In Vitro Simulation of Solid-Solid Dehydration, Rehydration, and Solidification of Trehalose Dihydrate Using Thermal and Vibrational Spectroscopic Techniques

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Purpose. The processes of dehydration, rehydration, and solidification of trehalose dihydrate were examined to simulate it in the drying and wetting states.

Methods. Techniques included differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and Fourier transform infrared (FT-IR) microspectroscopy combined with thermal analysis. Trehalose dihydrate was pressed on one KBr pellet (1KBr method) or sealed within two KBr pellets (2KBr method) for FT-IR measurement.

Results. On the DSC thermogram, the shoulder between 60°C and 80°C represented a transitional change because no weight loss occurred in this area of the TGA curve. The endothermic peak at 100°C represented dehydration of trehalose dihydrate to anhydrous trehalose; a 9.5% weight loss in the TGA curve occurred from 81°C. The thermal-dependent FT-IR spectra for trehalose dihydrate prepared by the 1KBr method changed markedly with temperature in the 1800–1500 cm^{-1} region during dehydration. IR peak intensity at 1687 cm^{-1} assigned to the bending vibrational mode of solid-like water declined with temperature and decreased sharply at 67°C. Another IR peak at 1640 cm^{-1} associated with the bending of liquid water increased at 67°C but disappeared at 79°C as a result of water evaporation. Both peaks for the sample prepared by the 2KBr method changed dramatically at 64°C; peak intensity at 1640 cm^{-1} remained constant above 64°C.

Conclusions. A new polymorphic transition of trehalose dihydrate was first evidenced at 64–67°C from both DSC curves and thermal-related FT-IR spectra. This transitional temperature reflected the thermal-dependent transformation from solid-like water to liquid water in the trehalose dihydrate structure during dehydration. During rehydration, trehalose anhydrate was rehydrated to the dihydrate, and liquid water in the dihydrate structure was again transformed to solid-like water. The polymorphic transition within this temperature range seems to correlate with the bioprotective effect of trehalose dihydrate in preserving protein stability.

KEY WORDS: trehalose dihydrate; dehydration; rehydration; solidification; polymorphic transformation; DSC; TGA; thermal FT-IR microscopic system.

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ABBREVIATIONS: DSC, differential scanning calorimetry; TGA, thermogravimetric analysis; FT-IR, Fourier transform infrared microspectroscopy.

INTRODUCTION

Trehalose is a stable, nonreducing disaccharide that does not react chemically with proteins or peptides. Trehalose protects biologic molecules from denaturation caused by lyophilization (1) and therefore is added as a cryoprotectant to biopharmaceutical products. At elevated temperatures, trehalose also preserves the native conformation of a dried protein and suppresses the aggregation of denatured proteins during storage (2,3). Although protein dehydration can induce partially irreversible conformational changes, the original structure can be restored in the presence of trehalose. Trehalose may have promising applications in pharmaceutical manufacturing because of these characteristics.

Trehalose, found in many plants and animals, is an important storage carbohydrate that functions not only as an energy source but also as a stress protectant. Trehalose effectively stabilizes proteins against denaturation caused by desiccation or freezing; it also prevents cell membrane damage (4,5). Anhydrobiotic organisms with high trehalose content can survive dehydration for a long time and can restore activity within minutes after rehydration. The mechanisms by which trehalose provides desiccation tolerance may be explained by the water-replacement hypothesis and the vitrification hypothesis (6,7). Crowe *et al.* (7) suggest that both of these actions are required for the stabilizing effects in anhydrobiotic systems, in which trehalose can perform both functions.

Other investigators have examined the desiccation tolerance of anhydrobiotic systems in the presence of trehalose; however, the functional mechanisms underlying the action of trehalose in these systems remain unknown. The polymorphic transition of trehalose has been extensively studied and may correlate with its bioprotective action (8–13). A unique, anhydrous form of trehalose may be responsible for the dehydration tolerance of anhydrobiotic organisms (14); thermodynamic properties of trehalose may play a protective role. In our preliminary study, a polymorphic transition, not found in other studies (8–13), was observed within the temperature range of 60–80°C, and a temperature range of 50–70°C is the critical point of protein denaturation for many proteins and peptides (15–17). Thus, in order to examine the functional mechanism of the protective effect of trehalose, the polymorphic interconversions of trehalose dihydrate, particularly within this temperature range, is warranted. Moreover, the polymorphic transformation of trehalose dihydrate within 50–100°C during the drying or wetting process is of great interest and worthy of investigating to correlate with the bioprotective function of trehalose dihydrate.

In the present study, we used thermoanalytic, thermogravimetric, and Fourier transform infrared (FT-IR) spectroscopy combined with thermal analysis to simulate and investigate the polymorphic transition of trehalose dihydrate in the dehydration, rehydration, and solidification processes, particularly in the temperature range of 50–100°C. Based on the study, we report that a unique polymorphic transition of trehalose dihydrate occurs near 64–67°C from both DSC curve and thermal-dependent FT-IR spectra, which seem to correlate with its protective function to protein stability. This transitional temperature reflected the thermal-dependent transformation from solid-like water to liquid water in the trehalose dihydrate structure during dehydration.

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MATERIALS AND METHODS

Materials

Trehalose dihydrate (T-0167) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The KBr crystals for the pellets were obtained from Jasco Parts Center (Jasco Co., Tokyo, Japan).

Thermal Analysis

The trehalose dihydrate sample was examined using differential scanning calorimetry (DSC; DSC-910, TA Instruments Inc., New Castle, DE) at a heating rate of 5°C/min with an open pan system in a stream of N_2 gas. Thermogravimetric analysis (TGA; TGA-951, TA Instruments Inc., New Castle, DE) was also performed at the same heating rate to measure the weight loss of the sample.

Thermal FT-IR Microspectroscopic Study

A trace powder of trehalose dihydrate was smeared on one piece of KBr pellet and then directly compressed with an IR spectrophotometric hydraulic press (Riken Seiki Co., Tokyo, Japan) under 200 kg/cm^2 for 15 s, and then the pressure was quickly removed (1KBr method). Another powder of trehalose dihydrate was smeared and sealed into two pieces of KBr pellets by compression with 200 kg/cm^2 for 15 s, and then the pressure was quickly removed (2KBr method).

Each compressed KBr disk was placed directly onto a micro–hot stage (DSC microscopy cell, FP 84, Mettler, Greifensee, Switzerland). The DSC microscopy cell was placed in an FT-IR microscopic spectrometer (Micro FTIR-200, Jasco, Japan) with a mercury cadmium telluride (MCT) detector. The system was operated in transmission mode. The temperature of the DSC microscopy cell was monitored with a central processor (FP 80HT, Mettler, Switzerland). The heating rate of the DSC assembly was maintained at 5°C/min under ambient conditions. The sample disk was equilibrated to the starting temperature (30°C) and then heated from 30°C to 120°C. The thermal-responsive IR spectra were recorded while the sample disk was heated on the DSC microscope stage (18).

Isothermal FT-IR microscopic time-scan measurements were also determined. Trehalose dihydrate prepared by the 1KBr method was heated to 81°C and then cooled to 25°C and time-scanned isothermally at 25°C for 120 min. Another sample disk was heated to 120°C, cooled to 25°C, and time-scanned isothermally at 25°C for 180 min. During the experiment, the sample disk was first equilibrated at 25°C and then time-scanned. Humidity was maintained at 70% RH. The time-scanned IR spectra were recorded.

RESULTS AND DISCUSSION

Trehalose dihydrate has been reported to have several polymorphs depending on the different thermal treatments (8–13). The thermodynamic characteristics of these polymorphs have been proposed to correlate with the peculiar properties of trehalose dihydrate in preserving the structure and function of biomolecules. This indicates that the polymorphic transition of trehalose dihydrate may be an important factor contributing to its protective efficacy in biologic system.

Thermal Dehydration of Trehalose Dihydrate

Thermal-dependent FT-IR spectra of trehalose dihydrate prepared by the 1KBr or 2KBr pellet method were plotted three-dimensionally (Figs. 1 and 2). The contour profiles of IR spectra at 3501, 3000–2800, 1687, and 1500–900 cm^{-1} changed markedly near 67°C for the 1KBr sample and at 64°C for the 2KBr sample. The peak at 3501 cm^{-1} was assigned to the stretching vibration of water molecules in the dihydrate of trehalose; the peaks within 3000–2800 cm^{-1} were caused by the CH stretching of trehalose; the peak at 1687 cm^{-1} corresponded to the bending vibration mode of the crystal water in trehalose dihydrate; the peaks below 1500 cm^{-1} belonged to the O-C-H, C-C-H, and C-O-H deformation modes and the C-C and C-O stretching vibration modes of trehalose (19). Two peaks at 998 and 957 cm^{-1} represented the asymmetric and symmetric stretching vibrations of the α-(1→1) glycosidic bond (14).

Peak intensity at 1687 cm^{-1} for the 1KBr sample decreased sharply at 67°C (Fig. 1). Simultaneously, another IR peak at 1640 cm^{-1} quickly appeared at 67°C but disappeared at 79°C. The thermal-dependent spectral changes at 1687 cm^{-1} and 1640 cm^{-1} for the 2KBr sample were similar to those of the 1KBr sample. However, the dramatic temperature change for the 2KBr sample occurred at 64°C, and the peak intensity at 1640 cm^{-1} remained constant after 64°C (Fig. 2). Water dehydrated from the dihydrate crystal may

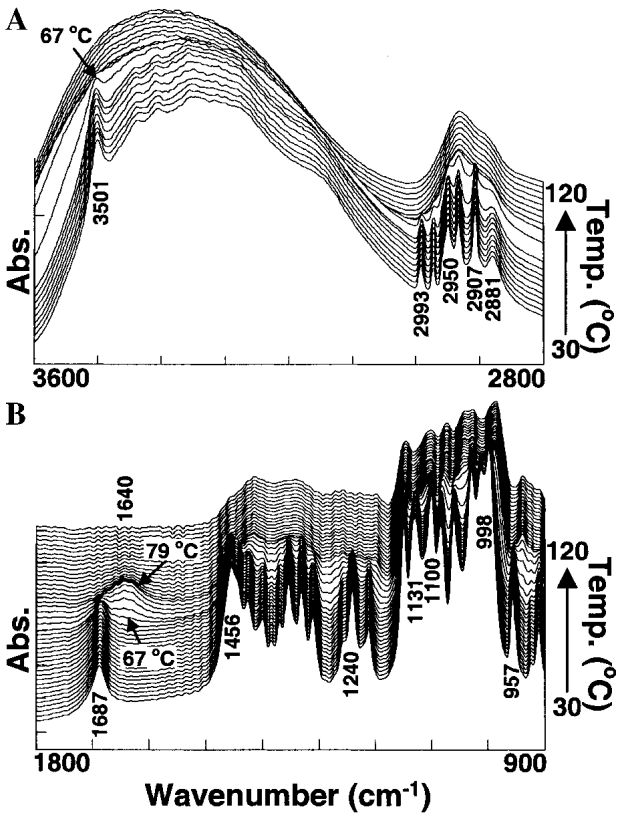

Fig. 1. Three-dimensional plots of the FT-IR spectra of trehalose dihydrate prepared by the 1KBr method within 3600–2800 cm^{-1} and 1800–900 cm^{-1} as a function of temperature.

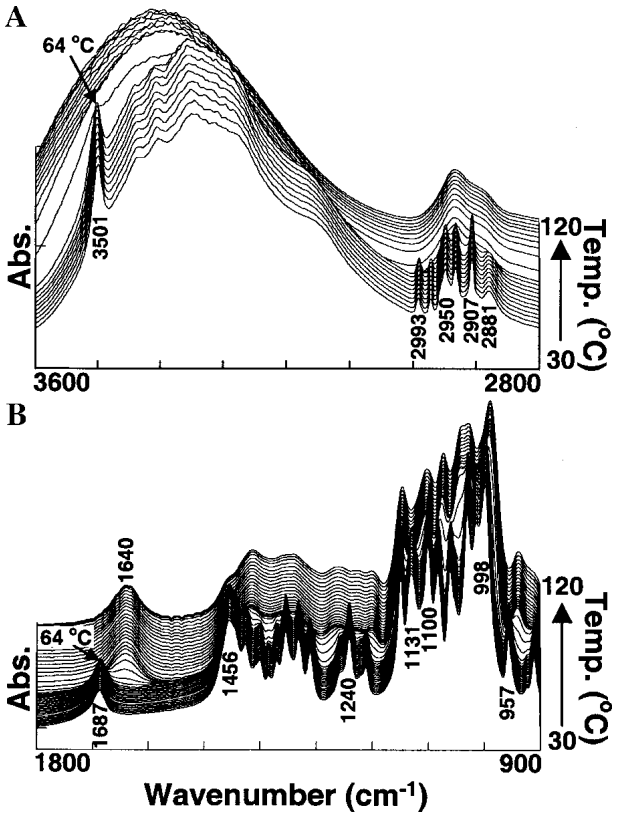

Fig. 2. Three-dimensional plots of the FT-IR spectra of trehalose dihydrate prepared by the 2KBr method within 3600–2800 cm^{-1} and 1800–900 cm^{-1} as a function of temperature.

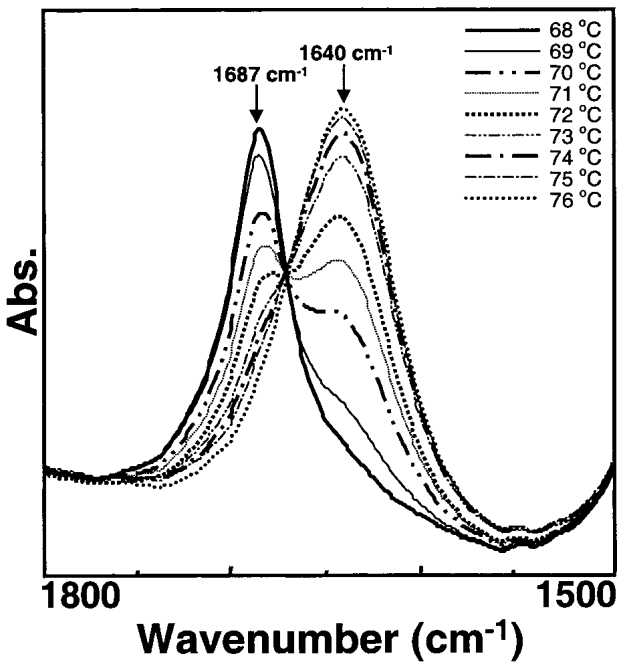

Fig. 3. The thermal-dependent changes in the IR spectral range of 1800–1500 cm^{-1} for trehalose dihydrate prepared by the 2KBr method.

have been sealed between the two KBr pellets, resulting in an IR spectrum for water at 1640 cm^{-1}. However, the water likely evaporated from the 1KBr pellet at 79°C, and the IR peak disappeared. The thermal-dependent changes in the IR spectral range of 1800-1500 cm^{-1} for trehalose dihydrate prepared by the 2KBr method are recorded (Fig. 3). A declining peak at 1687 cm^{-1} and a rising peak at 1640 cm^{-1} were observed simultaneously. Apparently, the thermal behavior of trehalose dihydrate was significantly different from the samples prepared by the 1KBr or 2KBr method.

The H-O-H bending vibration of water occurs in the IR spectral range near 1700 cm^{-1} to 1600 cm^{-1} (20). The bending band of liquid water appears at 1640 cm^{-1}, but that of solid-like water is located near 1700 cm^{-1}. In this study, if the IR peak at 1687 cm^{-1} were, in fact, associated with the bending vibrational mode of solid-like water, then the bound water molecule in the dihydrate structure of trehalose at ambient temperatures may have existed as a solid-state, similar to ice. When the temperature increased, the 1687 cm^{-1} peak shifted to 1640 cm^{-1} at 64°C for the 2KBr sample and at 67°C for the 1KBr sample. The water molecule in the trehalose dihydrate crystal underwent a thermal transition from a solid state to a liquid state. This phenomenon is similar to the melting of ice from the solid phase to the liquid phase. X-ray crystallographic studies of trehalose dihydrate confirmed that water played a critical structural role by hydrogen bonding with trehalose molecules. The average O…O distance was 2.8 Å, which approximates the O…O distance (2.76Å) of ice (21,22); the dihydrate of trehalose apparently exhibited a solid-like, ice cluster structure at ambient temperatures.

Thermal Transition of Trehalose Dihydrate

The DSC thermogram and TGA curve of trehalose dihydrate were constructed using an open pan system (Fig. 4). On the DSC thermogram, a shoulder peak was present near 60°C to 70°C, and a main endothermic peak at 100°C. No weight loss was evident in the TGA curve below 81°C, but a 9.5% weight loss occurred between 81°C and 120°C. The TGA weight loss at 81°C might have resulted from dehydration of water in the crystal. The total weight loss of 9.5% was almost equal to the loss of two moles of dihydrate water from trehalose dihydrate (molecular weight: 378.3). No weight loss occurred between 60°C and 80°C on the TGA curve; the subtle change in this temperature range on the DSC thermogram might have been associated with a polymorphic structural transformation of trehalose dihydrate. As compared with other DSC reports (8–14), the present study shows that trehalose dihydrate was first found to exhibit a polymorphic transition between 60 and 80°C in the DSC curve. Because the temperature range within 50–70°C is the critical point for denaturation of many proteins (15–17), the polymorphic transformation of trehalose dihydrate seems to reflect the protective function of trehalose dihydrate in preserving protein stability. The result may also be confirmed by the following temperature-dependent changes in IR spectra of trehalose dihydrate.

The thermodependent changes in IR peak intensity at 1687 cm^{-1} and 1640 cm^{-1} were recorded (Fig. 4). Peak intensity changed dramatically near 67°C for the 1KBr sample and near 64°C for the 2KBr sample; these temperatures were similar to those observed on the DSC thermogram and TGA curve. At 67°C, the 1KBr sample seemed to transition from solid-like water to liquid water. The onset temperature of the thermal transformation for the 2KBr sample was 64°C. The peak intensity for the 1KBr sample at 1640 cm^{-1}, which rep-

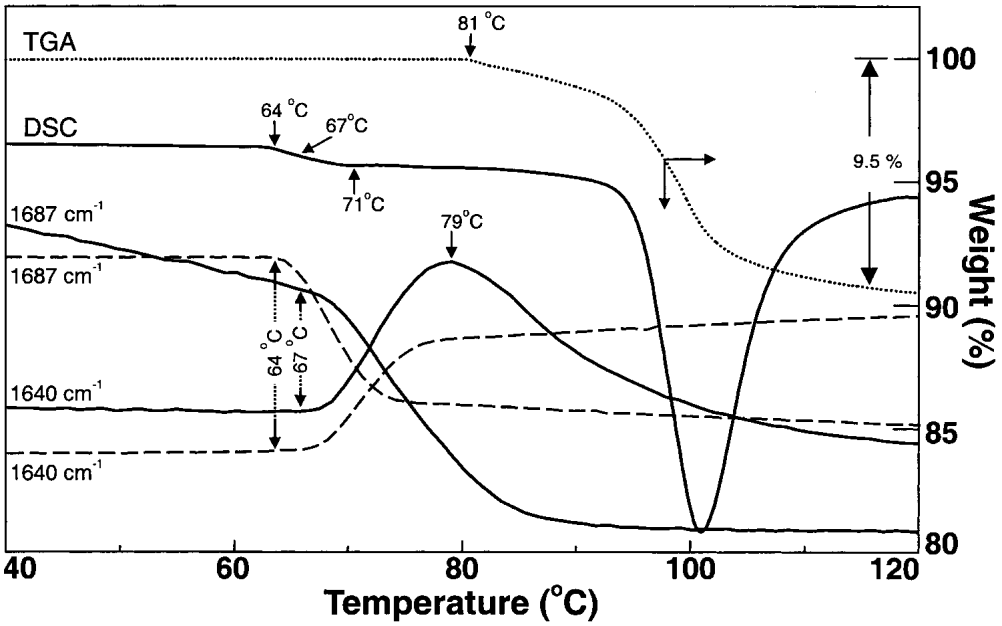

Fig. 4. The DSC thermogram, TGA curve, and thermodependent changes in IR peak intensity at 1687 cm^{-1} and 1640 cm^{-1} of trehalose dihydrate prepared by 1KBr (*solid line*) or 2KBr (*dash line*) method.

resented the bending vibration of liquid water, was gradually reduced at 79°C through water evaporation. This temperature was also consistent with the 81°C temperature on the TGA curve. The thermal-related IR spectra provided more critical information about molecular structure and the dehydration process in trehalose dihydrate than did the thermal analysis. A unique temperature-dependent transition of the H-O-H bending was observed in the trehalose dihydrate structure.

Isothermal Processes of Rehydration and Solidification

The anhydrous form of trehalose can easily rehydrate to form the dihydrate, leading to stabilization of the trehalose vitrification state (1,5). This dihydrate form may act as a water substitute and function to sustain life in anhydrobiotic organisms. In the present study, the rehydration and solidification processes of trehalose were investigated by isothermal FT-IR time-scan measurements.

Trehalose dihydrate prepared by the 1KBr method was preheated to 81°C, cooled to 25°C, and isothermally studied at 25°C, 70% RH for 120 min; isothermal three-dimensional FT-IR spectra were recorded (Fig. 5). The two peaks at 1640 cm^{-1} and 1687 cm^{-1} clearly changed; the other peaks retained their original shape. Under these isothermal conditions, the peak at 1640 cm^{-1}, representing the bending band of liquid water, decreased as the thermal time increased. Conversely, the 1687 cm^{-1} peak, which represented solid-like water, increased as the thermal time increased. Both lines tended to equilibrate beyond 70 min but reached an almost constant after 100 min. The isothermal conditions (25°C, 70% RH for 120 min) probably induced the rehydration process that transformed the liquid water to solid-like water in trehalose dihydrate.

FT-IR spectra were also recorded when trehalose dihydrate prepared by the 1KBr method was preheated to 120°C and then cooled to 25°C before being held isothermally at 25°C, 70% RH for 180 min (Fig. 6). The 120°C-preheated trehalose dihydrate should have been an anhydrous form of trehalose (Figs. 1 and 4). During the entire rehydration process, the IR peak intensity at 1640 cm^{-1} for the anhydrous sample quickly increased with time, reached a plateau within 50 min, and then gradually decreased to a constant level (Fig. 6). The plateau probably represented rehydration of trehalose anhydrate to the dihydrate. At this time, the liquid water in trehalose dihydrate was formed. The IR peak at 1687 cm^{-1}

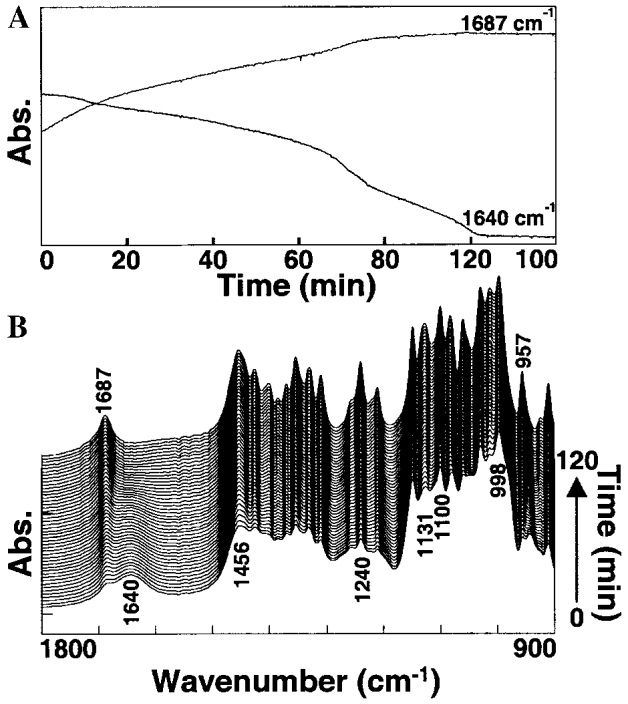

Fig. 5. Isothermal three-dimensional FT-IR spectra of trehalose dihydrate preheated to 81°C, cooled to 25°C, and isothermally studied at 25°C, 70% RH for 120 min.

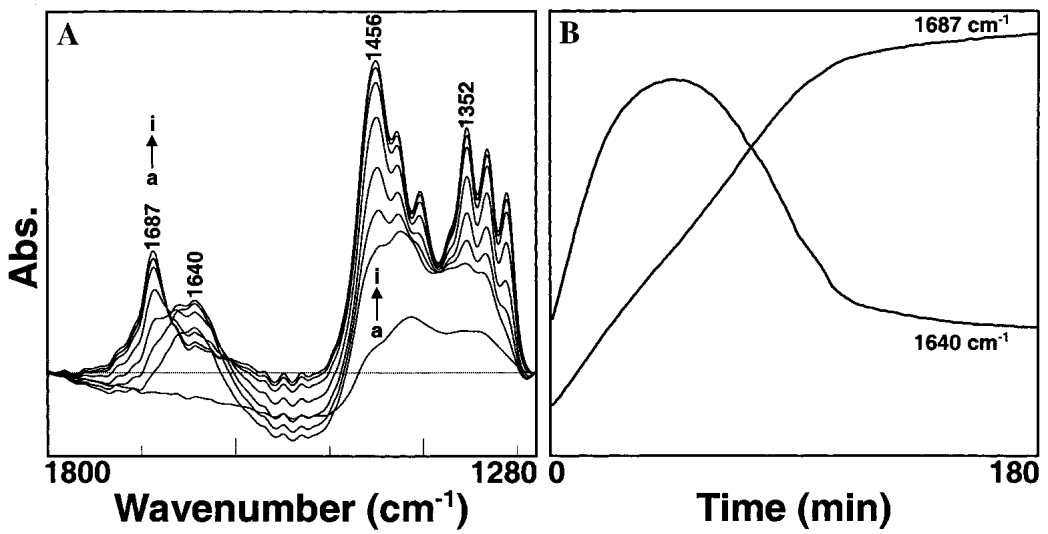

Fig. 6. Isothermal FT-IR spectra of trehalose dihydrate preheated to 120°C (A) and held isothermally at 25°C, 70% RH for 180 min (B). Key: a → i = 0 min → 180 min.

for the anhydrous sample increased linearly until 100 min and then reached equilibrium. Before 50 min, the linear portion of the 1687 cm^{-1} peak might have overlapped the IR peak at 1640 cm^{-1}. During the 50-min rehydration process, both the decrease in the peak intensity at 1640 cm^{-1} and the increase in the peak intensity at 1687 cm^{-1} might be explained by the transformation of liquid water in the hydrate structure to solid-like water. Rehydration of trehalose anhydrate to trehalose dihydrate likely occurs by two separate processes: the anhydrate is rehydrated to the dihydrate form, and liquid water in the dihydrate is transformed to solid-like water.

In conclusion, we successfully used DSC, TGA, and thermal FT-IR microspectroscopy to simulate and investigate the polymorphic transition of trehalose dihydrate in the dehydration, rehydration, and solidification processes, particularly in the temperature range of 50–100°C. A unique polymorphic transition of trehalose dihydrate was first found near 64–67°C from both DSC curve and thermal-dependent FT-IR spectra. This transitional temperature reflected the thermal-dependent transformation from solid-like water to liquid water in the trehalose dihydrate structure during dehydration. The dehydration of trehalose dihydrate might proceed as follows: trehalose dihydrate (solid-like water in dihydrate) is first transformed to trehalose dihydrate (liquid water in dihydrate) and then dehydrated to trehalose anhydrate. However, its rehydration process occurs by rehydration of anhydrous trehalose to the dihydrate form and solidification of the liquid water to solid-like water in the dihydrate structure. The polymorphic transition within this temperature range seems to be correlated with the protective effect of trehalose dihydrate to stabilize the protein structure.

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