Protein Denaturation in Dosage Forms Measured by Differential Scanning Calorimetry

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The stability of β-galactosidase dosage forms was studied by differential scanning calorimetry (DSC). It was found that the observed enthalpy of thermal denaturation was approximately in proportion to remaining enzyme activity, and denaturation temperature was related to protein stability. These results suggest that DSC can be used to determine native proteins in dosage forms and to clarify the factors affecting protein stability. The DSC method seems to be more convenient than conventional activity assay methods, and useful to follow protein denaturation during the manufacturing process and storage of dosage forms.

Keywords differential scanning calorimetry (DSC); thermal denaturation; β-galactosidase; stability; protein dosage form

In recent years novel drug delivery systems (DDS) of proteins and peptides, such as liposomes,[1,2] microcapsules,[3,4] erythrocyte ghosts,[5] and conjugation with macromolecules,[6,7] have been investigated increasingly. Instability of proteins is one of the main problems we have to overcome in the development of such systems. We usually face difficulties in preventing inactivation of proteins during the manufacturing process and storage of dosage forms. Though there are some reports on the effect of pharmaceutical additives on heat coagulation of proteins used as DDS matrices,[8] sufficient information can not easily be obtained on the stability of active proteins in DDS.

Proteins in dosage forms degrade *via* complex mechanisms.[9] Inactivation of proteins can result from the primary structure changes such as hydrolysis and deamidation, or tertiary structure changes (*i.e.*, denaturation) in which the conformation of molecule is converted to inactive forms (unfolded state).[10] A primary structure change is usually irreversible, but denaturation may be reversible or irreversible, depending on the structure of proteins.[11]

Active proteins in dosage forms have been determined by chemical- or bio-assay which is usually specific for each protein and requires much experimental effort. Therefore simple assay methods applicable to various kinds of proteins are desirable to follow protein degradation in dosage forms. Differential scanning calorimetry (DSC) has been used to detect thermal denaturation of proteins, and to study the tertiary structure and thermodynamic properties of proteins.[12–14] Many DSC studies have been carried out on the stabilizing effect of sugars and polyols on proteins.[15,16] The stability of small spherical proteins such as lysozyme in diluted (ideal) solutions has been studied by DSC.[17,18] DSC, however, has not been applied to stability studies of protein dosage forms.

In this paper, we show that DSC may be a convenient method to follow protein inactivation during the manufacturing process and storage of protein dosage forms. β-Galactosidase from *Aspergillus oryzae*,[19] which is used for the improvement of lactose intolerance, was chosen as a model protein. We discuss the stability of β-galactosidase based on calorimetric data obtained by DSC, and compare the DSC method with the conventional assay method. The usefulness of differential adiabatic scanning microcalorimetry to detect thermal denaturation of small amounts of proteins is also discussed.

Materials and Methods

Materials A powder formulation β-galactosidase (Deminase) was purchased from Fuso Yakuhin Kogyo Co. β-Galactosidase from *Aspergillus oryzae* lyophilized powder containing mannitol was purchased from Funakoshi Yakuhin Co. The activity of β-galactosidase was approximately 5 unit/mg for powder formulation and 100 unit/mg for lyophilized powder. 2-Nitrophenyl-β-D-galactopyranoside, bovine pancreas α-chymotrypsin and thrombin (for pharmaceutical usage) were from Wako Pure Chemical Industries Ltd., Boehringer Mannheim Yamanouchi Co. and Mochida Seiyaku Co., respectively. Kallidinogenase was Japanese Pharmacopoeia Reference Standard. All other chemicals were of the highest grade available. Buffer solutions used were 50 mM phosphate for pH 5.4 to pH 8.0 and 50 mM citrate-phosphate for pH 3.8 to 5.0. The buffer solutions were prepared at 25 °C.

Calorimetric Measurement by DSC Calorimetric measurements for β-galactosidase were carried out by using a Shimadzu heat-flux type differential scanning calorimeter, DSC-41M. Powder formulations and lyophilized powder were dissolved in the pH 5.4 buffer to get a 10% (w/w) solution of powder formulation and 1% (w/w) solution of lyophilized powder unless otherwise stated. Aliquots (25 μl) of samples were packed in a pre-autoclaved aluminum cell. The scan rate was 5 °C/min. Apparent enthalpy of thermal denaturation was calculated from the area under the denaturation curve by the microcomputer software (Shimadzu). Denaturation temperature was determined from the peak point of the curve.

Calorimetric measurement for protein solutions of low concentration (0.175—0.38%, w/w) was carried out by using a Privalov type differential adiabatic scanning microcalorimeter (DASM-4).[20,21] Protein concentrations of solutions were determined by the method of Lowry *et al.*[22] The scan rate was 1 °C/min. Calorimetric data were analyzed by the software prepared by Sinku-Riko Inc.

Enzyme Activity Measurement of β-Galactosidase β-Galactosidase powder formulations and lyophilized powder were dissolved in distilled water to 0.02 mg/ml and 1 μg/ml, respectively. β-Galactosidase activity was measured at 30 °C and pH 4.5 using 2-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate by the method of Tanaka *et al.*[19] with a minor modification (the concentration of ONPG was changed to 5.71 mM).[23] Relative activity was expressed as the ratio to the initial value. Activity measurement was not affected by guanidine hydrochloride (GuHCl) in the concentration range studied.

Kinetic Study on Inactivation of β-Galactosidase in Dosage Forms β-Galactosidase powder formulations were stored at 60 °C in direct packages. Relative humidity was adjusted to 49.9% with a saturated solution of sodium bromide. At appropriate intervals, the activity and enthalpy of denaturation were measured. Some precipitates were observed upon dissolution of the powder samples which had been stored for longer periods. In this case, the suspension of the powder was used for measurement of activity and enthalpy.

Kinetic Study on Inactivation of β-Galactosidase in Solutions β-Galactosidase lyophilized powder was dissolved in cold buffer (pH 5.4, 0.05 mg/ml). The solution was diluted to 1 μg/ml with various buffer solutions (pH 3.8—8.0) and stored at 50 °C. The effects of sugars and guanidine hydrochloride were studied at pH 7.4 and 5.4 at 50 °C. At appropriate intervals, aliquots of the solution were taken and used to measure the activity.

Results and Discussion

Determination of Active β-Galactosidase by DSC In order to apply DSC to determine the native protein in dosage forms, we studied the dependence of denaturation enthalpy observed by DSC on the amount of β-galactosidase. The denaturation enthalpy was measured for various amounts of β-galactosidase lyophilized powder, which was the most highly purified enzyme available, as well as β-galactosidase powder formulation. The enzyme is monomeric,[24] and the apparent molecular weight is about 105000.[19] Figure 1 shows observed denaturation enthalpy as a function of enzyme amount. Observed enthalpy was in proportion to the amount of β-galactosidase in both cases. The linear relationship indicates the utility of observed denaturation enthalpy measured by DSC to determine the native protein, though large standard errors were observed in the case of the powder formulation.

Inactivation of β-Galactosidase in Dosage Forms and Observed Denaturation Enthalpy The DSC method was applied to follow inactivation of β-galactosidase in the solid-state dosage form. Figure 2 shows the denaturation enthalpy of β-galactosidase powder formulation stored at 60 °C, 50% RH, as measured by DSC. The enzyme activity of the samples, which was simultaneously measured, is shown in Fig. 3. Both the observed enthalpy and the enzyme activity decreased with time, and a linear relationship was observed between them (Fig. 4). Though the observed enthalpy was not detected in the lower activity region because of the low sensitivity, it was suggested that remaining native protein in the β-galactosidase formulation can be determined by the DSC method. It should be noted that an inactivation pathway accompanied with a small enthalpy difference may not be detected by this DSC method.

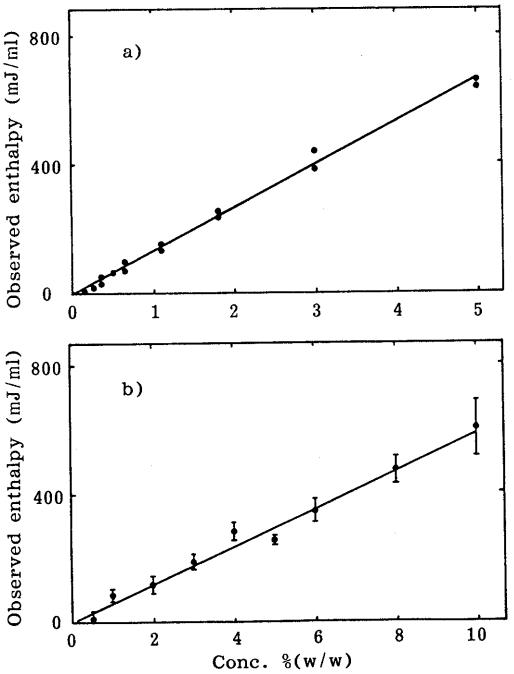

Fig. 1. Observed Denaturation Enthalpy of β-Galactosidase

β-Galactosidase lyophilized powder (a) and powder formulation (b) were dissolved to the desired concentration in pH 5.4 buffer. Aliquots (25 μl) of solution were placed in aluminum cells. The DSC scanning rate was 5 °C/min. (b) Each point represents the mean of triplicate measurements and the bar indicates the SD.

Denaturation Temperature and Stability of β-Galactosidase It has been reported that pH and some reagents such as polyhydric alcohols and denaturants can influence the rate of enzymatic activity loss.[25] The utility of the DSC method as a means to clarify these factors affecting the stability of protein was studied with β-galactosidase solutions.

Figure 5 shows the thermal denaturation temperature (Td) of β-galactosidase in solutions measured by DSC as a function of pH. The highest temperature was observed at

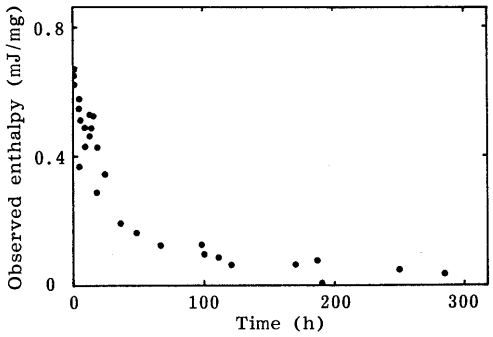

Fig. 2. Observed Denaturation Enthalpy Change of β-Galactosidase Formulation Powder

β-Galactosidase powder formulation was stored in a direct package at 60 °C, 50% RH. At appropriate intervals, powders were dissolved in pH 5.4 buffer (10%, w/w). Aliquots (25 μl) of the solution were placed in aluminum cells. The DSC scanning rate was 5 °C/min.

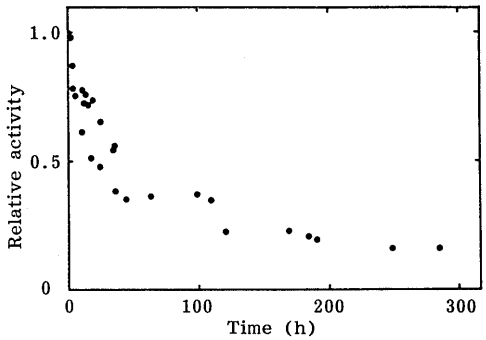

Fig. 3. Enzyme Activity Loss of β-Galactosidase Formulation Powder

β-Galactosidase powder formulation was stored in a direct package at 60 °C, 50%RH. At appropriate intervals, powders were dissolved in pH 5.4 buffer (0.02 mg/ml), and the activities were measured at 30 °C, pH 4.5 using ONPG as the substrate.

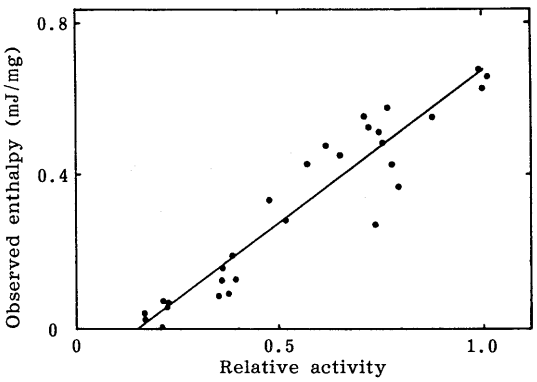

Fig. 4. Relationship between Remaining Activity and Observed Denaturation Enthalpy

β-Galactosidase powder formulation was stored in a direct package at 60 °C, 50% RH. Activity and denaturation enthalpy were measured as described in the legends to Figs. 2 and 3.

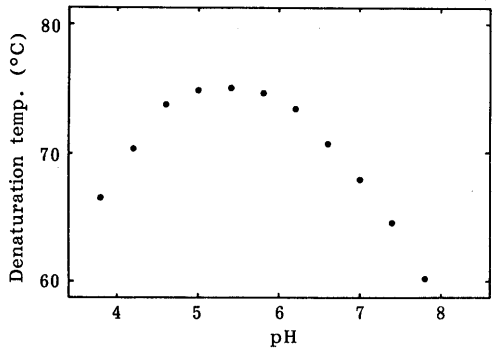

Fig. 5. Effect of pH on Denaturation Temperature of β-Galactosidase

β-Galactosidase lyophilized powder was dissolved in buffer of various pH values (1%, w/w). Aliquots (25 μl) of solution were packed in aluminum cells. The peak of the DSC curve was taken as the denaturation temperature.

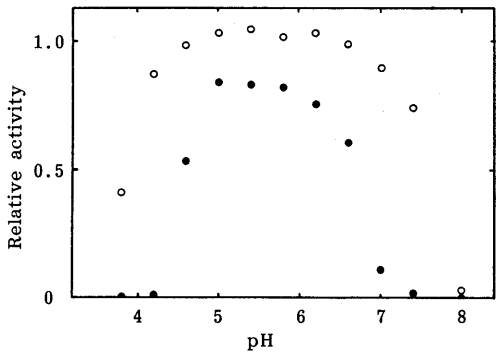

Fig. 6. Effect of pH on Inactivation of β-Galactosidase

β-Galactosidase lyophilized powder was dissolved in cold buffer (pH 5.4, 0.05 mg/ml). The solution was diluted to 1 μg/ml with buffer of various pH values and stored at 50 °C. Activities of β-galactosidase were measured at 1 h (○) and 96 h (●) at 30 °C, pH 4.5 using ONPG as the substrate.

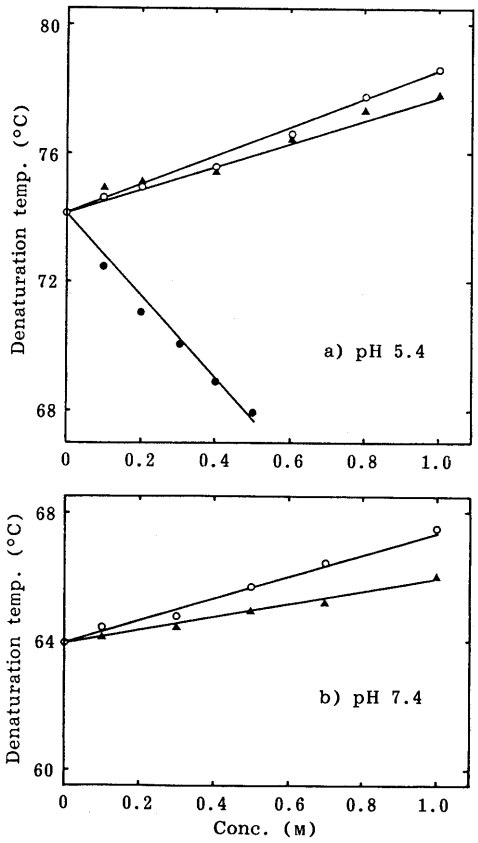

Fig. 7. Dependence of Denaturation Temperature of β-Galactosidase on Sugars and Denaturant

β-Galactosidase lyophilized powder was dissolved (1%, w/w) in buffer of pH 5.4 (a) and pH 7.4 (b) containing various concentrations of sucrose (○), sorbitol (▲) and guanidine hydrochloride (●). Thermal denaturation of β-galactosidase was detected by DSC, and the peak of the DSC curve was determined as the denaturation temperature.

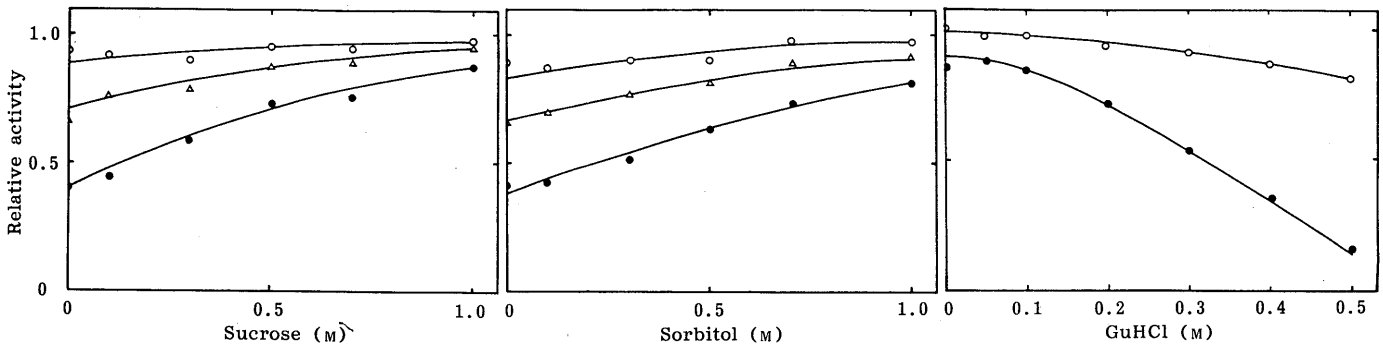

Fig. 8. Effect of Sucrose (a), Sorbitol (b) and Guanidine Hydrochloride (c) on Inactivation of β-Galactosidase

β-Galactosidase lyophilized powder was dissolved in cold pH 5.4 buffer (0.04 mg/ml). (a), (b): The solution was diluted (1 μg/ml) to pH 7.4 buffer containing additives, and stored for 20 min (○), 1 h (△) and 3 h (●) at 50 °C. (c): The solution was diluted (1 μg/ml) to pH 5.4 buffer containing guanidine hydrochloride, and stored for 1 h (○) and 24 h (●) at 50 °C.

pH 5.4. Stability of β-galactosidase in various pH solutions was studied at 50 °C. Figure 6 shows the remaining activity observed. β-Galactosidase was most stable in the pH range from 5.0 to 6.4 as reported at 30 °C.[19,26] The stability of β-galactosidase is related to the Td; the higher the Td is, the more stable the enzyme is. This indicates that the effect of pH on the stability can be clarified by the DSC method.

It has been reported in connection with α-chymotrypsin stability that sucrose increases the apparent activation energy of the unfolding process, and increases the denaturation temperature.[27] Figure 7a and b shows the effect of sugars on the Td of β-galactosidase at pH 5.4 and 7.4, respectively. The Td was elevated with the addition of sucrose and sorbitol. The effect of sucrose was greater than that of sorbitol. The effects of sucrose and sorbitol on the inactivation of β-galactosidase in solutions are shown in Fig. 8a and b, respectively. The activity loss decreased with increasing amount of sucrose and sorbitol. This was also the case at pH 5.4 (data not shown). On the other hand, guanidine hydrochloride, which is one of the most powerful denaturants, lowered the Td (Fig. 7a), and promoted activity loss (Fig. 8c). These results show that the effect of

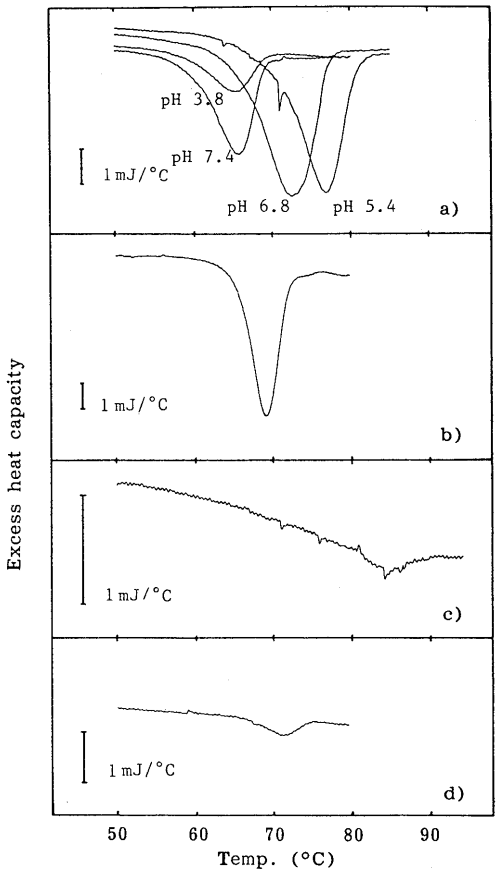

Fig. 9. DSC Curves of Proteins Obtained with the DASM-4

Proteins were dissolved in cold buffers and thermal denaturation was observed with the DASM-4 (scanning rate: 1 °C/min). a) β-Galactosidase from *A. oryzae* lyophilized powder: 0.175% (w/w) in pH 3.8 buffer, 0.252% (w/w) in pH 5.4 buffer, 0.235% (w/w) in pH 6.8 buffer, 0.179% (w/w) in pH 7.4 buffer. b) Bovine pancreatic α-chymotrypsin 0.38% (w/w) in pH 7.4 buffer. c) Kallidinogenase 200 I.U./ml in pH 7.4 buffer. d) Thrombin 0.0118% (w/w) in pH 7.4 buffer.

additives on the stability can be studied by measuring the Td. Understanding the effect of such additives and pH on protein stability in preformulation will allow for improved development strategies to minimize protein degradation during manufacturing process.

Calorimetric Measurement by Differential Adiabatic Scanning Microcalorimetry The heat-flux type DSC used for the detection of β-galactosidase denaturation described above requires protein concentrations of more than 1% (w/w) to get adequate information about the observed enthalpy. At such a high concentration, interaction of protein molecules may occur, resulting in aggregation. A more sensitive method is required to detect protein denaturation without the influence of aggregation. As a method to detect protein denaturation in diluted solutions, therefore, we studied the utility of differential adiabatic scanning microcalorimetry (DASM).

Figure 9 shows DSC curves of some proteins measured with an adiabatic scanning calorimeter. Denaturation curves of β-galactosidase and α-chymotrypsin were clearly observed at the concentration of 0.3% (w/w). Even for low concentration solutions of thrombin and kallidinogenase, the denaturation peak could be observed. It is suggested that DASM can provide useful information on protein denaturation of dosage forms containing small amounts of proteins.

In conclusion, it is suggested that DSC can be used to determine native protein in dosage forms and to clarify the factors affecting protein stability. The DSC method seems to be more convenient than conventional activity assay methods, and should be useful to follow protein denaturation during the manufacturing process and storage of dosage forms.

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