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Research Article

Title: Crystallization Characteristics of Amorphous Trehalose Dried from Alcohol

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Running Title: ANHYDROUS CRYSTALLIZATION OF TREHALOSE

ABSTRACT

Trehalose forms a glass that can be used to preserve labile substances under desiccation. The crystallization characteristics, namely crystallization temperature (T_{cry}) and isothermal crystallization behavior of amorphous trehalose, dried from alcohol (methanol, ethanol), was analyzed and the results were compared with those for the amorphous trehalose freeze-dried from water. The use of alcohol as a drying solvent lowered the T_{cry} by ~90˚C from the value for the case of an aqueous solvent. The formation of multiple forms of crystals and partial melting were suggested by the thermal analysis. Isothermal crystallization experiments showed that the alcohol-originated amorphous trehalose was eventually exclusively converted into ß-form crystals. The induction period (t_{ind}) before the start of isothermal crystallization was markedly shortened when alcohol was used as the solvent compared to water. The t_{ind} values for various amorphous sugar samples including the alcohol-originated ones could be correlated with difference between T_{cry} and the sample temperature.

Key words: trehalose, crystallization, anhydrous crystal, methanol, vacuum foam drying

1. Introduction

Trehalose (1-O-α-D-glucopyranosyl-α-D-glucopyranoside) is used as a stabilizing agent and an excipient in the food and pharmaceutical industries (Crowe, 2007; Elbein, Pan, Pastuszak, & Carroll, 2003; Jain, Roy, 2009; Kopjar et al., 2013; Neri et al., 2014; Ohtake & Wang, 2011; Umene, Hayashi, Kato, & Masunaga, 2015), and the commercial usage of trehalose appears to be increasing year by year (Cai et al., 2018; Walayat et al., 2020). Trehalose is commonly available in the form of a dihydrate crystalline material. The procedures and conditions for preparing crystalline trehalose dihydrate was commercially established (Kubota, Sawatani, Oku, Takeuchi, & Murai, 2004; Schiraldi, Di Lernia, & De Rosa, 2002).

Trehalose can exist in the form of anhydrous crystals and the crystallization behavior of trehalose under anhydrous conditions has been extensively investigated (Nagase et al., 2008; Ohashi, Yoshii, & Furuta, 2007a; Ohashi, Yoshii, & Furuta, 2007b; Pyszczynski & Munson, 2013; Sussich, Urbani, Princivalle, & Cesàro, 1998). The ß-form of trehalose crystals can be produced by heating dihydrate crystals at 85˚C under a vacuum (Nagase, Endo, Ueda, & Nagasaki, 2002) or at 70˚C in ethanol containing 2.5% water (Verhoeven et al., 2012). Amorphous trehalose, vacuum dried from an aqueous solution, was also converted into the anhydrous crystal in the α-form (Reisener, Goldschmid, Ledingham, & Perlin, 1962) while spray-dried (Surana, Pyne, & Suryanarayanan, 2004) or freeze-dried amorphous trehalose (Roe, & Labuza, 2005) does not undergo crystallization under anhydrous conditions. In addition, the aging of amorphous trehalose was found to facilitate the crystallization of the anhydrous form of trehalose (Surana, Pyne, & Suryanarayanan, 2004).

It should be noted that our previous study demonstrated that, when a sugar is amorphized somehow such as by the freeze-drying of an aqueous solution, the solubility of the resulting amorphous sugar in a simple alcohol such as methanol was much greater than the equilibrium solubility of the material (Satoh et al., 2016). An organic solution containing sugar can be converted into an amorphous sugar powder by allowing it to dry up before the occurrence of segregation. The alcohol-originated amorphous sugars have significantly lower glass transition temperatures than corresponding samples dried from an aqueous solution (Takeda et al., 2017) and thus would also be expected to show different crystallization characteristics.

This study reports on an examination of the crystallization characteristics of amorphous trehalose, dried from an organic solvent, as well as their glass transition behavior. Amorphous trehalose was prepared by drying an alcohol solution of

trehalose and the glass transition temperature (T_g), crystallization temperature (T_{cry}) and crystallization processes of the material under different conditions as well as the crystal form of crystallized trehalose were determined. Interactions between trehalose molecules in the dried amorphous matrix were also analyzed and the findings were compared between different types of solvent.

2. Materials and methods

2.1. Materials

Methanol and ethanol, used as solvent, were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Trehalose was a product of Nacalai Tesque Inc. (Kyoto, Japan). Dihydrate trehalose crystal powder was dissolved in distilled water to give a final concentration of 100 mg/mL. A 10 mL ~~solution~~ of a trehalose solution was frozen in a freezer (-20˚C) and further cooled in liquid nitrogen for ~5 min to give a completely frozen sample. The frozen samples were freeze-dried, as described in our previous study (Imamura et al., 2008a). Our previous studies showed that the resulting freeze-dried matrix of trehalose is fully amorphous (Takeda et al., 2017). The freeze-dried cake of amorphous trehalose was thoroughly dehydrated by storage under a vacuum over P_2O_5 at 37˚C for more than three days. The attained residual water content of the amorphous trehalose cake was below the detection limit (>0.002 g/g-dry matter) based on a Karl-Fischer titration analysis (Takeda et al., 2017).

2.2. Vacuum foam drying

A 10 ~~ten~~ mL portion of methanol was added to a glass vial that contained 1 g of amorphized trehalose cake and vigorously shaken with a vortex mixer for 10 seconds to homogeneously disperse (dissolve) the trehalose in methanol. Aliquots of 100 µL of the organic solution were transferred to 1.5 mL polypropylene sampling tubes and then vacuum-dried at ca. 1000 Pa for 90 min (initial drying) using a centrifugal concentrator (EYELA CVE-1100, TOKYO RIKAKIKAI Co., Tokyo, Japan) connected to a diaphragm type vacuum pump (FDU-1200, ULVAC Japan, Ltd., Tokyo, Japan). The foaming of the solution was not induced during the initial drying. After the initial drying, the sample solution was removed from the centrifugal concentrator and then punctured with a stainless steel needle (Hidaka et al., 2019; Satoh et al., 2017), followed by further vacuum drying (Second drying) for 30 min. The needle puncturing

(stimulation) reliably induced foaming immediately after the start of the secondary drying. The amount of the remaining organic solvent in the dried sample was estimated to be less than 0.01 g/g-dry matter, based on drying profiles reported in our previous study (Hidaka et al., 2019; Satoh et al., 2017).

2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) analyses of amorphous trehalose samples, obtained from alcohols (methanol, ethanol) as well as water, were conducted, using a TA Q2000 calorimeter (TA instruments Co., New Castle, DE) equipped with RCS90 cooling system (TA instruments Co.) according to the same procedures as was used in our previous study (Sekitoh et al., 2021; Takeda et al., 2017; Takeda et al., 2019). Namely, 1~5 mg of an amorphous trehalose sample was hermetically sealed in an aluminum pan and then scanned from -40˚C to 190˚C at a rate of 10˚C/min, in which an empty aluminum pan was used as a reference. The glass transition temperature of the sample, T_g, was determined as the onset of heat capacity change in the obtained DSC curve. The prewarming of the sample was omitted since it has been known to alter the characteristics of the amorphous sugar matrix dried from alcohol (Sekitoh et al., 2021; Takeda et al., 2019).

2.4. Powder x-ray diffractometry

The amorphous trehalose sample that had been dried from methanol (and then sealed in a DSC pan) was stored at 80˚C for 60 min to fully crystallize (isothermal crystallization). The resulting heated sample as well as the unheated sample were pulverized into fine powders and then placed on a sample holder of an X-ray diffractometer PANalytical X'PERT PRO MPD system with Cu-Kα radiation (PANalytical B.V., Almelo, Holland). X-ray diffraction spectra of the trehalose samples were measured at an X-ray tube voltage of 45 kV and a current of 40 mA. The trehalose samples, dried from alcohols or water and without being heated, were also analyzed by XRD and confirmed to be fully amorphous.

2.5. Fourier transform infrared ray spectroscopy

IR absorption spectra of amorphous trehalose samples that had been dried from different solvents were obtained using a Fourier transform IR spectrometer (FTIR,

Nicolet 4700, Thermo Scientific Inc., Madison, WI) with a diffuse diffraction technique in the same manner as was used in our previous study (Imamura et al., 2008b). Namely, a 1~2 mg sample of amorphous sugar was ground into a fine powder with an approximately 100-fold amount of powdered KBr. The resulting mixed powder was packed in a stainless steel cup for the diffuse reflection attachment (Gemini, Spectra Tech., Co., Shelton, CT) and then scanned 64 times from 600 cm^{-1} to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

The extents of formation of sugar-sugar hydrogen bonds in different amorphous sugar samples can be semi-quantitatively compared based on the peak frequency of the sugar O-H stretching vibration bands (Imamura et al., 2006; Kagotani et al., 2013; Takeda et al., 2019; Wolkers, van Kilsdonk, & Hoekstra, 1998). Hence, the measured IR spectra for dried amorphous trehalose samples were smoothed at 80 points and the peak wavenumber of the IR band due to sugar O-H stretching vibration was determined at around 3300 cm^{-1} (Kagotani et al., 2013; Takeda et al., 2019).

The FTIR measurement were at least triplicated for each sample, and the deviations in wavenumber was within 5 cm^{-1} of the average values.

3. Results and discussion

3.1. DSC measurements

The thermograms of amorphous trehalose samples, prepared by vacuum foam drying from alcohols and freeze-drying from water, are shown in Fig. 1. A single shift in the apparent heat capacity of the sample, corresponding to the glass-to-rubber transition, was detected in all the amorphous trehalose samples. The glass transition temperatures, T_g, for the trehalose samples were determined from the DSC thermograms and the results are listed in Table 1. As shown in Table 1, the T_g value of amorphous trehalose matrix is varied depending on the solvent. Namely, the T_g for the freeze-dried amorphous trehalose obtained from the aqueous solution was around 105 ˚C, which is in good agreement with previously reported values (Imamura et al., 2008c; Miller & de Pablo, 2000; Roos, 1993; Saleki-Gerhardt & Zografi, 1994; Surana, Pyne, & Suryanarayanan, 2004) although some data showed slightly higher (~10˚C) values (Miller & de Pablo, 2000; Saleki-Gerhardt & Zografi, 1994; Surana, Pyne, & Suryanarayanan, 2004); The methanol- and ethanol-solutions originated samples exhibited ~50 and ~30˚C lower T_g than the water-originated ones.

The amorphous trehalose samples dried from alcohols exhibited an exothermic peak at temperatures that were 30~50˚C higher than T_g (curves (a) and (b) in Fig. 1) as well as an endothermic peak in the temperature range above ~170˚C. These exothermic and endothermic peaks are assumed to be due to the crystallization and melting of trehalose, respectively. It should be noted that the thermograms for the methanol- and ethanol-originated samples exhibited an at-least-three-dented bottom of the 170~200˚C endothermic peak. This indicates that multiple forms of crystals are formed in the DSC upward scan, which has also been indicated in a previous study (Nagase et al., 2008; Pyszczynski & Munson, 2013; Sussich, Urbani, Princivalle, & Cesàro, 1998).

In the case of water as a solvent, the two exo- and endothermic peaks were significantly overlapped with each other. This results in markedly small ΔH_{cry} and ΔH_m values (Table 1), relative to those for the case of water as the solvent. Assuming that the ΔH_m for the water solvent is equal to the reported value (~156 J/g) (Cai et al., 2018; Jain & Roy, 2009), the overlapping magnitude is estimated to be approximately 130 J/g. It thus follows that the true value for the ΔH_{cry} for the water-originated sample would likely be around 150 J/g, which is in agreement with the reported value (~140 J/g) (Surana, Pyne, & Suryanarayanan, 2004). Compared to the true ΔH_{cry} value for the water solvent, the ΔH_{cry} values for the alcohol-originated samples were markedly low, implying that the corresponding exotherm involves not only (multiple forms of) trehalose crystallization but also melting. Actually, in the thermogram for the methanol-originated sample, the exotherm is accompanied by a small endothermic peak, which may be due to the endotherm for the melting of crystallized trehalose in the α (T_m=120˚C) (Pyszczynski & Munson, 2013; Sussich, Urbani, Princivalle, & Cesàro, 1998) and γ forms (Sussich, Urbani, Princivalle, & Cesàro, 1998)).

The onset temperatures (T_{cry} and T_m) and heats of the exothermic (ΔH_{cry}) and endothermic peaks (ΔH_m) as well as the reported values (Pyszczynski & Munson, 2013; Surana, Pyne, & Suryanarayanan, 2004; Sussich, Urbani, Princivalle, & Cesàro, 1998) are listed in Table 1. The data shown in Table 1 indicate ~~indicates~~ that the use of methanol or ethanol as a drying solvent significantly lowers the T_{cry} value and therefore facilitates the crystallization of trehalose, compared to the case of water as the solvent. The T_{cry} for the methanol-originated sample is slightly lower than that for the ethanol-originated one. The melting enthalpy, ΔH_m for the case where the sample was dried from alcohols is in the range of 140~160 J/g. This is entirely consistent with the reported value (Cai et al., 2018; Jain & Roy, 2009) and also roughly comparable to the melting enthalpy of sucrose (134 J/g) (Hurtta, Pitkanen, & Knuutinen, 2004) although

the existence of different forms of trehalose crystals was suggested by the DSC thermogram (Fig. 1), as described above.

Figure 2 shows the IR absorption O-H stretching vibration ($\nu_{O\text{-}H}$) bands for amorphous trehalose samples dried from different solvents. As shown in Fig. 2, the methanol-originated sample showed considerably lower peak wavenumbers for the $\nu_{O\text{-}H}$ band than the other two, suggesting a greater extent of hydrogen bond formation. The $\nu_{O\text{-}H}$ band for the ethanol-originated sample is positioned at a slightly higher frequency than that of the methanol-originated one (Fig. 2).

Our previous study suggested that the specific molar volume of a disaccharide (α-maltose) in methanol was approximately 30% smaller than that in water (Takeda et al., 2019). Trehalose molecules are also considered to have markedly smaller occupied volumes in alcohols than in water and possibly retain highly packed conformations, even when thoroughly dried (from alcohols). Consequently, the intimate hydrogen bonding may be an important feature of the amorphous trehalose matrix dried from alcohols by the smaller occupied volume of trehalose molecules, as indicated by the lower peak wavenumbers of the $\nu_{O\text{-}H}$ band (Fig. 2).

When the methanol- and ethanol-originated samples are compared, the T_g, T_{cry} and IR peak wavenumbers of the $\nu_{O\text{-}H}$ band are slightly higher in the case where methanol is used as a drying solvent than in the case of ethanol while they are clearly lower than when dried from water (Table 1 and Fig. 2). Although detailed reasons for this are unknown at the present stage, this may possibly be related to the molar bulk densities of methanol and ethanol. Namely, ethanol has ca. a 44% smaller molar density (17.2 mmol/mL) than methanol (24.7 mmol/mL) (Merck index No. 5816 and 212, 10th ed), which might allow trehalose molecules to be less packed in ethanol than in methanol. Consequently, intermediate characteristics between those of the methanol- and water-originated samples might be exhibited when ethanol was used as the drying solvent.

Considering the findings obtained from the FTIR analysis (Fig. 2) and based on the "free volume theory (Boyer & Spencer, 1944; Frenkel, 1955)," the markedly lower T_g value for the alcohol-originated samples than that for the water-originated samples can be explained as follows. Namely, the dependence of the specific molar volume of the trehalose molecule on temperature can be schematically represented by two lines, corresponding to glassy and rubbery states as shown in Fig. 3, and the crossing point temperature is the T_g value for amorphous trehalose. As described above, the specific volume of trehalose molecules in the alcohol-originated amorphous matrix is considered to be smaller than that in the water-originated sample, which would shift the line for the

glassy state downward (dotted line in Fig. 3). Assuming that the specific molar volume in the rubbery state is independent of the solvent type, it follows that the crossing point between the glassy- and rubbery-state lines (namely T_g) will shift toward lower temperature (Fig. 3).

3.2. Isothermal crystallization behavior

As indicated by Fig. 1, the crystallization of an amorphous trehalose matrix is markedly facilitated when it is first dried from methanol and ethanol. Hence, we next conducted isothermal crystallization tests of the alcohol- and water-originated amorphous trehalose. Figure 4 shows XRD patterns for the amorphous trehalose sample that had been vacuum foam dried from methanol and then heated for 60 min at 80˚C. The resulting XRD spectrum after the isothermal crystallization test indicates the presence of several sharp peaks, and the peak positions are completely consistent with those for ß form of anhydrous crystals of trehalose (Sussich, Urbani, Princivalle, & Cesàro, 1998). This suggests that ß-form of trehalose anhydrous crystal is exclusively obtained in the isothermal crystallization. However, when the entire crystallization exotherm was integrated during the isothermal crystallization test, the value was found to be ca. 37±5 J/g, which is approximately one fourth that for ΔH_m and even less than the ΔH_{cry} (Table 1). The isothermal crystallization of anhydrous trehalose therefore appears to involve multiple thermal events, including the formation of multiple forms of trehalose crystals and their subsequent melting, although all of the trehalose molecules are eventually converted into the ß form. This insight is consistent with the observation for the ascending DSC scanning (Fig. 1 and Table 1).

The isothermal crystallization processes of the amorphous trehalose samples are shown in Fig. 5. The crystallization processes were approximated by using the following modified Avrami equation (Avrami, 1939; Avrami, 1940; Avrami, 1941; Erofeev, 1946; Imamura et al., 2010; Imamura, Kinugawa, Kagotani, Nomura, & Nakanishi, 2012; Kedward, MacNaughtan, Blandshard, & Mitchell, 2008; Kedward, MacNaughtan, & Mitchell, 2000), having the induction period, t_{ind} (min):

$$C(t) = \exp[-\{k_{cry}(t-t_{ind})\}^n] \quad (1)$$

where $C(t)$ denotes the degree of crystallinity at the time point of t (min), and k_{cry} and n are originally regarded as the rate constant and dimension of crystal growth, respectively (Avrami, 1939; Avrami, 1940; Avrami, 1941; Erofeev, 1946). The kinetic

parameters for the crystallization of the different samples and temperatures were determined by the Avrami plot of ln$C(t)$ vs ln(t-t_{ind}) (Fig. 5(b)) and the results are summarized in Table 2. As shown in Table 2, the induction time for the crystallization, t_{ind}, is drastically shortened with increasing temperature. It should be noted that the t_{ind} values for the methanol- and ethanol-originated samples are in the same range as those for the water originated sample (Table 2) irrespective of the significantly lower tested temperature range. This demonstrates that the use of alcohols as a solvent significantly facilitates crystal nucleation.

On the other hand, since the multiple thermal events including crystallization and melting may occur during isothermal crystallization, as described above, the obtained k_{cl} and n values are only qualitative rather than reflecting the actual crystal growth kinetics. However, the determined n values are roughly 5±1 (-), except for the case of water as a solvent and heating temperature of 170˚C (Table 1 and Fig. S1(a)). These large n values (>4) may be attributed to the overlapping of multiple phase transitions of trehalose that can allow the increase in the "specific" surface area of the crystal phase with the advance of the crystallization (Ohashi, Yoshi, & Furuta, 2007b). The isothermal crystallization of water-originated amorphous trehalose shows an exceptionally small ~~k_{cry}~~ n value at 170˚C (3.2±0.4), which may possibly be related to the testing temperature being quite close to the T_m value (Table 1).

The crystal growth rate has been accepted to increase with (T-T_g) value (Truong & Wang, 2017). However, the plot of k_{cry} values (Table 2) as a function of (T-T_g) (Fig. S1(b)) exhibits clearly different curves for each solvent although the k_{cl} value for each solvent-originated sample usually increases with increasing (T-T_g). This finding may suggest that the crystal growth kinetics is strongly affected by the solvent type and thus trehalose molecule conformation before being dried. The exceptionally small k_{cl} value for water-originated amorphous trehalose at (T-T_g)=63˚C (Fig. S1(b)) may be due to the melting overimposed to crystallization, similarly to the small n value (3.2±0.4) as described above.

The amorphous sugar matrix, obtained by the freeze-drying the aqueous solution of sugar, is comprised of pores and thin pore walls (a few micrometers in thickness) (Abdul-Fattah,2007; Imamura et al., 2010), and the pore walls contain huge cavities that take up (sorb) water molecules (Imamura, Kagotani, Nomura, Kinugawa, & Nakanishi, 2012; Kagotani et al., 2013). Our previous study demonstrated that, when compressed at several hundred MPa, the pores and cavities in freeze-dried amorphous sugar are reduced and, concomitantly, the t_{ind} is markedly shortened (Imamura et al., 2010; Imamura, Kinugawa, Kagotani, Nomura, & Nakanishi, 2012). Based on these findings,

we conclude that the matrix pores and cavities may serve as a discontinuous area that interferes with the nucleation of sugar crystals and subsequent growth; The continuity of the sugar matrix is increased when the matrix pores and cavities are diminished due to the compression, resulting in sugar crystallization being facilitated (Imamura, Kinugawa, Kagotani, Nomura, & Nakanishi, 2012). On the other hand, in the case of vacuum foam drying, the sugar in alcohol exists in the dissolved (liquid) and rubbery state until it is converted into a glassy solid (Hidaka et al., 2019). The flowable state of a sugar solution during vacuum foam drying is thought to result in minimizing the discontinuities in the sugar matrix and thus facilitate the subsequent crystallization of the sugar. Consequently, the amorphous trehalose, vacuum-foam-dried from alcohol, may exhibit markedly a smaller t_{ind} value (Table 2) as well as a lower T_{cry} than a sample that was freeze-dried from water (Table 1).

Our previous studies revealed that the induction period before the crystallization for amorphous sucrose and α-lactose, prepared by freeze-drying, could be correlated with the difference between the crystallization temperature (T_{cry}) and the sample temperature (Imamura, Kinugawa, Kagotani, Nomura, & Nakanishi, 2012; Kinugawa et al., 2015). Figure 6 summarizes the t_{ind} values for the amorphous trehalose samples prepared in this study as well as those for freeze-dried amorphous sucrose and lactose. As shown in Fig. 6, the t_{ind} values for amorphous trehalose that was dried from different solvents, are plotted around the curve representing the t_{ind}-(T_{cry}-T) relationship for sucrose and α-lactose (Imamura, Kinugawa, Kagotani, Nomura, & Nakanishi, 2012; Kinugawa et al., 2015). Accordingly, the induction period for crystallization can be predicted from the T_{cry} value, irrespective of the type of the original solvent used as well as the sugar type.

The T_{cry} can be regarded as the temperature at which a critical nucleus that can grow is formed without any delay, and the probability for the critical nucleus formation is thus expected to decrease with decreasing temperature; In principle, when the sample temperature is lowered to T_g, the induction period would become infinite. Considering these, the existence of a common relationship between t_{ind} and (T_{cry}-T) would means that the decrease in t_{ind} with increasing sample temperature from T_g to T_{cry} is in the same order, irrespective of the type of solvent and sugar type as well as water sorption state, etc.

Our previous study (Sekitoh et al., 2021; Takeda et al., 2019) indicated that the thermal annealing of the amorphous sugar, vacuum foam dried from alcohols, markedly increased the T_g value. Considering this, the T_{cry} also is expected to be altered by thermal annealing, and furthermore, the temperature where amorphous sugar is solidified, namely drying temperature, may change (increase) the T_g and possibly T_{cry}

values. Further study on the influence of the drying condition (especially, drying temperature) on the thermal characteristics of the amorphous trehalose matrix is required to increase the options to produce the amorphous sugar-based products.

4. Conclusions

Amorphous trehalose was prepared by vacuum foam drying from alcohols (methanol and ethanol), and the T_g, T_{cry}, and crystallization behavior of the samples were compared with those for aqueous freeze-dried trehalose. The trehalose samples produced from methanol and ethanol showed markedly lower T_g values than the water-originated sample, which can be explained by their high molecular packing states, as evidenced by FTIR analysis. The T_{cry} and induction periods (t_{ind}) before the start of the isothermal trehalose crystallization were markedly lower and shorter in the case of samples that were dried from alcohols compared to samples that were freeze-dried from water. This indicates that the use of alcohol as a solvent facilitates the formation of the critical nucleus of anhydrous trehalose crystal that can then grow. Plots of t_{ind} against (T-T_{cry}) for the amorphous trehalose obtained from different solvents (methanol, ethanol, and water) coincide well with those reported for different sugar types, sample treatments (rehumidification and compression), and related processes. The isothermal crystallization eventually exclusively produced ß form crystals, but different types of anhydrous trehalose crystals appear to have been temporarily produced during the overall process. Hence, for an understanding of the detailed crystallization process of anhydrous trehalose, the formation and melting of the individual forms of crystals will need to be examined.

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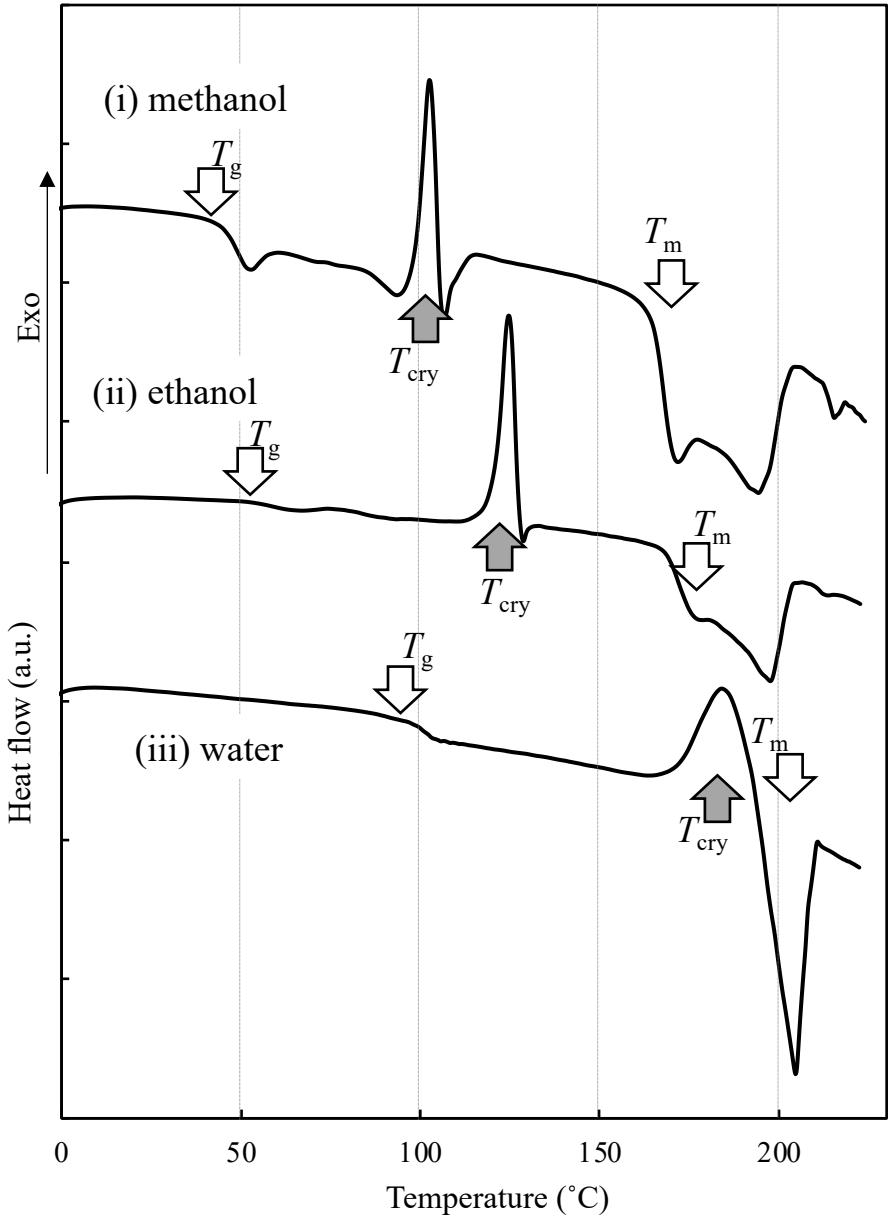
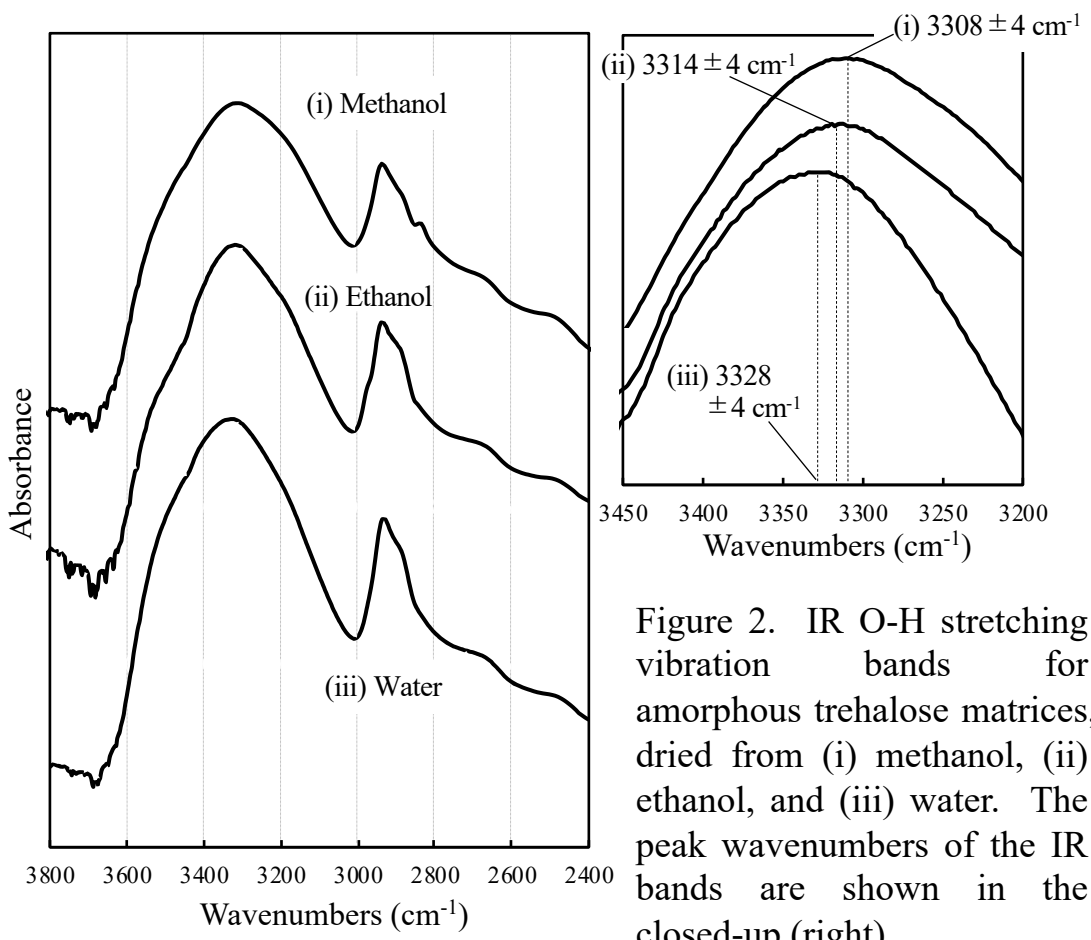


Fig. 1. DSC thermograms for amorphous trehalose matrices, vacuum-foam dried from (i) methanol and (ii) ethanol and (iii) for freeze-dried aqueous trehalose solutions. Arrows in the graph denote T_g, T_{cry}, and T_m.



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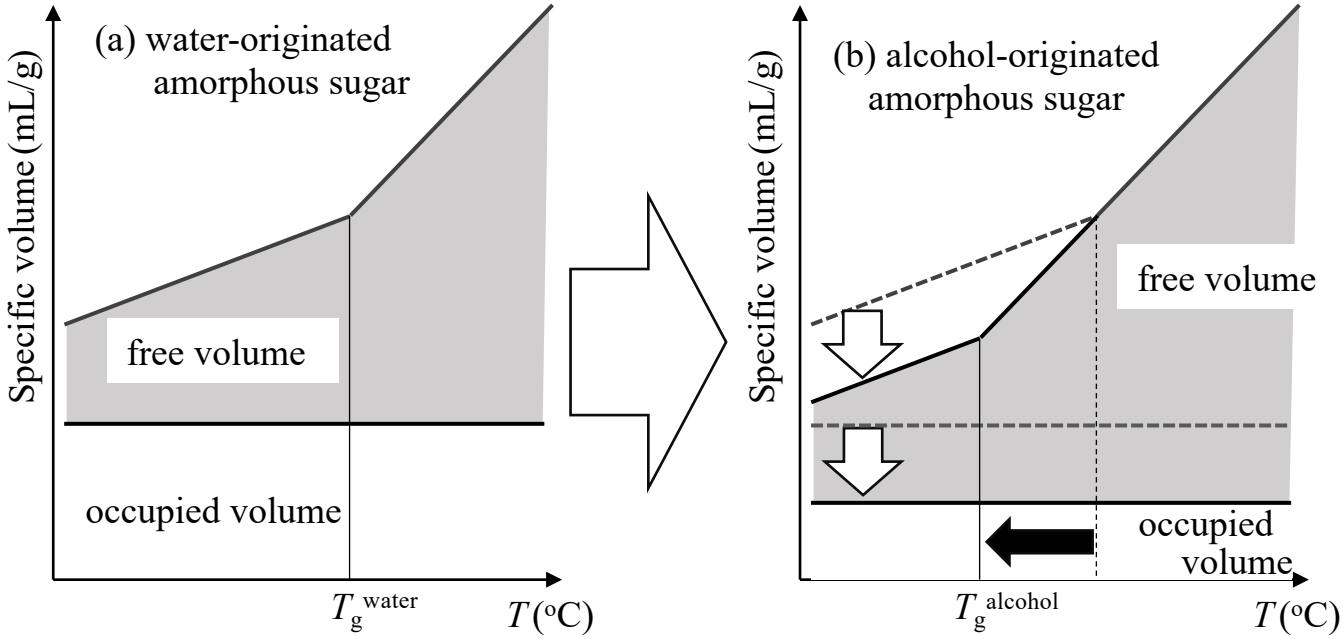
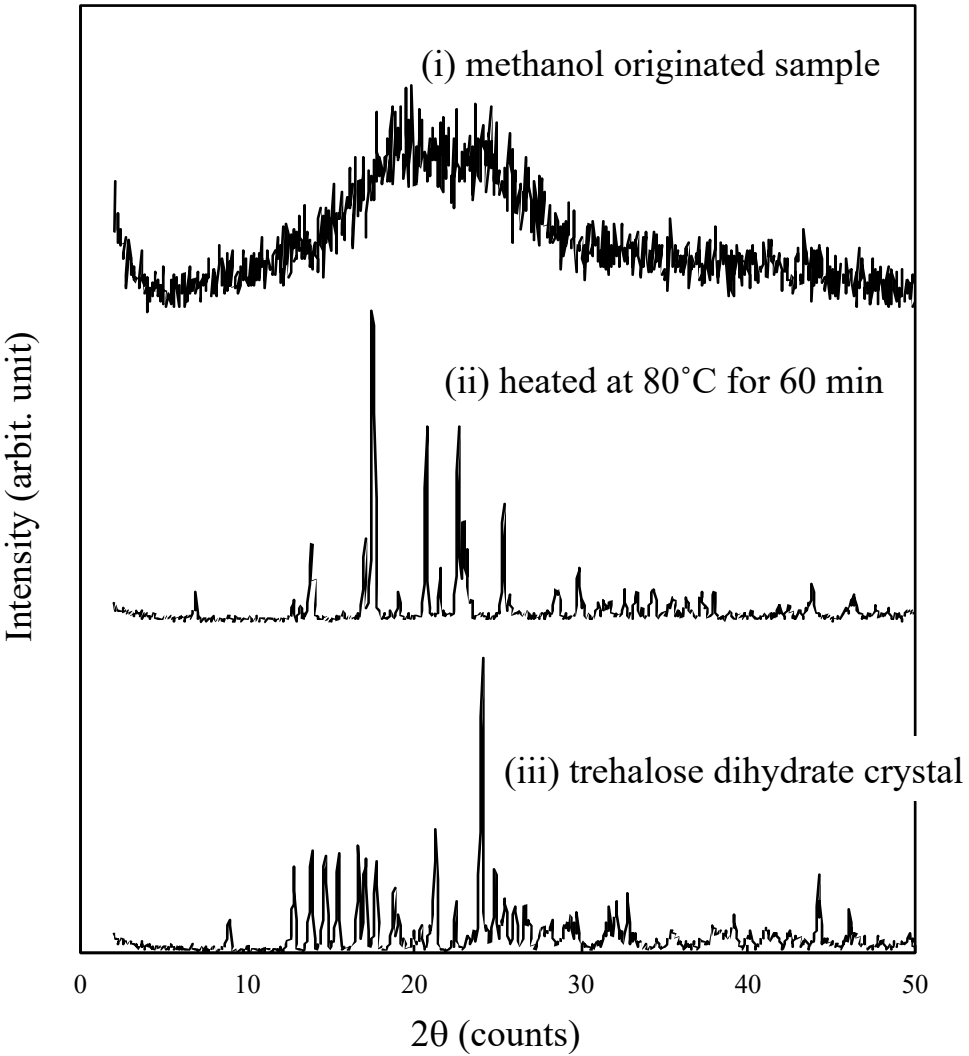


Fig. 3. Schematic relationship between temperature and the specific volume of an amorphous sugar matrix, dried from (a) water or (b) alcohol. According to free volume theory (Boyer & Spencer, 1944; Frenkel, 1955), the sugar specific volume is comprised of an occupied volume and a free volume. The free volume increases with increasing temperature, and the occupied volume for the alcohol-originated amorphous sugar appears to be markedly smaller than that for the water-originated one.



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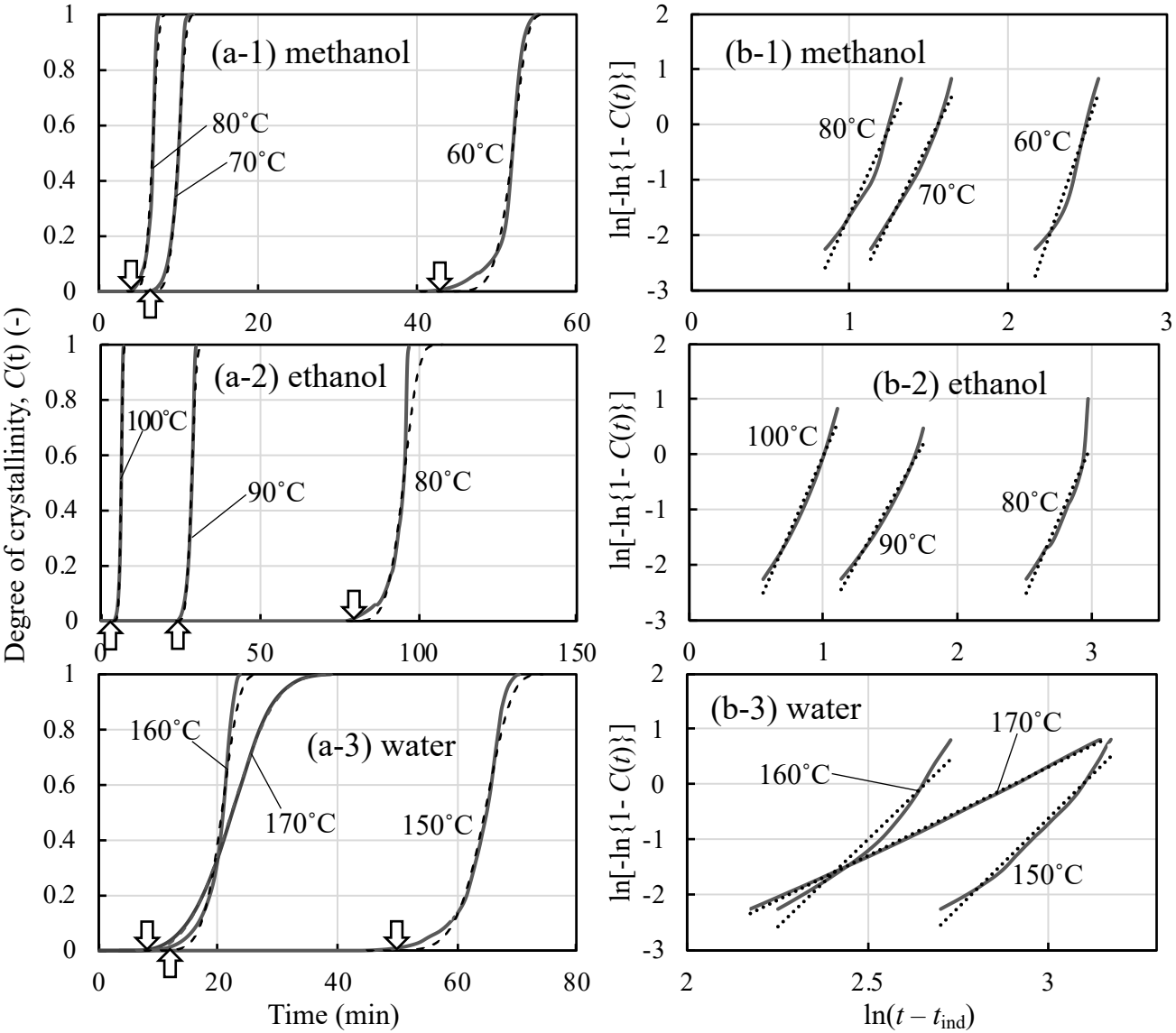

Fig. 5. Isothermal crystallization of amorphous trehalose, vacuum foam dried from (a-1) methanol and (b-1) ethanol, at different temperatures and their Avrami plots (b-1, 2). Results for the trehalose sample obtained by freeze-drying from water were also shown (a-3, b-3). Arrows in graphs (a-1~3) indicate the induction times, t_{ind}, and dotted lines in graphs (a-1~3) and (b-1~3) are the approximated ones.

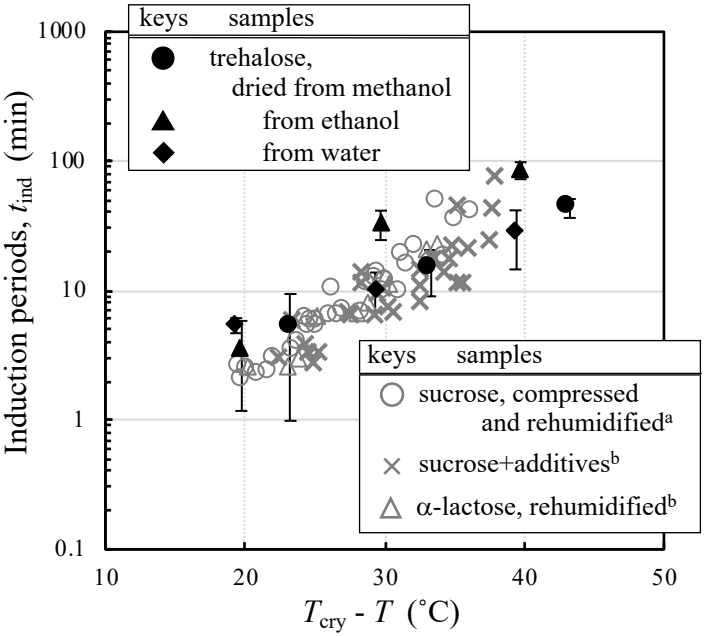

Fig. 6. Relationship between induction periods, t_{ind}, and crystallization temperature, T_{cry}. T refers to the temperature in the isothermal crystallization test. The values for the sucrose and α-lactose samples (gray keys) are cited from our previous reports ([a] Imamura, Kinugawa, Kagotani, Nomura, & Nakanishi, 2012; [b] Kinugawa et al., 2015).

Table 1

Temperatures and enthalpies for crystallization (T_{cry}, ΔH_{cry}) of amorphous trehalose and melting (T_m, ΔH_m) as well as the T_g values. The amorphous trehalose samples were prepared by being dried from different solvents (methanol, ethanol, and water).

Solvent	T_g (˚C)	T_{cry} (˚C)	ΔH_{cry} (J/g)	T_m (˚C)	ΔH_m (J/g)
Methanol	44±2	103±5	47±8	177±7	156±10
Ethanol	56±3	120±8	52±8	178±10	141±14
Water	107±6 105,[a] 115,[b,d] 100,[c] 117[e]	184±6 189,[e] 185,[f] 180,[g]	24±9 140[e]	208±2 215,[e,g] 213[f]	24±10 156[h,i]

[a] Imamura et al., 2008c; [b] Miller & de Pablo, 2000; [c] Roos, 1993;

[d] Saleki-Gerhardt & Zografi, 1994; [e] Surana, Pyne, & Suryanarayanan, 2004;

[f] Pyszczynski & Munson, 2013; [g] Sussich, Urbani, Princivalle, & Cesàro, 1998;

[h] Cai et al., 2018; [i] Jain & Roy, 2009

Table 2

Induction time (t_{ind}), apparent crystallization rate constants (k_{cry}), and Avrami constants (n), determined from isothermal crystallization processes of amorphous trehalose, dried from different solvents (methanol, ethanol, and water), at different temperatures

Solvent	Temp. (˚C)	t_{ind} (min)	k_{cry} (min^{-1})	n (-)
Methanol	60	44±7	0.104±0.070	5.9±2.3
	70	15±6	0.188±0.034	5.5±0.6
	80	5.2±4.2	0.395±0.297	5.8±0.4
Ethanol	80	85±13	0.064±0.018	4.3±1.4
	90	33±8	0.181±80.007	5.2±1.4
	100	3.5±2.3	0.436±0.097	4.8±1.1
Water	150	28±14	0.040±0.007	5.2±1.2
	160	10±3	0.055±0.018	5.2±1.1
	170	5.4±0.7	0.045±0.016	3.2±0.4

***Conflict if Interest form**

Conflict of interest: No conflicting relationship exists for any author.

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Figure legends

Fig. 1. DSC thermograms for amorphous trehalose matrices, vacuum-foam dried from (i) methanol and (ii) ethanol and (iii) for freeze-dried aqueous trehalose solutions. Arrows in the graph denote T_g, T_{cry}, and T_m.

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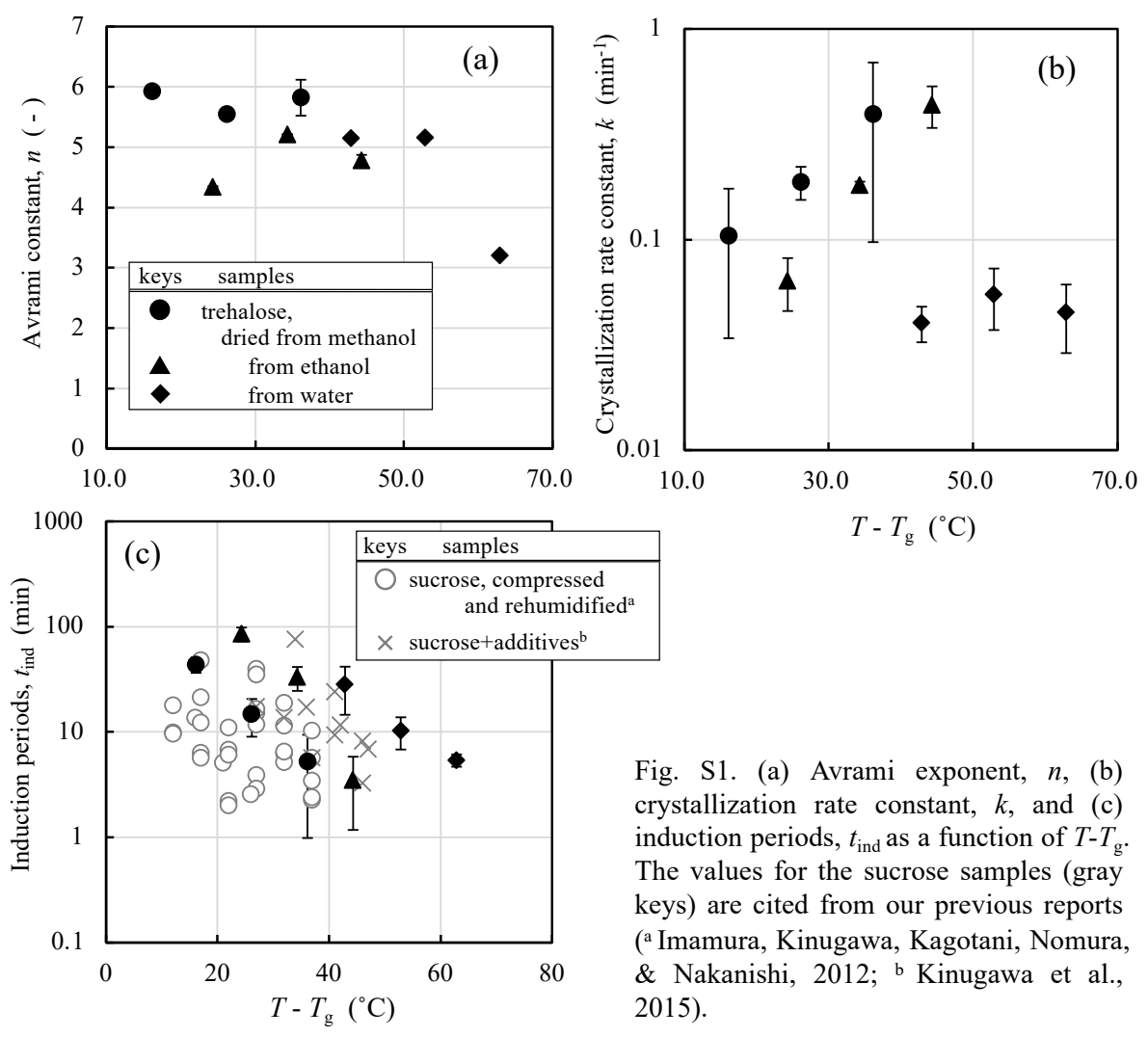

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***Credit Author Statement**

Takanari Sekitoh: Methodology, Validation, Investigation, Writing-Original Draft
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