The values of parameter β_a obtained by fitting the aggregation data to Eq. (1) exhibited a rapid change when temperature increased beyond the NMR relaxation-based critical mobility temperature (T_{mc}). This finding suggested that protein aggregation behavior was largely affected by glass transition because T_{mc} is the temperature at which the glass transition begins to be detected by NMR relaxation measurements (7).

The purpose of the present study is to examine whether the empirical KWW equation is applicable to not only protein aggregation but also to protein denaturation in lyophilized formulations. β-Galactosidase (β-GA) is used as a model protein, and polyvinylalcohol (PVA) and MC are used as model excipients. The possibility of predicting the storage stability of lyophilized protein formulations based on the temperature dependence of the estimated parameters corresponding to τ_{KWW} and β_{KWW} (τ and β) is discussed.

MATERIALS AND METHODS

Materials

β-GA from *Aspergillus oryzae* (10 U/mg) was kindly provided by Amano Pharmaceutical Co. (Nagoya) and purified by dialysis against phosphate–citrate buffer (pH 4.5, 2.5 mM) followed by freeze-drying (80 U/mg). PVA (average molecular weight of 13,000 to 23,000) and MC (136-07172) were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Wako Pure Chemical Industries Ltd. (Osaka), respectively.

Preparation of Lyophilized Formulations

Four hundred microliters of 2.5% polymer solution containing β-GA (50:1 w/w) was frozen in a polypropylene sample tube (10 mm diameter) 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). 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. Lyophilized cakes showed no visible evidence of collapse. The enzyme activity of lyophilized β-GA formulations containing PVA and MC was 99.9 ± 0.6% and 95.9 ± 0.6% of that in the solution before freeze-drying, respectively. The amount of aggregated protein produced during freeze-drying was 5.7 ± 0.6% and 8.8 ± 0.8% in the presence of PVA and MC, respectively.

Lyophilized β-GA formulations were stored at 15°C for 24 h in a desiccator with a saturated solution of $LiBr \cdot H_2O$

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0724-8741/03/1000-1655/0 © 2003 Plenum Publishing Corporation

[6% relative humidity (RH)], $LiCl \cdot H_2O$ (12% RH), potassium acetate (23% RH), and $K_2CO_3 \cdot 2H_2O$ (43% RH).

The NMR relaxation-based critical mobility temperature (T_{mc}) of lyophilized formulations containing PVA and MC was determined to be 35°C at 23% RH and 55°C at 43% RH, respectively, as described previously (7).

Determination of Inactivation and Aggregation of β-GA

Lyophilized BGG formulations in screw-capped polypropylene tubes were stored at temperatures ranging from 10°C to 70°C (±0.1°C) and 6% to 43% RH. The samples were removed at appropriate intervals and dissolved in distilled water to make 1 μg/ml GA solution. Activity was determined at 30°C and pH 4.5 using 2-nitrophenyl-β-D-galactopyranoside as a substrate (8).

Lyophilized samples were dissolved in 1.7 ml of 200 mM phosphate buffer (pH 6.2) and injected into a size-exclusion chromatograph as described previously (6). 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. Typical chromatograms are shown in Fig. 1. The amount of protein within an intact molecule of a given size was measured based on the peak height of its chromatogram.

RESULTS

Protein Aggregation

Figure 2 shows the time course of protein aggregation in a lyophilized β-GA formulation containing PVA. The data were fitted to Eq. (1) using nonlinear regression, providing τ_1 and β_1 values that correspond to τ_{KWW} and β_{KWW} in Eq. (1), respectively. The obtained regression curve was plotted as a function of the time scaled to τ_1 in Fig. 3. Protein aggregation at 6% RH/10–70°C and 23% RH/10–40°C exhibited a linear regression curve, indicating that aggregation is describable by first-order kinetics with a β_1 value of unity. In contrast, protein aggregation at 23% RH/50–70°C exhibited a nonlinear regression curve with a β_1 value less than 1, indicating that aggregation diverged from first-order kinetics under these conditions.

Figure 4 shows the time course of protein aggregation in lyophilized β-GA formulation containing MC, and Fig. 5 shows the regression curve plotted as a function of the time scaled to τ_1. Aggregation exhibited a linear regression curve at 12% RH/20–30°C, and a nonlinear regression curve at 12% RH/40–70°C and 43% RH/20–70°C.

Table I shows the τ_1 and β_1 values obtained for protein aggregation. For the PVA formulation, β_1 tended to decrease from unity when temperature increased beyond T_{mc} (35°C at 23% RH and higher than 70°C at 6% RH). In contrast, β_1 was less than unity even at temperatures below T_{mc} (55°C at 43% RH and higher than 70°C at 12% RH) for the MC formulation, except for aggregation at 20–30°C/12% RH. In addition, a relatively large decrease in β_1 was observed around T_{mc} at 43% RH.

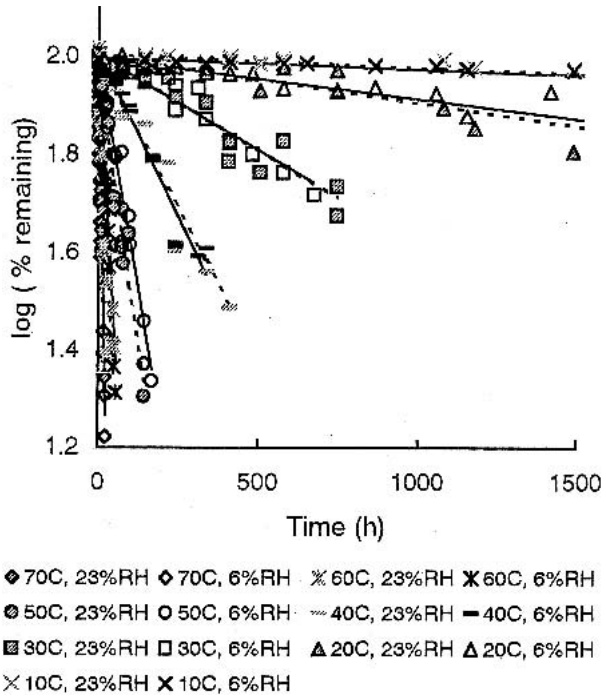

Fig. 2. Protein aggregation in lyophilized β-GA formulation containing PVA as a function of time.

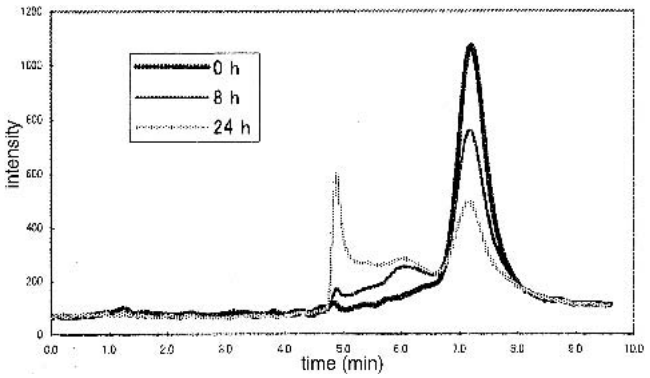

Fig. 1. Typical size exclusion chromatograms of lyophilized β-GA formulation containing PVA stored at 60°C and 23% RH.

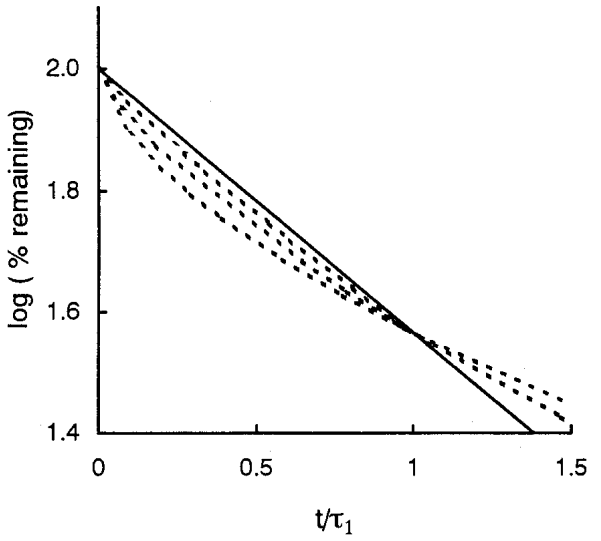

Fig. 3. Regression curves for protein aggregation in lyophilized β-GA formulation containing PVA as a function of time scaled to τ_1.

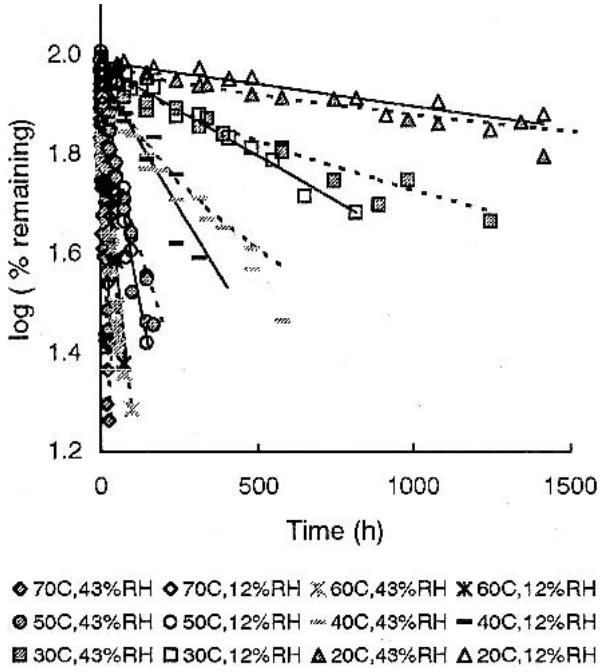

Fig. 4. Protein aggregation in lyophilized β-GA formulation containing MC as a function of time.

Protein Inactivation

An equation using τ_2 and β_2 instead of τ_{KWW} and β_{KWW} in Eq. (1) was used for fitting protein inactivation data. Figures 6 and 7 show the time courses for inactivation of β-GA in lyophilized formulations containing PVA and MC, respectively, plotted as a function of the time scaled to τ_2. Protein inactivation was describable by first-order kinetics with a β_2 value of unity at all the temperatures examined at 6% RH and 23% RH for the PVA formulation. In contrast, the MC formulation exhibited a nonlinear regression curve at 60–70°C/43% RH.

Table II shows the τ_2 and β_2 values obtained for protein inactivation. The β_2 value largely decreased when temperature increased beyond T_{mc} for the MC formulation, whereas the β_2 value remained unity even at temperatures above T_{mc} for the PVA formulation.

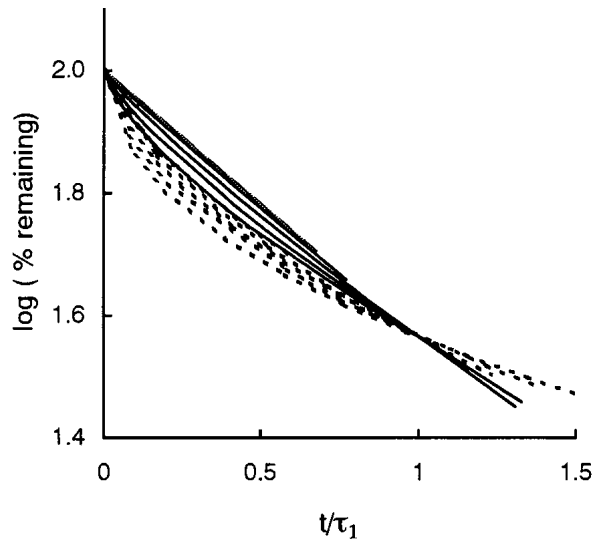

Fig. 5. Regression curves for protein aggregation in lyophilized β-GA formulation containing MC as a function of time scaled to τ_1.

Temperature Dependence of Aggregation and Inactivation

Time required for 10% aggregation or inactivation (t_{90}) was calculated from the τ (τ_1 or τ_2) and β (β_1 or β_2) values according to Eq. (2). Figures 8 and 9 show the temperature dependence of t_{90} obtained for protein aggregation and inactivation in lyophilized formulations containing PVA and MC, respectively. The temperature dependence of t_{90} for protein inactivation in the PVA formulation was linear both at 6% RH and 23% RH. A linear temperature dependence was also observed for the t_{90} of protein aggregation at 6% RH (at which T_{mc} was higher than the temperature range shown in Fig. 8), but a change in the temperature dependence gradient was observed near T_{mc} for protein aggregation at 23% RH.

$$t_{90} = \tau \exp\left(\frac{\ln(-\ln 0.9)}{\beta}\right) \quad (2)$$

On the other hand, the t_{90} of aggregation and inactivation in the MC formulation exhibited a linear temperature dependence at 12% RH (at which T_{mc} was higher than the temperature range shown in Fig. 9) but diverged from linearity when temperature increased beyond T_{mc} at 43% RH.

DISCUSSION

Inactivation and Aggregation in the PVA Formulation

Protein inactivation in the PVA formulation was describable by first-order kinetics at all the conditions examined, even at temperatures above T_{mc} (Table II). In contrast, protein aggregation in the PVA formulation was describable by first-order kinetics only in the temperature range below T_{mc} (Table I). These findings suggest that in the glassy PVA formulation at temperatures below T_{mc}, native β-GA undergoes first-order inactivation to denatured protein, which further undergoes aggregation.

When glass transition begins to occur with increasing temperature, and PVA molecules with partially enhanced mobility begin to appear at temperatures around T_{mc}, the diffusion rate of protein molecules is expected to exhibit a greater average value and a larger distribution. This may bring about decreases in β_1 for protein aggregation at temperatures above T_{mc}. Similar decreases in β have been observed for aggregation of γ-globulin in lyophilized formulations containing dextran and MC (6).

Compared with aggregation in the PVA formulation, appearance of PVA molecules with partially enhanced mobility at temperatures around T_{mc} did not change β_2 values for inactivation. This finding suggests that changes in mobility of the PVA molecules caused by the glass transition do not affect protein inactivation as intensely as aggregation that involves intermolecular collisions.

The t_{90} calculated from τ and β for protein aggregation was smaller than that for protein inactivation (Fig. 8). If protein inactivation is followed by aggregation, t_{90} for aggregation should be larger than t_{90} for inactivation. Therefore, it is suggested that the lyophilized formulation contained a certain amount of denatured protein even before storage. Because no

Table I. τ_1 and β_1 Obtained for β-GA Aggregation by Curve-Fitting to the KWW Equation

Temperature (°C)	PVA				MC			
	6% RH		23% RH		12% RH		43% RH	
	τ_1 (h)	β_1	τ_1	β_1	τ_1	β_1	τ_1	β_1
10	1.87×10^4	1	1.74×10^4	1	—	—	—	—
20	5.29×10^3	1	4.56×10^3	1	4.89×10^3	1	7.92×10^3	0.688
30	1.13×10^3	1	1.11×10^3	1	1.20×10^3	1	2.13×10^3	0.697
40	3.23×10^2	1	3.56×10^2	1	4.02×10^2	0.954	5.83×10^2	0.624
50	1.15×10^2	1	89.2	0.875	1.10×10^2	0.873	1.37×10^2	0.650
60	38.7	1	27.9	0.724	54.2	0.779	39.4	0.541
70	13.9	1	10.3	0.607	21.4	0.699	10.4	0.486

activity was lost, but approximately 6% of aggregation was observed during the freeze-drying process as described in the Materials and Methods section, the smaller t_{90} for aggregation may be explained by assuming that the β-GA powder used in the present study originally contained denatured protein that underwent aggregation during freeze-drying and the initial stages of storage.

Inactivation and Aggregation in the MC Formulation

Inactivation and aggregation behaviors of β-GA in the MC formulation were substantially different from those in the PVA formulation. Divergence from first-order kinetics was observed for protein inactivation at temperatures above T_{mc} (Table II). Increases in molecular mobility caused by glass transition appear to affect protein inactivation in the MC formulation. This may be explained by assuming that MC does not possess the ability to stabilize protein, in contrast with PVA, which interacts with protein and prevents β-GA from inactivation with increases in molecular mobility.

Although protein aggregation in the PVA formulation diverged from first-order kinetics only at temperatures above T_{mc}, protein aggregation in the MC formulation diverged from first-order kinetics even at temperatures below T_{mc} (Table I). This may be attributed to the presence of protein molecules with different degrees of deformation resulting from stresses created during the freeze-drying process, as previously suggested for aggregation of γ-globulin in lyophilized formulations containing dextran and MC (6). β-GA in the MC formulation may be considered to undergo inactivation and aggregation in the manner in which each denatured protein with a different degree of deformation that has been produced during the freeze-drying process undergoes further deformation in a sequential manner during storage, and highly deformed protein is susceptible to aggregation. Thus, the time required for protein to aggregate is expected to have a distribution represented by β_1 and an average represented by τ_1 even at temperatures below T_{mc}. The finding of significant activity loss of β-GA during freeze-drying with MC, as described in the Materials and Methods section, suggests that protein molecules with different degrees of deformation are formed during the freeze-drying process, supporting the above interpretation.

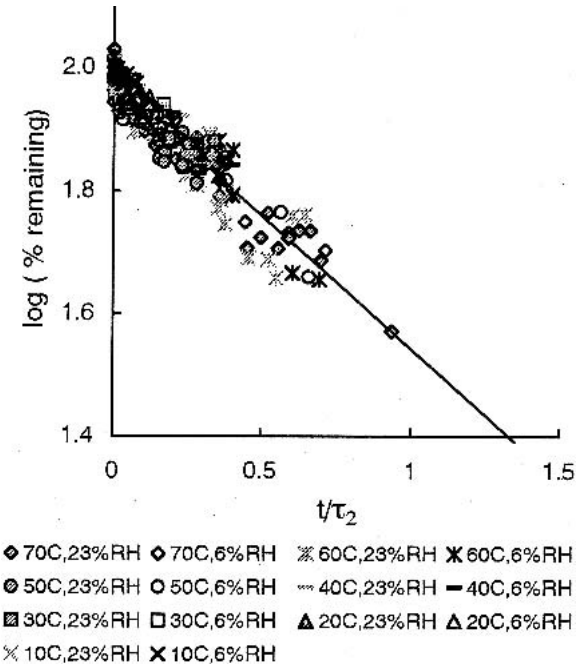

Fig. 6. Regression curves for inactivation of β-GA in lyophilized formulation containing PVA as a function of time scaled to τ_2.

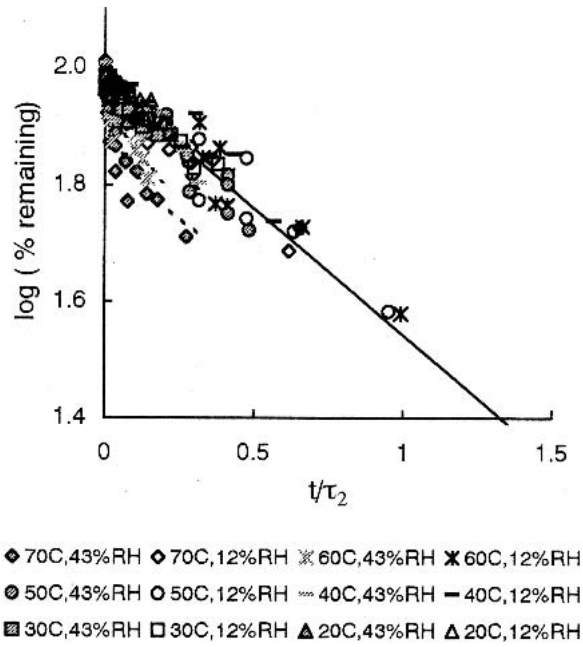

Fig. 7. Regression curves for inactivation of β-GA in lyophilized formulation containing MC as a function of time scaled to τ_2.

Table II. τ_2 and β_2 Obtained for β-GA Inactivation by Curve-Fitting to the KWW Equation

Temperature (°C)	PVA				MC			
	6% RH		23% RH		12% RH		43% RH	
	τ_2 (h)	β_2	τ_2	β_2	τ_2	β_2	τ_2	β_2
20	1.41×10^4	1	1.36×10^4	1	9.13×10^3	1	2.49×10^4	1
30	4.43×10^3	1	4.01×10^3	1	2.15×10^3	1	6.97×10^3	1
40	8.40×10^2	1	9.48×10^2	1	5.50×10^2	1	1.82×10^3	1
50	2.55×10^2	1	3.12×10^2	1	1.51×10^2	1	3.47×10^2	1
60	79.5	1	74.5	1	72.5	1	2.05×10^2	0.579
70	22.4	1	28.7	1	27.6	1	56.0	0.419

If protein molecules with different degrees of deformation resulting from stresses during the freeze-drying process exist in the MC formulation, the β value for aggregation should be less than unity under all the temperature and humidity conditions examined. However, β_1 of unity was obtained for aggregation at 12% RH/20–30°C (Table I). This may be attributed to very slow aggregation rates at these temperatures, even for highly deformed protein molecules. The distribution of the aggregation time constant that arises from the presence of protein molecules with different degrees of deformation is expected to become apparent as the aggregation rate increases with increasing temperature.

Prediction of Shelf Life Based on the τ and β Parameters

The τ values obtained for protein aggregation and inactivation (Tables I and II) correspond to the τ_{KWW} in Eq. (1) and represent the time required for 37% inactivation/aggregation regardless of the values of β, which is a measure of the distribution of inactivation/aggregation time constants. In contrast, τ_Γ calculated according to Eq. (3), which has been used for comparing the relaxation behavior of amorphous materials having different τ_{KWW} and β_{KWW} values (3), appears to be a useful parameter for representing inactivation/aggregation behaviors. However, τ_Γ corresponds to the time required for a larger amount of protein to inactivate/aggregate, as β becomes smaller. Therefore, in the case of degradation giving a small β value, τ_Γ represents the time before which most protein molecules have undergone inactivation/aggregation. Furthermore, τ_Γ unexpectedly increases with increasing temperature when β varies rapidly with temperature, as previously reported (6).

$$\tau_\Gamma = \frac{\tau_{KWW}}{\beta_{KWW}} \Gamma\left(\frac{1}{\beta_{KWW}}\right) \tag{3}$$

where Γ is the gamma function defined as:

$$\Gamma(n+1) \equiv \int_0^\infty e^{-x} x^n dx$$

Compared with τ_Γ, the time required for 10% inactivation/aggregation (t_{90}), which is also derived from τ and β according to Eq. (2), may be a more appropriate measure for evaluating inactivation and aggregation behaviors of protein pharmaceuticals. The t_{90} obtained for aggregation in the PVA formulation and that obtained for aggregation and inactivation in the MC formulation exhibited a change in temperature dependence gradient near T_{mc} (Figs. 8 and 9). This change in the gradient can be attributed to rapid changes in β resulting from changes in molecular mobility around T_{mc}.

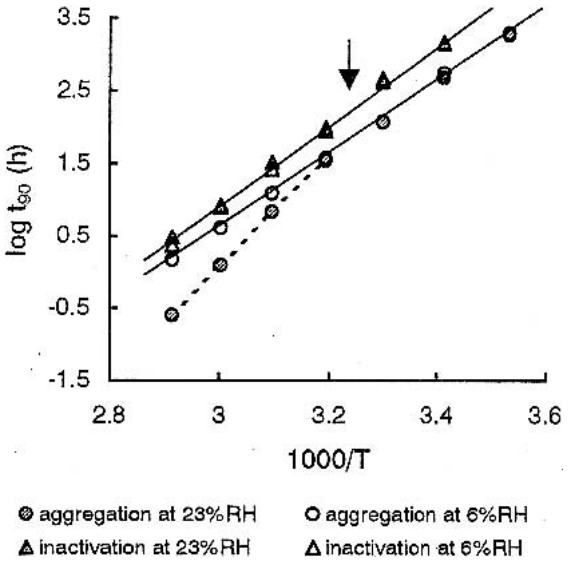

Fig. 8. The time required for 10% aggregation or inactivation (t_{90}) in lyophilized formulation containing PVA. Arrow indicates critical mobility temperature (T_{mc}) at 23% RH as determined by NMR relaxation.

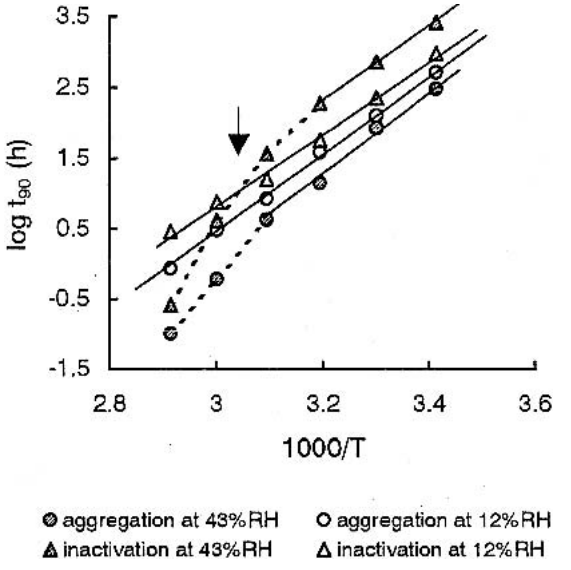

Fig. 9. The time required for 10% aggregation or inactivation (t_{90}) in lyophilized formulation containing MC. Arrow indicates critical mobility temperature (T_{mc}) at 43% RH as determined by NMR relaxation.

On the other hand, gradual decreases in β observed for aggregation in the MC formulation at the temperatures below T_{mc} did not bring about a change in temperature dependence gradient of t_{90}. The obtained linear temperature dependence indicates that t_{90} can be predicted by extrapolating the values obtained at higher temperatures even if β varies with temperature.

Rapid decreases in β with temperature are not expected to occur unless molecular mobility changes rapidly as a result of glass transition. Furthermore, rapid changes in molecular mobility are not necessarily expected to result in nonlinear temperature dependence of t_{90}, as exemplified by β-GA inactivation in the PVA formulation.

CONCLUSIONS

Inactivation and aggregation of β-GA in lyophilized formulations containing PVA and MC were describable with the empirical KWW equation, regardless of whether the temperature was above or below T_{mc}, and regardless of whether protein molecules with different degrees of deformation resulting from stress created during lyophilization exist in the formulation. The t_{90} calculated from obtained τ and β parameters was found to be a useful parameter for evaluating the stability of lyophilized β-GA formulations. The prediction of t_{90} by extrapolation can be made in the temperature range in which β did not rapidly vary with temperature.

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