Usefulness of the Kohlrausch–Williams–Watts Stretched Exponential Function to Describe Protein Aggregation in Lyophilized Formulations and the Temperature Dependence Near the Glass Transition Temperature

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Purpose. We studied the feasibility of using the Kohlrausch–Williams–Watts stretched exponential function (KWW equation) to describe protein aggregation in lyophilized formulations during storage. Parameters representing "mean aggregation time" (τ_a) and stretched exponential constant (β_a) were calculated according to the KWW equation by assuming that the time required for protein molecules to aggregate (τ) varies because of the fact that protein aggregation occurs at a rate that depends on the degree of protein deformation resulting from stresses created during freeze-drying. The temperature dependence of the parameters near the glass transition temperature was examined to discuss the possibility of predicting protein aggregation by accelerated testing.

Methods. Protein aggregation in lyophilized bovine serum γ-globulin (BGG) formulations containing dextran or methylcellulose, at temperatures ranging from 10 to 80°C, was followed by size-exclusion chromatography.

Results. Non-exponential BGG aggregation in lyophilized formulations could be described by the KWW equation. The τ_a and β_a parameters changed abruptly around the NMR relaxation-based critical mobility temperature for formulations containing dextran and methylcellulose. In the glassy state, in contrast, the τ_a parameter of these formulations exhibited continuous temperature dependence. The parameter τ_Γ, as calculated from τ_a and β_a, reflected differences in τ values between the two excipients.

Conclusions. The results indicate that the parameter β_a is reflective of physical changes wihtin lyophilized formulations. Within the temperature range, during which no abrupt changes in β_a were observed, knowledge regarding the $\tau_a$and β_a parameters allows the rate of protein aggregation to be predicted. The parameter τ_Γ was found to be useful in comparing the protein aggregation behavior of formulations having different τ_a and β_a values.

KEY WORDS: protein aggregation; lyophilized formulation; temperature dependence; molecular mobility; Kohlrausch–Williams–Watts stretched exponential function.

INTRODUCTION

One of the most common degradation pathways of lyophilized protein formulations involves protein aggregation (1,2). Knowledge regarding the temperature dependence of protein aggregation rates in lyophilized formulations is important in evaluating the feasibility of using accelerated stability testing. The temperature dependence of the chemical and physical degradation rates of pharmaceuticals having small molecular weights in lyophilized formulations often exhibits a distinct break near the glass transition temperatures (T_g) in association with changes in molecular mobility at the T_g (3–6). Similarly, the temperature dependence of protein aggregation should change around the T_g because protein aggregation involves collisions between protein molecules, which is closely related to molecular mobility.

Protein aggregation in lyophilized formulations cannot be described by simple kinetics because it is a complicated process comprising several sequential steps requiring high-order structural changes. Furthermore, protein within lyophilized formulations may not adopt a single molecular structure, but rather, a number of structures that have been deformed to various degrees resulting from stresses created during the freeze-drying process. Therefore, quantitatively determining protein aggregation rates in lyophilized formulations is a great challenge.

If, during storage of their formulations, protein molecules having different degrees of deformation are assumed to aggregate at a rate dependent on their degree of deformation (i.e., structure perturbation), the time required for aggregation should exhibit a distribution; protein molecules that are only slightly deformed should aggregate via a number of sequential deformation steps, whereas protein molecules that are significantly deformed should aggregate through a fewer number of deformation steps. Protein aggregation rates that vary according to their degree of structural deformation may be described by the Kohlrausch–Williams–Watts stretched exponential function (KWW equation).

The KWW equation (Eq. 1) has been used to describe non-exponential relaxation processes within various amorphous pharmaceuticals (7–9). The non-exponential behavior of these relaxation processes can be explained by assuming that these relaxation processes are inherently non-exponential, or that individual exponential relaxation processes, each having separate characteristic relaxation times, supperimpose (10–13). The latter assumes that heterogeneity within molecular dynamics gives rise to a distribution of relaxation times, and that τ_{KWW} represents an "average" relaxation time, and β_{KWW} a measure of the distribution of relaxation times.

$$\phi(t) = \exp(-(t/\tau_{KWW})^{\beta KWW}) \quad (1)$$

where $\phi(t)$ is a relaxation function and t is time. The use of the KWW equation has also been proposed to describe protein degradation when the protein exists in a number of configurations, each configuration degrading in first order fashion with a different rate constant (14).

We studied the feasibility of using the KWW equation to describe protein aggregation in lyophilized formulations assuming that protein molecules in lyophilized formulations exist in a number of configurations and there is a distribution in the time required for protein aggregation. Bovine serum γ-globulin (BGG) was used as a model protein, and dextran and methylcellulose as model excipients of the formulations. Parameters representing "mean aggregation time" (i.e., reciprocal of aggregation rate constant) and the "stretched expo-

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nential constant" were obtained as τ_a and β_a, respectively. The possibility of predicting protein aggregation rates in lyophilized protein formulations using accelerated stability testing, is discussed based on the temperature dependence of the parameters, τ_a and β_a, at temperatures near the T_g and NMR relaxation-based critical mobility temperature (T_{mc}) (15).

MATERIALS AND METHODS

Materials

BGG (G5009) and dextran (D-4133, average molecular weight, 42,000) were purchased from Sigma Chemical Co. (St. Louis, MO). Methylcellulose (136-07172) were obtained from Wako Pure Chemical Industries Ltd. (Osaka).

Preparation of Lyophilized Formulations

Ninety microliters of 10% w/v BGG solution was added to 20 g of a 2.5 % w/w dextran or methylcellulose solution. Four-hundred microliters of the solution was frozen in a polypropylene sample tube (10-mm diameter) by immersion in liquid nitrogen for 10 minutes and then dried at a vacuum level below 5 Pa for 23.5 hours in a lyophilizer (Freezevac C-1, Tozai Tsusho Co., Tokyo), as previously described (15). The shelf temperature was between –35 and –30°C for the first hour, 20°C for the subsequent 19 hours, and 30°C for the last 3.5 hours. Lyophilized cakes showed no visible evidence of collapse.

Lyophilized BGG formulations were stored at 15°C for 24 hours in a desiccator with a saturated solution of LiCl H_2O (12% relative humidity [RH]) and NaBr $2H_2O$(60.2%RH). The T_{mc} and T_g of formulations containing dextran with a water activity of 0.6 were 35°C and 58°C, respectively. The T_{mc} of formulation containing methylcellulose was 25°C at a water activity of 0.6, although T_g could not be determined due to the complicated DSC thermogram. At a water activity of 0.1, the T_{mc} and T_g were higher than 80°C for both formulations containing dextran and methylcellulose.

Determination of BGG Aggregation by Size Exclusion Chromatography

Lyophilized BGG formulations in screw-capped polypropylene tubes were stored at temperatures ranging from 10°C to 80°C (±0.1°C). The samples were removed at appropriate intervals, dissolved in 1.7 ml of 200 mM phosphate buffer (pH 6.2) and injected into a size exclusion chromatography (16). 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.

Estimation of Parameters of τ_a and β_a

Parameters τ_a and β_a were estimated by curve fitting according to the KWW equation. Curve fitting was performed using a same initial values of τ_a and β_a at different temperatures. When convergence was not attained, curve fitting was repeated using a varied initial value.

RESULTS

Lyophilized BGG formulations containing dextran or methylcellulose underwent protein aggregation during storage at temperatures between 10 and 80°C. Figure 1 shows some typical size exclusion chromatograms which indicate that intact protein molecules can transfer to larger sizes. The amount of protein within an intact molecule of a given size was measured based on the peak height of its chromatogram. All results for formulations containing dextran and methylcellulose, respectively, are shown as a function of storage time in Figs. 2 and 3. Solid lines within the figures represent curve fitting according to the KWW equation, assuming that the time required for protein molecules to aggregate (τ) has a distribution.

Figures 4 and 5 show the estimated τ_a and β_a parameters, respectively. At a water activity of 0.1, formulations containing dextran and methylcellulose can be considered to exist in the glassy state at the temperatures studied, since both the T_{mc} and the T_g are higher than 80°C under this condition (15). The estimated τ_a, the average time required for BGG to aggregate, exhibited continuous temperature dependence in both formulations. The estimated τ_a, a measure of the distribution of τ, in the formulation containing dextran, decreased continuously as the temperature increased, whereas no significant change in β_a was observed in the formulation containing methylcellulose.

At a water activity of 0.6, glass transition should occur within the temperature range studied, since the T_{mc} values of formulations containing dextran and methylcellulose under

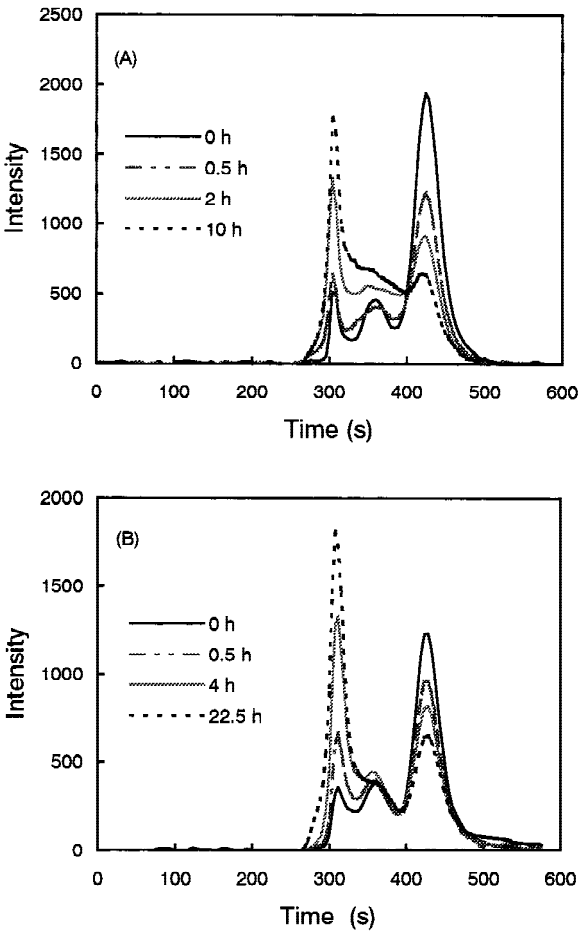

Fig. 1. Size exclusion chromatograms of lyophilized BGG formulations containing dextran (A) and methylcellulose (B) with a water activity of 0.6 after storage at 70°C for various periods.

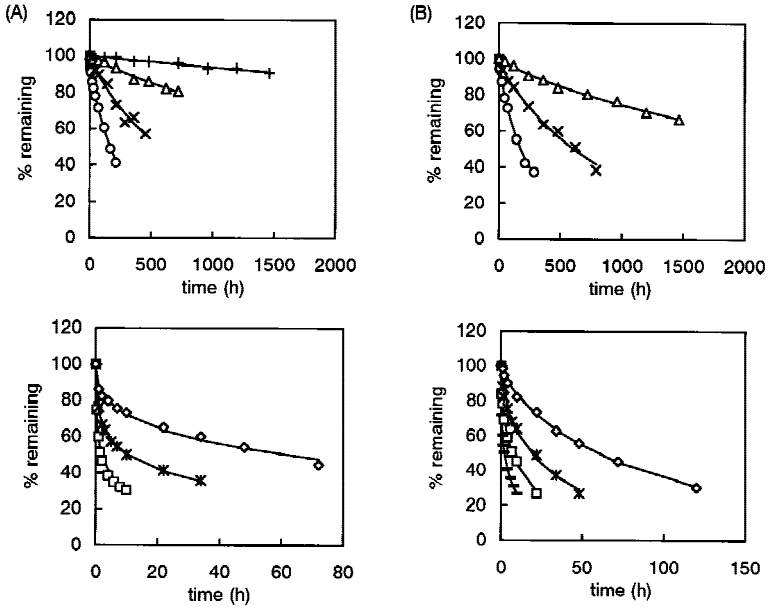

Fig. 2. Time courses of BGG aggregation in lyophilized formulations containing dextran with water activities of 0.6 (A) and 0.1 (B). 10 (+), 20 (△), 30 (X), 40 (○), 50 (◇), 60 (✶), 70 (□) and 80°C (–).

this condition are 35°C and 25°C, respectively (15). For the formulations containing dextran and methylcellulose, the estimated β_a changed abruptly between 40–50°C and 20–30°C, respectively. The estimated τ_a for these formulations exhibited temperature dependence with a distinct break at approximately 50°C and 20°C, respectively.

With regard to the relaxation processes that occur within various amorphous materials, a "mean" relaxation time, τ_Γ, has been calculated from their τ_{KWW} and β_{KWW}values using Eq. 2. The τ_Γ parameter is considered to be a single relaxation time constant that allows the relaxation behavior of amorphous materials having different τ_{KWW} and β_{KWW} values to be compared (10,13). The obtained τ_Γ values were close to the "median" relaxation time for cases involving lognormal distributions (13). Similarly, the "mean" time for BGG to aggregate, τ_Γ, was calculated as an average kinetic parameter from the τ_a and β_a values shown in Figures 4 and 5, and the results are shown in Figure 6.

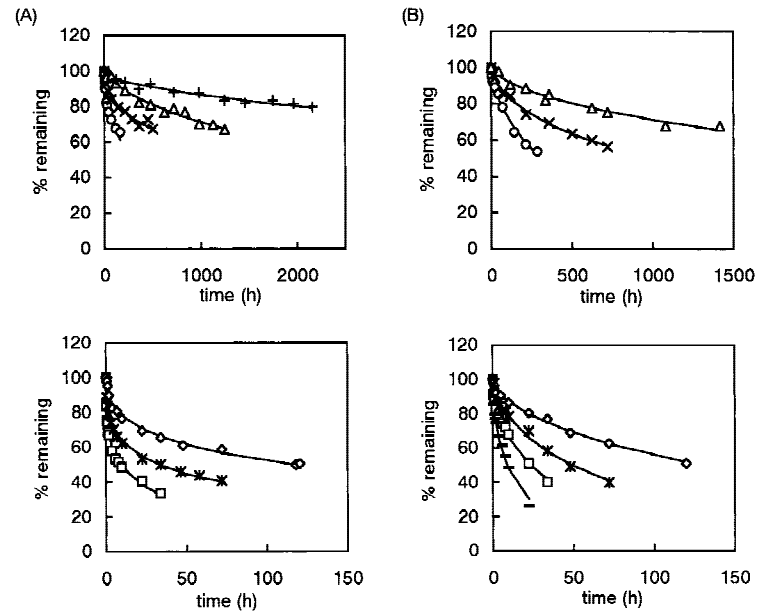

Fig. 3. Time courses of BGG aggregation in lyophilized formulations containing methylcellulose with water activities of 0.6 (A) and 0.1 (B). 10 (+), 20 (△), 30 (X), 40 (○), 50 (◇), 60 (✶), 70 (□) and 80°C (–). Standard deviations were less than 3%.

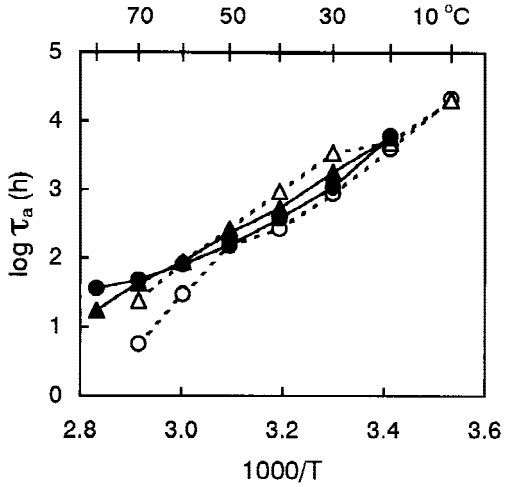

Fig. 4. Temperature dependence of τ_a. Formulations containing dextran with a water activity of 0.1 (●)and 0.6 (○); Formulation containing methylcellulose with a water activity of 0.1 (▲) and 0.6 (△).

$$\tau_\Gamma = \frac{\tau_{KWW}}{\beta_{KWW}} \Gamma\left(\frac{1}{\beta_{KWW}}\right) \quad (2)$$

where Γ is the gamma function defined as:

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

At a water activity of 0.6, τ_Γ exhibited temperature dependence with a distinct break at approximately 50°C for the formulation containing dextran, and 20°C for the formulation containing methylcellulose, similar to the trend observed for τ_a. The break observed in the temperature dependence of τ_Γ was unusual in that an increase in temperature caused a decrease in the temperature dependence of τ_Γ. At a water activity of 0.1, τ_Γ exhibited a continuous temperature dependence similar to τ_a. Significant differences in τ_Γ were observed among the formulations containing dextran and methylcellulose.

DISCUSSION

Protein aggregation during the storage of lyophilized BGG formulations, namely the decrease in the amount of intact BGG of a given size, which was measured by size exclusion chromatograms (Fig. 1), could be described by the KWW equation. Because intact BGG of a given size, namely the BGG monomer, includes both native BGG and partially deformed BGG, the non-exponential decrease in its amount can be interpreted by assuming that lyophilized formulations contain BGG molecules having different degrees of deformation resulting from stresses created during the freeze-drying process. A kinetic model shown in Scheme 1 may be applicable to protein aggregation in such formulations. In the model, each configuration that has been produced during the freeze-drying process undergoes further deformation in sequential manner during storage, and highly deformed configuration is susceptible to aggregation. If deformation occurs in a comparable rate to aggregation, the apparent rate constant for aggregation of configuration i ($k_{app\ (i)}$) depends on its degree of deformation brought about during freeze-drying process. Thus, the time required for BGG to aggregate via deformation (τ) has a distribution. τ_a and β_a can be considered to be the average of τ, and a measure of the distribution of τ, respectively.

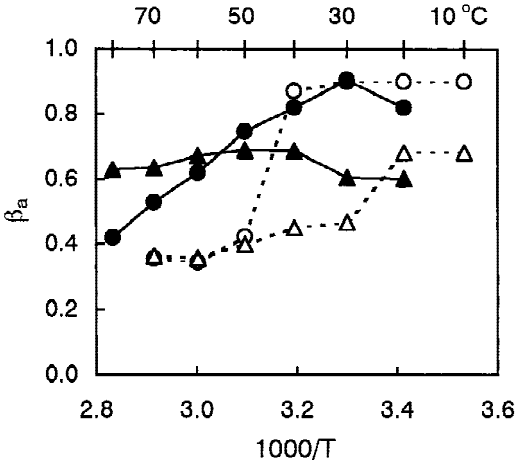

Fig. 5. Temperature dependence of β_a. Formulations containing dextran with a water activity of 0.1 (●) and 0.6 (○); Formulation containing methylcellulose with a water activity of 0.1 (▲) and 0.6 (△).

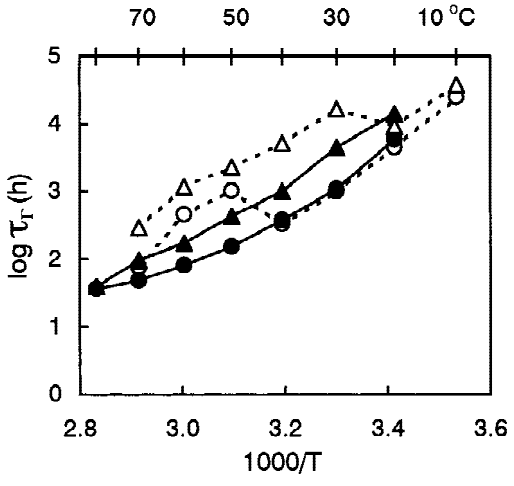

Fig. 6. Temperature dependence of τ_Γ. Formulations containing dextran with a water activity of 0.1 (●) and 0.6 (○); Formulation containing methylcellulose with a water activity of 0.1 (▲) and 0.6 (△).

Discontinuous Temperature Dependence of τ_a and β_a Around T_{mc}

In formulations containing dextran and methylcellulose, the β_a decreased abruptly at temperatures above T_{mc}, at a water activity of 0.6, indicating that an abrupt change in the distribution of τ occurred (Fig. 5). The abrupt decrease in β_a at temperatures above T_{mc} may be explained by the following speculation. Increasing temperature as the glass transition is passed enhances both deformation and aggregation in association with increase in molecular mobility. Since aggregation involves intermolecular collisions, the rate of aggregation step may increase to a greater degree than the rate of deformation steps. Therefore, the apparent rate constant for aggregation

$$C_1 \rightarrow C_2 \rightarrow \text{---} \quad C_i \rightarrow \text{---} \rightarrow C_n$$
$$\downarrow k_{agg}$$

Scheme 1. A kinetic model for protein aggregation.

of configuration *n* (i.e., configuration with the largest degree of deformation) ($k_{app(n)}$) may increase to a greater degree than that of configuration *1* (i.e., configuration with the least degree of deformation) ($k_{app(1)}$). Thus, the difference between $k_{app(1)}$ and $k_{app(n)}$ may become larger with increasing temperature as the glass transition is passed, resulting in the abrupt increase in the distribution of τ (i.e., decrease in β_a). Thus, the observed temperature dependence of β_a may be explained by assuming the kinetic model shown in Scheme 1. However, it may be more practical to assume that not only configuration *n* but also configurations with larger degrees of deformation (configurations *n – 1, n – 2*—) can aggregate.

The parameter β_a, which was found to reflect the physical changes of lyophilized formulations (i.e., glass transition), can be considered a useful parameter in examining the temperature range within which prediction of protein aggregation can be made by accelerated testing.

The temperature dependence of τ_a and τ_Γ exhibited a distinct break around 50°C and 20°C for formulations containing dextran and methylcellulose, respectively, at a water activity of 0.6. These breaks in the temperature dependence may reflect changes in the temperature dependence of molecular mobility at temperatures above and below T_{mc}. The unusual change in τ_Γ around T_{mc} may indicate that the distribution of τ changes qualitatively at T_{mc}, in association with an abrupt change in molecular mobility. Thus, distribution at temperatures above T_{mc} cannot be described by the same distribution model as that below T_{mc}. $\tau\Gamma$ can probably reflect such qualitative changes in the distribution of τ, because this parameter is determined using β_a as well as τ_a.

Temperature Dependence of τ_a and β_a in the Glass State

The parameters τ_a and τ_Γ, as determined for formulations in the glass state with a water activity of 0.1, exhibited continuous temperature dependence without any breaks within the temperature range studied (Figs. 4 and 6). Although no significant difference in τ_a was observed between the formulations containing dextran and methylcellulose, τ_Γ varied significantly between these formulations. The τ_Γ of the formulation containing methylcellulose was larger than that of the formulation containing dextran (Fig. 6). This difference among the two polymer excipients may result from differences in the stabilizing effect provided through interaction with protein. τ_Γ appears to distinguish such differences more sensitively than τ_a because τ_Γ can reflect the differences in β_a between these formulations.

The continuous temperature dependence of τ_a for formulations containing dextran and methylcellulose in the glassy state, suggests that it is possible to extrapolate the τ_a values obtained under accelerated conditions in order to determine τ_a values at lower temperatures. Thus, it may be possible to predict the rate at which protein aggregation might occur, on the basis of estimated τ_a and β_a values. In addition, the $\tau\Gamma$ parameter, as calculated from τ_a and β_a, can be considered useful in comparing protein aggregation behavior among formulations having different τ_a and β_a values.

CONCLUSIONS

Non-exponential protein aggregation of lyophilized BGG formulations containing dextran or methylcellulose could be described by the KWW equation that has been used to describe the non-exponential relaxation processes of various amorphous materials. The parameters, τ_a and β_a, were obtained by assuming that the time required for protein molecules to aggregate (τ) has a distribution due to the fact that protein aggregation occurs at a rate which is dependent upon the degree of protein deformation resulting from stresses created during freeze-drying.

The parameter β_a was found to reflect physical changes within lyophilized formulations and it can be considered a useful parameter in determining the outer limits of the temperature range in which stability can be predicted by accelerated testing. It was suggested that protein aggregation in the glassy state can be predicted on the basis of estimated τ_a and β_a values. Furthermore, protein aggregation behavior with different τ_a and β_a values can be compared by means of parameter τ_Γ.

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