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An anhydrous polymorphic form of trehalose

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Abstract

An anhydrous polymorphic form of α,α-trehalose was prepared from trehalose dihydrate by two different drying methods: (1) heating under vacuum; and (2) heating in hot air. Preparation of this anhydrous form by vacuum heating showed good reproducibility. This form was characterized by X-ray powder diffraction analysis and differential scanning calorimetry. This anhydrous form was converted to an amorphous phase at 127 °C and was found to be hygroscopic. At 43% relative humidity at 25 °C, this form rapidly reverted to dihydrate, while the amorphous phase remained unchanged. When an amorphous phase coexisted with this form, the rate of water adsorption to the amorphous phase was slower than that to the amorphous phase alone. These properties of this anhydrous form of α,α-trehalose may explain the effects of trehalose in dehydration tolerance of plants and insects in the desert. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

α,α-Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a nonreducing disaccharide of glucose and is a useful cryoprotectant for liposomes[1] and proteins.[2] Trehalose is currently being considered as a new powder inhaler excipient.[3] Trehalose is found at particularly high concentrations in the so-called 'anhydrobiotic organisms'.[4] These organisms can survive without water for long periods, and upon hydration they rapidly resume active metabolism.[4,5] Two hypotheses have been proposed to account for the stabilizing effect: (1) direct interaction of saccharides with membranes[4,6] and proteins;[2,7] and (2) vitrification of saccharides.[8–10] Crowe et al. suggested that both glass formation of saccharide and its direct interaction with membranes and proteins were required for the stabilizing effect.[11] The special properties of trehalose may not be explained by these hypotheses alone because other saccharides interact with membranes[1] and proteins,[7] and they also undergo vitrification.[8,12]

Upon adding appropriate amounts of water to amorphous trehalose and swelling for 5 h at 65 °C, all or some fraction of amorphous trehalose could be transformed to trehalose dihydrate.[13] Based on these results, we concluded that trehalose intercalated between the phospholipid bilayers and formed phospholipid complexes stoichiometrically.[13] This interaction between trehalose and phospholipid caused loose packing of the hydrocarbon chains of the phospholipid and resulted in a decrease in the gel–liquid crystal transition temperature of the phospholipid.[14,15] The special properties of trehalose as a biostabilizer are considered to be due to its ability to form dihydrate crystals, which provides an increase in glass transition temperature of the remaining amorphous trehalose.[16] However, several saccharides may actually have more or less ability to form hydrates[12] because these saccharides usually exist as hydrate forms; e.g., α-lactose monohydrate, α-melibiose monohydrate, raffinose pentahydrate. Therefore, these properties alone cannot explain the special properties of trehalose in anhydrobiotic organisms.

Two crystal structures of trehalose have been reported. The dihydrate,[17,18] designated here as T_h, is the most common form and is stable at room temperature. The anhydrate,[19] designated here as T_β, can be pre-

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pared by heating the dihydrate under appropriate conditions. Recently, an anhydrous form of trehalose prepared by leaving the dihydrate at 323 K for 48 h under vacuum, Form II,[20] was characterized by FT-Raman, FT IR, and ^{13}C NMR spectroscopies and by X-ray powder diffraction analysis. Taylor et al. questioned the formation of this Form II based on comparison with their FT-Raman data of trehalose dihydrate.[21] These authors concluded that this Form II is not a new crystalline form but is the disordered product of dehydrated dihydrate. More recently, two polymorphic forms of trehalose, T_α[22–24] and T_γ,[23] were characterized by X-ray powder diffraction and/or DSC. T_α is an anhydrous form prepared by heating the dihydrate at 85 °C for 4 h under vacuum and can only be obtained by heating below 100 °C under vacuum.[23] Sussich et al. reported that T_γ was actually a mixture of the dihydrate and the anhydrous form.[25] The detailed physicochemical properties of Form II and T_α, however, have not been reported.

Here, we report an anhydrous polymorphic form of α,α-trehalose, designated as trehalose κ (T_κ). T_κ can be obtained from trehalose dihydrate (T_h) by vacuum heating and heating in hot air. We discussed the relationship between T_κ and other anhydrous forms previously reported.

2. Experimental

Materials and methods.—Trehalose dihydrate (T_h) and the anhydrous form (T_β) were obtained as kind gifts from Hayashibara Biochem. Lab., Inc., Okayama, Japan. Potassium carbonate and sodium bromide were purchased from Wako Pure Chemical Industries Ltd. The materials were used without further purification. Water was purified using the Milli-Q Labo (Millipore Ltd.). T_h was ground in a mortar and pestle and sieved to obtain a particle size of less than 150 μm. An amorphous phase (T_r) of trehalose was prepared by heating T_h to 140 °C at a rate of 2 °C/min. T_κ was prepared from T_h using two drying methods: (1) vacuum heating and (2) heating in hot air. In the vacuum heating method, T_h was loaded into sample holders of glass or silicon plates for X-ray powder diffraction (XRD) and aluminum pans for differential scanning calorimetry (DSC). T_κ was prepared by heating T_h in the sample holders to 100 °C at 4.5 °C/min under vacuum (7 torr) and further heating at this temperature for 30 min under vacuum. One aluminum pan was immediately sealed under a stream of nitrogen gas, and sample weight was measured with an electronic balance. The other aluminum pan was carried to an electronic balance, and changes in sample weight were monitored on the electronic balance under appropriate conditions. The sample plate was immediately mounted onto the

goniometer head under a stream of nitrogen gas, and the XRD of the T_κ was measured under appropriate conditions. Dihydrates of potassium carbonate and of sodium bromide were crystallized in water. Relative humidity (RH) of 43 and 58% were obtained from the saturated potassium carbonate dihydrate and sodium bromide dihydrate solution, respectively, at 25 °C. In the hot air heating method, the T_h sample was heated by hot air. Hot air temperature was determined with a digital surface thermometer.

X-ray powder diffraction analysis.—X-ray powder diffraction (XRD) was recorded using a RINT 1400 X-ray diffractometer (Rigaku Co., Tokyo, Japan). XRD was carried out at 4°/min with a diffraction angle (2θ) from 3 to 40° at 50 kV, and 150 mA for the sample holder of the glass plate, and at 60 kV, and 200 mA for the sample holder of the silicon plate. If dry conditions were required, XRD was performed under a stream of nitrogen gas (5 L/min). If moist conditions (43 and 58% RH) were required, XRD were performed under a stream of air with these relative humidity values. The crystal data for T_h[18] and T_β[19] were used for computation of the idealized diffraction patterns for these forms. The simulation program in the software package RIETAN[26] supplied with the Rigaku analysis system was used for analysis. Crystallinity of the sample was estimated by the multi-peak separation method, and the Ruland method using the 'Crystallinity analysis software package' supplied with the Rigaku Co. In the Ruland method, the XRD of the T_κ and T_h samples was carried out from 3 to 136° (2θ) at 20 °C.

Calorimetric measurements.—Calorimetric measurements were performed with a Rigaku DSC 8240D under Thermo Plus 2 thermal analysis system (Rigaku Co., Tokyo, Japan), at a heating rate of 2 °C/min from 20 to 220 °C without gas flow. Alumina was used as a reference. The transition temperature was determined based on the peak temperature. Calibrations were carried out using tin, lead and indium. To clarify thermal events in the sample, XRD was carried out. In the case of T_κ, T_κ was heated to 140 °C at a scan rate of 2 °C/min in the aluminum-sealed pan, then cooled rapidly to 20 °C. The pan was opened, T_κ was loaded onto a silicon plate, and XRD was performed at 20 °C under a stream of nitrogen.

3. Results

X-ray powder diffraction of T_κ prepared by vacuum heating.—Fig. 1 shows the X-ray powder diffraction (XRD) of T_κ prepared by the vacuum heating method. Based on the fact that T_κ prepared by this method always showed very similar XRDs, we concluded that preparation of T_κ by vacuum heating showed good reproducibility. The XRD of T_h shown in this figure

was that of the T_{κ} sample before heating. The XRDs of T_{κ}, the well-known anhydrous form (T_{β}) and the amorphous phase (T_r) were all measured at 20 °C under a stream of nitrogen gas, while the XRD of T_h was performed in air. The decrease in weight of T_{κ} corre-
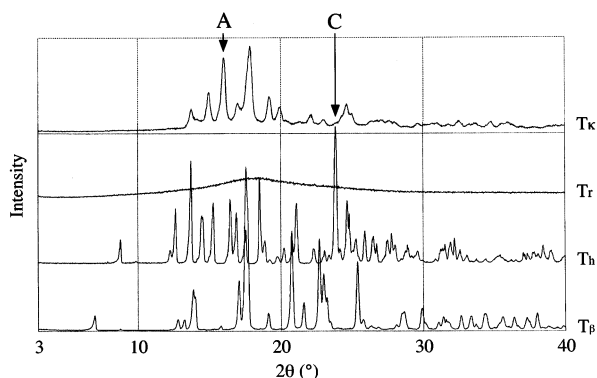

Fig. 1. XRD patterns of three polymorphic forms (T_{κ}, T_h and T_{β}) and an amorphous phase (T_r) of trehalose. T_{κ} was prepared by vacuum heating. A and C indicate diffraction peaks at 16.1 and 23.9°, respectively.
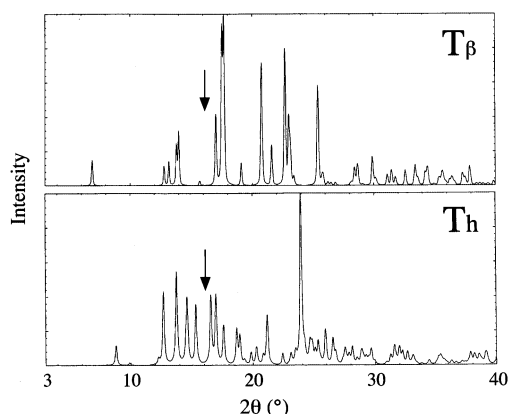

Fig. 2. Computer-generated diffraction patterns of T_h and T_{β}. Arrow indicates 16.1°.
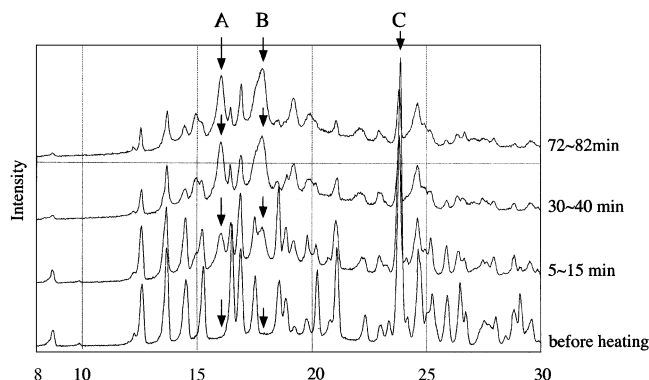

Fig. 3. Diffraction patterns obtained at various time intervals during heating of T_h at 120 °C. A, B and C indicate diffraction peaks at 16.1, 17.9 and 23.9°, respectively. A single measurement required 10 min.

sponded to a loss of two water molecules per molecule of trehalose, indicating that T_{κ} is an anhydrous form of α,α-trehalose. The crystallinity of T_{κ} was calculated to be 73% and $70 \pm 12\%$ by the multi-peak separation method and the Ruland method, respectively. The crystallinity of T_h was calculated to be 100% and $91 \pm 5\%$ by the multi-peak separation method and the Ruland method, respectively. The low crystallinity (ca. 70%) of T_{κ} suggested that T_r was simultaneously formed.

The stability of T_{κ} was examined under the following conditions. The XRD of T_{κ} did not change at 20 °C under a stream of nitrogen gas for 24 h, and no increase in the weight of the T_{κ} sample was observed under the same conditions, suggesting that T_{κ} was stable at room temperature under dry conditions. No changes were observed in the XRD or sample weight of T_{κ} at 100 °C in a chamber for 24 h, suggesting that T_{κ} did not transform to any other forms with heating at 100 °C while exposed to the atmosphere.

The XRD of T_h was not consistent with those of T_h reported in the literature,[20,22] suggesting that T_h shows a preferred orientation. That is, it is possible that some diffraction peaks of T_h could not be detected. Therefore, we compared the XRD of T_{κ} with computer-generated diffraction patterns of T_h and T_{β}, which are shown in Fig. 2. T_h and T_{β} did not show a diffraction peak at 16.1°. In the XRD of T_{κ}, the strongest peak of the T_h sample at 23.9° (see peak C) disappeared, and a strong peak at 16.1° (see peak A) appeared. Thus, T_{κ} could be distinguished from the known forms, T_h and T_{β}. By comparing the XRD of T_{κ} with the halo pattern of an amorphous phase (see T_r in Fig. 1), it was clear that T_{κ} was not the disordered product of dehydrated T_h.

An XRD of T_h with random orientation could be obtained by sieving to obtain a particle size less than 32 μm. The XRD of T_{κ} prepared by T_h with random orientation was similar to that of T_{κ} in Fig. 1. The XRD of T_{κ} prepared by T_h without sieving was also similar to that of T_{κ} in Fig. 1. T_{κ} showed the same XRD independent of orientation of the original T_h, suggesting that T_{κ} did not show the preferred orientation.

X-ray powder diffraction of T_{κ} prepared by hot air heating.—Heating T_h with a normal heating rate (1–60 °C/min) generally caused the transformation to T_r.[23,27] More rapid heating of T_h in hot air caused the transformation to T_{κ}. Fig. 3 shows XRDs obtained at various time points during the heating of T_h at 120 °C by hot air. Peaks at 16.1° (peak A) and 17.9° (peak B) characteristic of T_{κ} appeared with hot air heating and gradually increased in intensity, while the peak intensity at 23.9° (peak C) characteristic of T_h decreased with heating. T_{κ} showed no diffraction peak at 23.9° and T_h showed no diffraction peak at 16.1°. Fig. 4 shows changes in peak intensities at 16.1° (peak A) and 23.9°
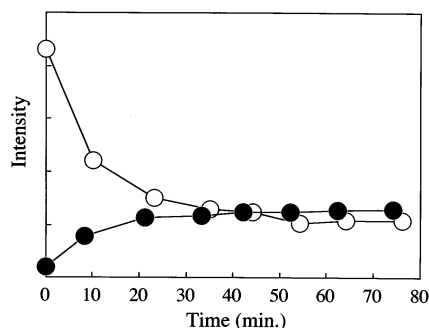
Fig. 4. Changes in intensities of peaks at 16.1° (●) and 23.9° (○) with time at 120 °C.

(peak C) with time at 120 °C. It was apparent that T_h was transformed to T_κ between 0 and 50 min, and after this time, there was hardly any further transformation. With heating in hot air, transformation of T_h was incomplete and T_h remained unchanged. The crystallinity of the XRD (72–82 min) was calculated to be 46% by the multi-peak separation method. This transformation from T_h to T_κ was accompanied by considerable loss of crystallinity, indicating that considerable amount of amorphous phase was simultaneously converted from T_h by hot air heating.

Recovery of T_κ to the original form, T_h.—To examine the hygroscopic nature of anhydrous trehalose, we exposed three anhydrous forms, T_κ, T_β and the amorphous phase (T_r), to conditions of 25 °C and 43% relative humidity (RH) after heating the samples to 100 °C at 4.5 °C/min under vacuum and further heating at this temperature for 30 min under vacuum. Heating induced the transformation from T_h to T_κ, while heating did not cause any change in T_β and T_r. Fig. 5(A) shows the time course of increase in the sample weight in air with 43% RH. In this figure, when the increase in the sample weight corresponded to an increase of two water molecules of per molecule of trehalose, the ordinate (increase in the sample weight) was 100. The XRDs of these samples after exposure to 43% RH for

1 h are shown in Fig. 5(B). These XRD measurements were performed under a stream of air with 43% RH. The weight of T_β was increased to 7.7 within 6 min, and thereafter no change in weight was observed. Exposure of T_β to 43% RH did not cause any change in the XRD. No change in the XRD of T_r was observed, although an increase in the weight of T_r was detected. These results for T_β and T_r indicate that the increase in the sample weight was caused by adsorption of water. The increase in the weight of T_r with time could be fitted by the Lungmuir's equation of adsorption rate:

$$v = v_e[1 - \exp(-\varphi(t - t_0))] \tag{1}$$

where v was the increase in the weight of T_r by water adsorption, v_e was the equivalent value of v, t was the measurement time (min), t_0 was the time at which adsorption began and φ corresponded to the rate constant of the adsorption. Poisson's r and χ^2 for this fit were 0.9917 and 7.29, respectively. The values of v_e and φ were 31.7 and 0.14 (min^{-1}), respectively.

We maintain that an amorphous phase is simultaneously formed during preparation of T_κ, assuming that the following conditions are satisfied: (1) T_κ is transformed to T_h according to a first-order reaction. (2) T_r formed with T_κ is hygroscopic as well as pure T_r and shows the same value of v_e obtained from Eq. (1). The increase in the weight of T_κ over time could be fitted to the following equation

$$S = [T_h] + v = [T_h]_e[1 - \exp(-k(t - t_0))]$$
$$+ (100 - [T_h]_e)(v_e/100)$$
$$\times [1 - \exp(-\psi(t - t_0))] \tag{2}$$

where S is the increase in the sample weight, $[T_h]$ is the increase in the sample weight corresponding to the transformation reaction from T_κ to T_h, v is the increase in the sample weight corresponding to the adsorption of water to T_r, $[T_h]_e$ is the equivalent value of $[T_h]$, k is the rate constant of this transformation, t is the measurement time (min), t_0 is the time at which adsorption
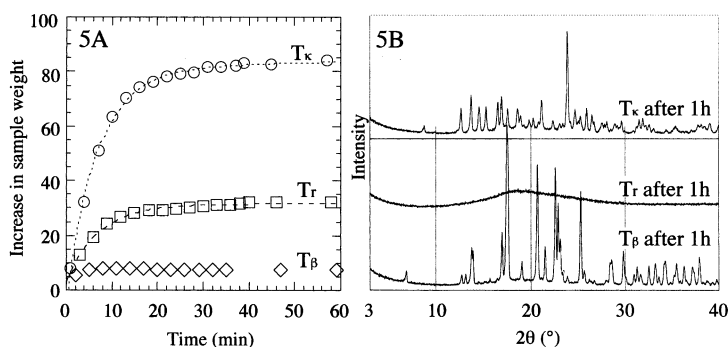

Fig. 5. Changes over time in sample weights of T_r, T_β and T_κ at 25 °C and 43% relative humidity (A) and XRD of these samples after 1 h (B). Before use, T_r and T_β were heated to 100 °C at 4.5 °C/min under vacuum, with further heating at this temperature for 30 min under vacuum.
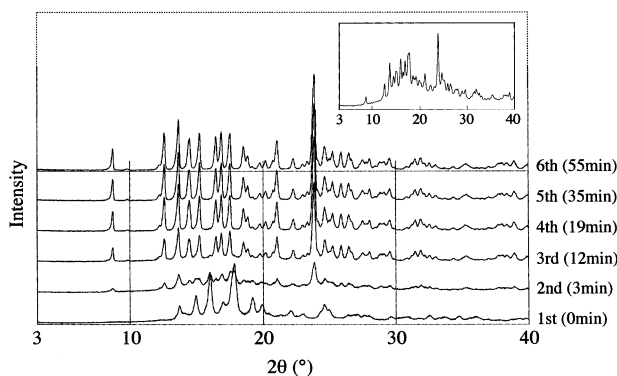
Fig. 6. Changes in the XRD of T_κ prepared by vacuum heating with time at 25 °C and 43% relative humidity. The small frame shows the 2nd XRD.
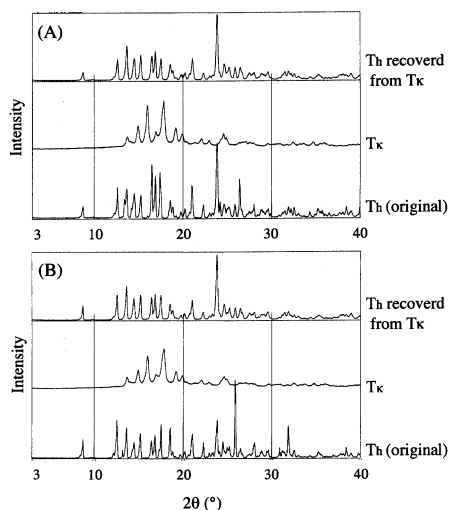

Fig. 7. Comparison of the orientation between the XRD of T_κ prepared from two T_h with different preferred orientations and the XRD of T_h recovered from the two T_κ.

began, and ψ corresponds to the rate constant of the adsorption of water to T_r formed with T_κ. Poisson's r and χ^2 for this fitting were 0.9992 and 11.12, respectively. $[T_h]_e$, k and ψ were 79.8, 0.15 and 0.015 (min^{-1}), respectively. ψ (= 0.015) of T_r formed with T_κ was smaller than that ($\varphi = 0.14$) of T_r alone, which suggested that the hygroscopic rate of T_r coexisting with T_κ was about tenfold slower than that of T_r alone. From the value of $[T_h]_e$, the amount of T_r coexisting with T_κ was estimated to be 20.2%.

T_κ prepared by vacuum heating in the other aluminum pan was also exposed to 43% RH for 1 h. The pan was sealed, and DSC was performed by scanning at 2 °C/min. This DSC showed the endothermic peak at 97.2 °C, corresponding to the transformation from T_h to T_r. By comparing the ΔH of this transition with that of original T_h, we estimated the amount of T_h recovered from T_κ to be 81.6%.

We examined the recovery of T_κ to the original form, T_h, at 25 °C and 43% RH. Fig. 6 shows the XRDs obtained over time for the T_κ prepared by vacuum heating. The second XRD is considered to be that of a mixture of T_κ and T_h. The third XRD differs from that of T_κ and is very similar to that of T_h shown in Fig. 2. After the third measurement, the XRD hardly changed except for increases in peak intensity.

The calibration curve for the amount of T_r was obtained by changes in peak intensity at 13.6° (2θ) of sample with various proportions of T_r and T_h. T_h used in this experiment was sieved with a 32 μm mesh to obtain an XRD with random orientation. The calibration curve was linear;

Integrated peak intensity $= 1.61 \times 10^5$ (fraction of T_h)

$$+ 6.47 \times 10^3 \qquad (3)$$

The correlation coefficient r was 0.994. We obtained the amount of T_h at equilibrium from the sixth XRD in Fig. 6 using this calibration curve. This value was 82%, which is similar to that (79.8%) estimated by Eq. (2). This similarity supports the correctness of the assumption in Eq. (2).

Although T_r was not transformed to any other form after 2 days at 43% RH at 25 °C, T_r was easily converted to T_h at 58% RH. Under these conditions, diffraction peaks of T_h were detected in the halo pattern of T_r within 5 min, and the intensity increased and was hardly changed after 80 min. When T_κ prepared by vacuum heating was exposed to 58% RH for 30 min, the increase in the weight of T_κ corresponded to two water molecules per molecule of trehalose. After exposure of T_κ to 58% RH at 25 °C for 30 min, the XRD and DSC of this sample were measured. The amount of T_h in T_κ after 30 min obtained from calibration curve of Eq. (3) was 99.6%. Analysis of ΔH corresponding to the transformation from T_h to T_r showed that T_h corresponding to 100% of the weight of the original T_h recovered by passing through T_κ and T_r.

The third XRD in Fig. 6 is consistent with the computer-generated diffraction pattern of T_h (see Fig. 2), which is considered due to transformation of T_h in random orientation in the sample. To examine changes in the orientation of T_κ and T_h recovered form T_κ, we used two original T_h's with different orientations. Fig. 7 shows the XRD of original T_h with the preferred orientation and changes in XRD by vacuum heating and subsequent hydration. Two original T_h's showed different XRDs due to the preferred orientation. The XRD of T_κ, however, was similar regardless of orientation and was similar to that of T_κ in Fig. 1, suggesting that T_κ showed a random orientation. This similarity was observed in other T_κ samples, as indicated in the previous section. Two diffraction patterns of T_h recovered from T_κ were similar and showed random orienta-

tions. Half-width of the peak at 16.1° (2θ) of T_h recovered from T_κ was larger than that of the original T_h by 0.036°. These results indicated that the mean diameter of the crystal particles of T_h recovered form T_κ was smaller than that of the original T_h. This observation corresponded to the finding that the XRD of T_h with random orientation could be obtained by sieving to obtain a particle size less than 32 μm.

Thermal analysis of T_κ form.—Differential scanning calorimetry (DSC) of T_κ prepared by vacuum heating are shown in Fig. 8. The DSC of T_h in the open pan showed two endothermic peaks at 100 and 210 °C and an exothermic peak at 166 °C. T_β in the open pan showed an endothermoic peak at 205 °C. These thermal events of T_h were consistent with those previously reported.[22]

For T_κ, endothermic peaks at 127 °C (onset temp., 122.3 °C, ΔH, 5.4 kJ/mol) and at 121 °C were observed in sealed and open pans, respectively. Endothermic peaks at 127 °C were characteristic of T_κ. The XRD analysis indicated that T_κ was transformed to an amorphous phase (T_r) at these temperatures. The endothermic peak at 93 °C in the open pan was due to the formation of T_h by moisture in the air. The exothermic peak at about 193 °C was due to transition from T_r to T_β. T_κ was stable at room temperature without exposure to the atmosphere because no thermal events were observed below 127 °C in the sealed pan.

4. Discussion

The decrease in weight for T_κ corresponds to a loss of two water molecules from T_h, indicating that T_κ is an anhydrous polymorphic form of α,α-trehalose. Due to the hygroscopic nature of T_κ and transformation from T_κ to T_h if experiments are not performed under appropriate dry conditions, it is possible that T_κ would be

Fig. 8. DSC thermograms of three polymorphic forms (T_κ, T_h and T_β). Heating rate was 2 °C/min. T_κ was prepared by vacuum heating.

regarded as a different anhydrous form. Here, we discuss the correlation between T_κ and other anhydrous forms. Three anhydrous forms of α,α-trehalose (From II,[20] T_α[22–24] and T_γ[23]) have been characterized by XRD and DSC. T_κ could be distinguished from T_γ because T_γ was a mixture of T_h and T_β.[25]

We could not precisely compare the XRD of T_κ (see Fig. 1) with that of Form II (see Fig. 8 in Ref. 20) because the horizontal axis (2θ-value or d-value) was not shown in this previous report. The XRD of T_κ is different from that of Form II; however, the XRD of Form II was similar to that of partially hydrated T_κ, i.e., a mixture of T_κ and T_h (see the small frame in Fig. 6). The hygroscopic nature of Form II was recently reported.[28] Form II may be partially converted to T_h during XRD measurements.

For T_α prepared under the same conditions, two different results for XRD and/or DSC were reported by Sussich et al.[22–24] One, referred to here as T_α-1, was described in a preliminary report,[22] and the other, T_α-2, was described in a full paper.[23,24] The XRD of T_κ is similar to that of T_α-1 (see Fig. 2 in Ref. 22) and is different from that of T_α-2 (see Fig. 5 in Ref. 23). In contrast, the DSC of T_κ is similar to that of T_α-2 (see Fig. 3 in Ref. 23 and Fig. 2 in Ref. 24) and is different from that of T_α-1 (see Fig. 1 in Ref. 22). The DSC data of T_κ (onset temp., 122.3 °C; ΔH, 5.4 kJ/mol) were similar to those of T_α-2 (onset temp., 120.9 $\pm$ 1.3 °C; ΔH, 5.8 $\pm$ 0.3 kJ/mol[24]). From the results of XRD analysis of T_α-2, Sussich et al. proposed a hypothetical structure that has the same lattice parameters as those of T_h, but one that does not have any crystal water molecules.[23] In the computer-generated diffraction pattern for this anhydrous structure, the diffraction peak at 9° (2θ) disappeared. As the diffraction peak at 9° (2θ) was observed in the diffraction pattern of T_α-2 (see Fig. 5 in Ref. 23), the diffraction pattern of T_α-2 does not correspond to this hypothetical structure. Since it was recently reported that T_α was hygroscopic,[29] it is reasonable that samples of T_α-2 for XRD were partially hydrated during XRD measurement. Therefore, we suppose at present that T_α and Form II are identical to T_κ.

The XRD of T_κ is quite different from that of T_h. We must, however, consider the fact that differences in diffraction pattern do not always indicate differences in packing mode, as seen in polymorphic forms of ($\pm$)-12-hydroxystearic acid.[30,31] In this instance, the XRD of two polymorphic forms are apparently different, but the two packing modes are very similar. Therefore, we did not describe the detailed changes in packing by the transformation because we have not determined the crystal structure of T_κ.

The effects of trehalose on anhydrobiosis result from its glassy state.[11] The transition temperature (T_g) from the glassy to the rubbery state is sensitive to water
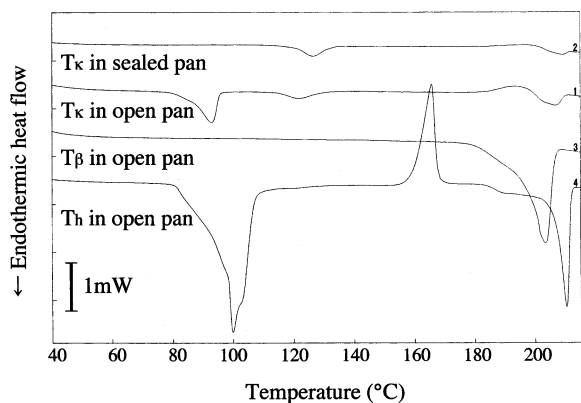
content.[32] Addition of water causes a rapid decrease in T_g of trehalose. As a result, the abilities of trehalose to stabilize membrane and protein are lost. The special properties of trehalose are due to shielding of the remaining glassy trehalose from the added water by transformation from an amorphous phase to T_h.[11] The results of the present study indicate that heating of T_h in hot air induces production of T_κ. Many plants and insects in desert regions are exposed to hot dry air during the summer. It is possible that T_κ and T_r of trehalose are produced in these organisms under conditions of extreme dehydration. We clarified that at 43% RH, the hygroscopic rate of T_r coexisting with T_κ was about tenfold slower than that of T_r alone. If this mixture of T_κ and T_r were exposed to moisture, T_κ would be transformed to T_h more rapidly than by water adsorption to T_r. As a result, no decrease in T_g of the glassy phase would occur. Thus, the special properties of this form may explain the special effects of trehalose in dehydration tolerance of plants and insects in the desert.

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