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Trehalose amorphization and recrystallization

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ABSTRACT

The stability of the amorphous trehalose prepared by using several procedures is presented and discussed.

Amorphization is shown to occur by melting (T_m = 215 °C) or milling (room temperature) the crystalline anhydrous form TRE-β. Fast dehydration of the di-hydrate crystalline polymorph, TRE-h, also produces an amorphous phase. Other dehydration procedures of TRE-h, such as microwave treatment, supercritical extraction or gentle heating at low scan rates, give variable fractions of the polymorph TRE-α, that undergo amorphization upon melting (at lower temperature, T_m = 130 °C). Additional procedures for amorphization, such as freeze-drying, spray-drying or evaporation of trehalose solutions, are discussed. All these procedures are classified depending on the capability of the undercooled liquid phase to undergo cold crystallization upon heating the glassy state at temperatures above the glass transition temperature (T_g = 120 °C). The recrystallizable amorphous phase is invariably obtained by the melt of the polymorph TRE-α, while other procedures always give an amorphous phase that is unable to crystallize above T_g. The existence of two different categories is analyzed in terms of the transformation paths and the hypothesis that the systems may exhibit different molecular mobilities.

1. Introduction

An overall view of the literature results provides numerous hints toward a better understanding of amorphous/glassy states,[1,2] and of their role in many technologically relevant processes, for example, in pharmaceutics.[3] It is also worth mentioning that physical properties of amorphous carbohydrates (either in the anhydrous or in the low-moisture state) have been always of great interest. The abundant literature on trehalose has also concerned several aspects of molecular mobility of the trehalose–water systems[4–8] and of structural transitions between crystalline (hydrate and anhydrous) forms and amorphous form[9–11], particularly for its role in many phenomena related to biologically important events such as that of bioprotection.[12]

The relevance of trehalose mostly lies in its endogenous presence in organisms like bacteria, rotifers, tardigrades, nematodes, and even larger animals before entering in a dormant state and in the following 'resuscitation'. Despite the enormous amount of work and experiments carried out in the last three decades, the mechanism by which trehalose acts is still unsolved. There are, although, several hypothesis and the formation of glassy layers of sugar molecules seems to be the most widely credited from the physical point of view than the direct interaction of the sugar with protein and lipid surfaces. A number of results show a remarkable damping of protein dynamics when biomolecules are coated with trehalose anhydrous layers;[13–15] therefore, the high efficiency of trehalose has been ascribed to the high value of the glass transition temperature, an explanation that has been rejected however by other researchers in view of the minor effect of sugar oligomers or dextrans with higher T_g values. In two separate studies[16,17] the authors concluded, on the basis of results on fusion and aggregation of vesicles, that vitrification is not the only mechanism by which trehalose and other sugars impart their bioprotection, but that also direct interaction has to be invoked; indeed, it was suggested that three combined factors (T_m depression, direct interaction and glassy state formation) are involved in membrane stabilization. In another approach, according to Wolfe and Bryant[18] many of the differences among the effects of different solutes can be explained by the differences in the crystallization, vitrification, volumetric, partitioning, and permeability properties of the solutes. Koster et al.[19] showed that when sugars vitrified near or between fluid phase bilayers, the lipid transition occurred over a broader temperature range (ca. 20 °C) and with a lower transition enthalpy when compared to the gel-to-fluid transition measured in non-vitrified samples, making furthermore a distinction between low and high molecular weight glasses.

A different mechanism has been proposed by Sussich et al.,[12] in which one of the polymorphs of trehalose, the species called TRE-α, is involved. This proposal originates from a thoughtful study of the

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interconversion among the several phases summarized in a 'dynamic' diagram that included not only the 'static' temperature/transformation coexistence lines but also their dependence on the time required for the transformations and the rate of vapor flowing out of the cell.[20] The analysis has been made on the effect of scan rate on the process of water escaping during the first thermal step (vapor effusion) and on the way water residence time is tuned by heating scans (water plasticization time). It has been proposed that modulation of the two streaming variables (temperature scanning and water flowing) is the key action for the production of a given trehalose form. The authors concluded that both the polymorph TRE-α and the glassy state may co-exist in the protection stage. The last claim calls for a better understanding of the inter-relation between the crystalline polymorph TRE-α and the glassy or amorphous state of trehalose, as well as for a scrutiny of the several procedures known for the preparation of the glassy form. Some papers have indirectly dealt with this subject;[21] most of them dealt with the different pathways that produce the dehydration of dihydrate crystalline trehalose.[20–28] Only recently a closer look at the question of crystallization from the amorphous state of trehalose has been investigated.[29]

Therefore, in the following sections the different procedures for producing amorphous trehalose are analyzed, focusing in particular on the stability of the non-ordered (undercooled liquid) phase toward non-isothermal crystallization, as revealed by a common investigation method, such as DSC. Then, a rationalization of the phenomenological behavior of amorphous trehalose prepared by using different procedures is attempted. The recognition of the molecular signature of the several paths has been used as the key to understand that the mobility of the undercooled liquid apparently depends on the phase and temperature from which the liquid is obtained, leaving the further characterization of these molecular differences to forthcoming papers.

2. Experimental

2.1. Materials

Trehalose dihydrate (TRE-h) was purchased from Sigma Chemical Co. and was used without further purification. The anhydrous crystalline forms, TRE-α and TRE-β, were prepared[22,24] by keeping the dihydrate samples under vacuum at 85 °C for 4 h and at 130 °C for 4 h, respectively.

Preparation of undercooled liquid from the melt of TRE-β anhydrous form (procedure 1) was carried out in situ directly in the calorimeter, by heating the crystalline sample above the melting point (ca. 220 °C). The sample was held isothermally for 1 min at this temperature to ensure complete melting of all potential nuclei, before fast cooling at room temperature ($dT/dt > 50$ °C/min).

Preparation of undercooled liquid from the melt of TRE-α anhydrous form (procedure 2) was carried out in situ directly in the calorimeter, following the procedure for the preparation of TRE-α and continuing the heating scan above its melting point (up to ca. 135 °C). The sample was held isothermally for 1 min at this temperature.

The procedure of amorphization from the di-hydrate TRE-h (procedure 3) has been taken from the previous study[20] elucidating the interplay of scanning rate and of water effusion time on the transformation fate of TRE-h in the several anhydrous forms, either crystalline or not.

For procedure 5 (microwave treatment) a small amount (20–50 mg) of powdered TRE-h was spread on the glass sample container and treated for a given time (2–20 min) at a given power (150–850 W) in a commercial microwave oven (Whirlpool MD 101, Sweden). After the treatment in the microwave oven, the samples were directly analyzed by DSC at 20 K min^{-1}.

The procedure of freeze-dry (procedure 6) followed the common laboratory practice. Freeze-dried samples were prepared by quenching small aliquots of trehalose solutions (about 2 mL of 10% trehalose in water) at low temperature (−70 °C) and drying in a freeze-drier (Freeze Dryer System, Edwards High Vacuum International, USA) for 24 h. The samples were analyzed by DSC without further thermal treatment.

The procedure 7 consisted in the evaporation of water from a drop of solution (ca. 10–15 mg) carried out isothermally at 35 °C in the calorimeter. Trehalose solutions at concentration from 4% to 16% of trehalose were prepared in NaCl 0.1 M and in pure water. Although different for the conditions used, this procedure will be discussed together with the results of dehydration by air-spraying taken from the literature.[30]

Preparation of undercooled liquid by milling (procedure 4), and by supercritical extraction (procedure 8) have not been carried out in our laboratory and the results of literature are reported for comparison (see Refs. 31 and 32, respectively).

2.2. Calorimetric measurements

Calorimetric measurements were carried out either with Perkin Elmer Pyris 1 or DSC6. The thermal unit of Pyris 1 was thermostatted with an external thermocryostat in which the coolant was kept at −30 °C; a nitrogen flux (20 mL/min) was used as a purge gas for the furnace. The thermal unit of Perkin–Elmer DSC 6 was thermostatted with an external thermocryostat in which the coolant was kept at 0 °C; a nitrogen flux (20 mL/min) was used as a purge gas for the furnace. Temperature scans were run on samples weighing between 5 and 15 mg, and sealed in aluminum Perkin–Elmer DSC pans, pierced according to Ref. 20; an empty aluminum pan was used as a reference. The instruments were calibrated using standard procedures. The PYRIS software version 3.81 from Perkin–Elmer was used with the WINDOWS NT 3.5.

3. Results

3.1. Layout of trehalose physical transitions

Although this work deals with the glassy/amorphous state and the two crystalline forms of trehalose that have already been reported in literature, it is of paramount importance that all transitions from one form to another are well characterized. The transition behaviors are repeatedly reproduced in our laboratory since the different polymorphs were prepared and their transformations studied in detail.[22] Figure 1 reports the thermal behavior of the several polymorphs of trehalose at a scan rate of 20 K min^{-1}. In addition to the dehydration thermogram of TRE-h (100–110 °C) and melting of TRE-β (215 °C), the melting of two forms, TRE-α (126 °C) and TRE-γ (118–122 °C), is reported. The latter form transforms upon melting into TRE-β; it was thereafter characterized as a mixture of two crystalline forms and probably made up of TRE-h crystals encapsulated in layers of TRE-β.[24,29] Dehydration studies have been extensively pursued in our laboratory and subsequent results on transformation paths and phase properties have been discussed in detail elsewhere.[20,33] In particular, let us recall that TRE-h thermal behavior and the product thereof strongly depend on the heating rate and also on the time when water remains in contact with trehalose crystals.[20] The residence time is modulated, among other parameters, by the hypothetical size of the cell pores. By means of calorimetric investigations, the easiness of water molecules to leave the crystal environment has been correlated to the diameter of the hole on the calorimetric cell caps. Scan rate

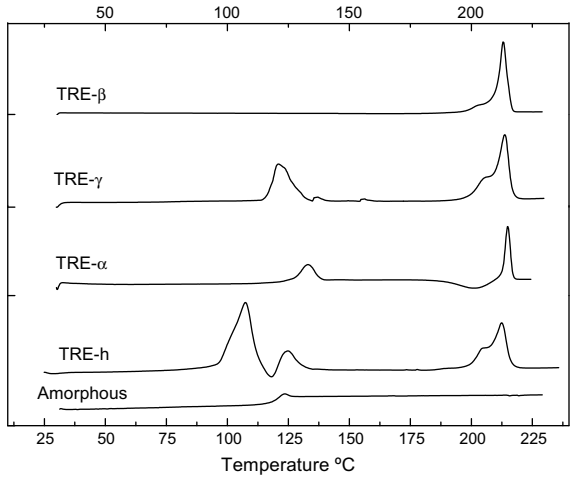

Figure 1. Thermal behavior of trehalose polymorphs at scan rate 20 K min^{-1}. The thermograms show from bottom to top: (1) the glass transition, (2) the dehydration process of TRE-h under regime of water effusion in a pin-hole cell, (3) the melting of TRE-α, followed by cold crystallization to TRE-β and its melting, (4) the dehydration of TRE-γ with solid-transition to TRE-β and its melting, (5) the melting of pure TRE-β.

Table 1
Summary of transition temperatures and thermodynamic data for trehalose polymorphs[a]

Transition	T_{tr}/K (°C)[b]	ΔH/kJ mol^{-1}	ΔS/ J K^{-1} mol^{-1}
Glass transition: $T_g \rightarrow T_{am}$	398 (125)	(0.48 J K^{-1} g^{-1})[d]	
Dehydration of T_h: $T_h \rightarrow$anhydrous	373 (100)	113.5 (full)[c]	304[c]
Dehydration of T_h: $T_h \rightarrow T_\gamma$	373 (100)	52[c]	139[c]
Dehydration of T_h: $T_h \rightarrow T_\alpha$	<373 (<100)	113[c]	300[c]
Melting of T_α: $T_\alpha \rightarrow T_{am}$	399 (126)	ca. 10[a,d]	14
Transition of T_γ: $T_\gamma \rightarrow T_\beta$	393 (120)	51.3	130
Melting of T_β: $T_\beta \rightarrow T_L$	478 (205)	50	107

[a] Taken from Sussich et al.[20,25]
[b] Onset temperature.
[c] The value includes the two water molecules undergoing vaporization: TRE-h (crys)→TRE-*i* (solid) + 2H_2O (g).
[d] Taken from Willart et al.[27]

determines the vapor effusion and indirectly, water residence time upon heating scans (water plasticization). Modulation of these two streaming variables (temperature scanning and water flowing) is therefore the key action for the production of a given trehalose form, which can be TRE-α, TRE-γ, TRE-β, or the amorphous state. For these reasons, in our opinion, previous studies in the literature were often in conflict not only with temperature values of transitions but also with the proper definition of transition and species formed (see Ref. 20).

In conclusion, once operational parameters are fixed, transition temperatures, transition enthalpies and non-equilibrium cold crystallization phenomena have well-defined values and contribute to the fingerprinting of trehalose polymorphs (Table 1).

3.2. Trehalose amorphization

Several different starting physical forms of trehalose and different operational protocols of temperature (scanning or isotherm) and energy type have been exploited. Although not necessarily exhaustive, the list (see Table 1, first and second column) shows quite a large variety of procedures that involve different paths to produce amorphization of trehalose. For sake of possible completeness, these results are here presented and discussed, including those previously published in our or others' laboratories (namely milling, spray-drying, and supercritical fluid extraction) (Table 2).

3.2.1. Undercooled liquid after melting TRE-β

Glassy/amorphous phases are very commonly obtained by cooling the liquid more or less abruptly from melting temperatures. Trehalose samples prepared by abruptly cooling the melt to low temperature (room temperature) always give a DSC thermogram typical of a glassy state and show the heat capacity step at the glass transition temperature, $T_{g\,onset}$ = 120 °C[22] and further heating above T_g show no other phase transitions such as cold crystallization from the undercooled liquid or melting of the crystalline TRE-β (Fig. 2). Experiments carried out under several time-scales have always shown[20] that amorphous trehalose from the melt of TRE-β is unable to undergo crystallization in the temperature range between T_g and T_m.

Confidence on the correct procedure for trehalose amorphization is given by reproducibility and reversibility of the glass transition, by absence of irreversible (e.g. caramelization) or relaxation processes in the temperature modulated scan and by visual observation of samples. The value of T_g of 120 °C (onset) is claimed as that of pure glassy trehalose, when using reasonably controlled experimental conditions (i.e., scan rate of 1 K min^{-1}).

3.2.2. Amorphization from the melt of TRE-α

According to polymorphism rules, TRE-α is a monotropic form of TRE-β, inasmuch TRE-β is the most stable form at all temperatures, while TRE-α is stable only at low temperatures and transforms into a melt at the transition temperature (Tmid-point = 126.1 at scan rate of 1 K min^{-1}). However, upon melting of TRE-α the liquid phase is effectively an undercooled liquid metastable with respect to TRE-β. Under these conditions the undercooled liquid melting from TRE-α can crystallize into TRE-β in the suitable temperature range (i.e., between T_g and T_m), irrespectively of whether the liquid is first cooled at lower temperature (ambient) or it is heated continuing the scan. The behavior of the first type is shown in Figure 2, where the heating scan of the glass formed

Table 2
Principal routes producing amorphous trehalose

Starting phase morphology	Procedure	Temperature (scan or isotherm)	Process critical parameters and problems	Amorphous cold-cryst.	Refs.[a]
(1) TRE-β	Cooling from melt	Scan above T_m (T_m = 215 °C)	High T; low exposure time	N	20, 22, 24
(2) TRE-α	Cooling from melt	Scan above T_m (T_m = 130 °C)	Troublesome prep. of TRE-α	Y	20, 22, 24
(3) TRE-h (2H_2O)	Dehydration with collapse	Scan above T_{tr} (T_{tr} = 100 °C)	Scan and water effusion rates	Y/N	20, 22, 23, 24, 27, 42, 45
(4) TRE-β	Milling	Isotherm[b] (mechanical energy)	Time, moisture	N	26, 31
(5) TRE-h (2H_2O)	Microwave oven	Isotherm[b] (EM absorption)	Sample geometry power diffusion	Y/N	This paper, 35
(6) TRE (aq soln)	Solution air-dry	Isotherm (T > 25 °C)	Time, moisture	N	This paper
(7) TRE (aq soln)	Solution freeze-dry	Isotherm (T < 0 °C)	Time, volumes	N	This paper, 36
(8) TRE-h (2H_2O)	Supercritical extraction	Isotherm SFE dehydration	Controlled cond.	Y	32

[a] References are mainly related to the production and to the stability or transformation of the amorphous form.
[b] Although not controlled, the temperature of the process is considered approximately constant.

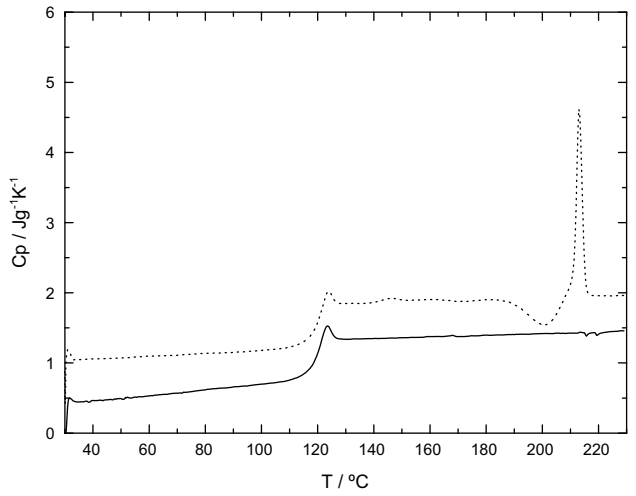

Figure 2. DSC heating thermograms of quenched melt (T_m = 215 °C) of TRE-β (full line) and of quenched melt (T_m = 130 °C) of TRE-α (dot line). Curves are arbitrarily shifted in the *y*-axis for sake of clarity. Scan rate 20 K min^{-1}.

from the melt of TRE-α crystalline phase (at $T \approx 135$ °C) clearly shows cold crystallization forming TRE-β. Analogously, a continuous scan, producing first TRE-α from TRE-h in situ, has always shown evidence of cold-crystallization phenomena.[20] Reproducibility of cold crystallization of the amorphous produced after melting of TRE-α is shown in Figure 3. Samples have been prepared by isothermal dehydration of TRE-h at three different temperatures and analyzed with a low scan rate (1 K min^{-1}) in order to enhance any possible difference in the crystallization kinetics.

Crystallization from the amorphous state obtained from the melt of TRE-α is therefore necessarily ascribed to some structural 'organization' of this amorphous state that is characterized by a higher mobility than the amorphous state obtained by cooling the melt from TRE-β.[27] The paths producing undercooled liquid from the melt of TRE-β and that produced from the melt of TRE-α apparently give different amorphous phases. Differences could be intuitively allocated in the radial distribution functions and in the orientation patterns of molecules in the two amorphous forms that are quenched from different melting temperatures. Indeed, polymorph TRE-α is believed to be a low-density rather weakly interacting molecular crystal, either because of the low melting point and melting energy or because of its involvement in the reversible dehydration-rehydration process.[22,24]

The hypothesis of some close structural correlation of the two crystalline forms of TRE-α and TRE-h has been advanced;[22,27] however, since the preparation of TRE-α is often accomplished with evidence of some amorphous (glassy) fraction, accurate X-ray structure determination has been elusive.

3.2.3. Heating TRE-h at several scan rates

As mentioned above, the dehydration process of TRE-h and the products thereafter formed depend on the interplay of water effusion rate and scan rate.[20] As far as the production of different polymorphs from TRE-h is concerned, only two limiting conditions are discussed here.

When open pans are used, non-isothermal dehydration produces TRE-α at low scan rates (sr < 5 K min^{-1}) and mainly an amorphous phase at high scan rates (sr > 10 K min^{-1}). Figure 4 shows the DSC profiles in two limiting conditions (sr 1 and 30 K min^{-1}). Apparently, the conditions used in the experiments reported in Figure 4 might jeopardize the rationale of this work. A slow scan rate the protocol seems to leave enough time (t > 60 min) to cross the $T_g - T_m$ window and let the amorphous (produced at about 130 °C from TRE-α) to undergo cold crystallization.

At high scan rates, the time to cross the same temperature window is of about 3 min. It could be argued that amorphous states obtained by dehydration at high scan rate could therefore hardly recrystallize (by cold crystallization) since the effective residence time in the region suitable for cold crystallization is too short to induce crystalline ordering. Independently of the scan rate, however, crystallization has never occurred from amorphous TRE prepared at high scan rates as above from TRE-h, nor from the melt of TRE-β. Furthermore, with the exception of quoted differences for TRE-h,[20] the thermal behavior of polymorphs TRE-α, TRE-β, TRE-γ, and amorphous TRE depends on the scan rate in the well-known and expected way, that is, a regular shift of the transition peaks to higher temperatures is observed with increasing scan rates.[25] By resuming the results of dehydration process in a large range of experimental conditions, the different trehalose forms produced imply the occurrence of different processes.

3.2.4. Milling

Mechanical milling is a known method applied to metallic systems for the production of composite metal powders with

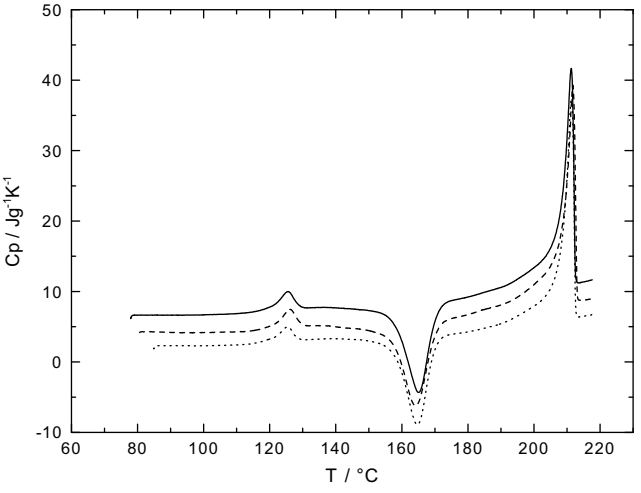

Figure 3. DSC heating thermograms of TRE-α samples prepared in situ by isothermal dehydration of TRE-h, at three different temperatures: 78 °C (full line), 80 °C (dash line), and 85 °C (dot line). Curves are arbitrarily shifted in the y-axis for sake of clarity. Scan rate 1 K min^{-1}.

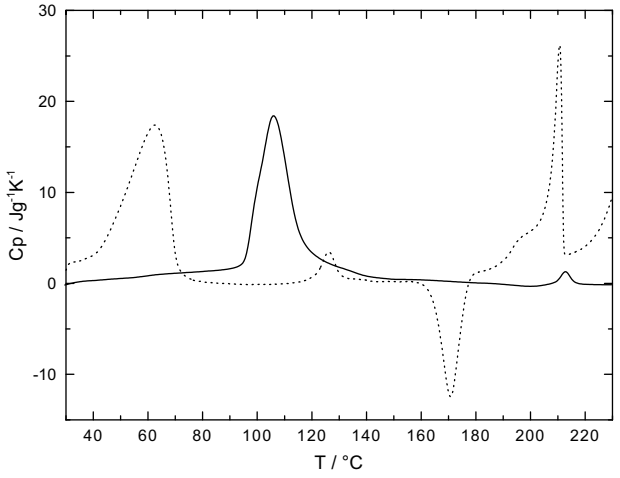

Figure 4. DSC heating thermograms of TRE-h in open pan at scan rate 30 K min^{-1} (full line) and 1 K min^{-1} (dot line).

controlled microstructure, especially in the process called mechanical alloying. The milling procedure (with cylindrical rods) has been recently used on trehalose (TRE-β) and has been claimed to be a convenient technique for the vitrification through a low-temperature route by using well-dried anhydrous crystalline form as a starting material.[26] In another work[31] a more conventional ball milling apparatus has been used. These authors compare amorphous products obtained by milling TRE-β with those obtained by two other different routes, that is, by rapid quench of its liquid phase and by drastic dehydration of TRE-h.

As an important outcome of these works, it has been confirmed that X-ray diffraction patterns of samples milled at different times are not sufficient to ensure the absence of crystalline phase and 'total' amorphization of the initial phase. Indeed, even if samples that have been milled for quite long times provide X-ray diffraction patterns that are indistinguishable from that obtained by quenching the melt, a substantial difference does exist in the DSC thermograms: quenched liquid does not show thermal effects above the glass transition, while samples milled at moderately long times ($t < 20$ h) present the characteristic cold crystallization followed by melting.

The discussion of these literature results implies a full understanding of the different procedures. In the milling procedure used by Nagahama and Suga,[26] the sample does not reach the complete amorphization and the enthalpy of crystallization, ΔH_c, is not more than the 80% of that measured by Villart et al.[31] Furthermore, a scaled comparison of the DSC plots of the two papers (Fig. 2 of Ref. 31 and Fig. 1b of Ref. 26, both at scan rate of 5 K min^{-1}) shows a significant shift toward lower temperatures of all thermal transitions (glass transition, cold crystallization, and melting). Particularly for cold crystallization, this effect can only be ascribed to the presence of residual nuclei in the amorphous matrix. More recently, analysis of milled samples by CP-MASS NMR reveals that residual crystalline fractions can be detectable even when the X-ray halo pattern characteristic of the amorphous phase is shown.[34]

The different behavior resulting from different milling times[31] is taken as an evidence for the existence of residual nuclei of the TRE-β, which survived to the milling for 20 h. Therefore, the stability of the amorphous phase TRE-am to recrystallization (cold crystallization) is interpreted in terms of a low nucleation rate of TRE-β in the undercooled liquid to produce nuclei of this phase in a wide temperature range (at scan rate of 5 K min^{-1}). If this conclusion is taken literally, apparently another evidence is given that the amorphous trehalose phase, here designated as TRE-am1, produced by methods 1 and 4 (Table 1) is structurally different from that produced by method 2 (TRE-am2).

3.2.5. Microwave treatment

A commercial pulsed microwave oven has been used to feed energy into small amounts (20–50 mg) of powdered TRE-h by exploring several powers (150, 350, 500, and 850 W) and exposure times (2–20 min). After treatment in the microwave oven, the samples were analyzed by DSC at 20 K min^{-1}.

Independently of power, the first effect of short exposure times to microwave is to slightly modify the usual splitting of dehydration peak into one sharp peak still at about 100 °C and another broader peak located at about 125 °C (full curve in Fig. 5). A similar shape, including the negative dip between the two peaks was previously observed under conditions (scan rate and pinhole) that produced the so-called γ-form and thereafter identified as a mixture of TRE-h and TRE-β. Only for samples heated at 350 W (for about 8 min or longer), at 500 W (above ca. 6 min) or at 850 W (about 4 min) the DSC features clearly indicate amorphization (see dot curve in Fig. 5). Samples treated with the above conditions do not present dehydration peaks (although small bumps can still be seen), but show, together with an 'imperfect' glass transition signature, a well-defined cold-crystallization phenomenon before the final melting. With the above values of power and time necessary for a complete dehydration (approximately 3–3.5 kJ), an apparent excess of energy seems necessary to extract the water molecules from TRE-h (however, part of this energy is spent in heating the sample).

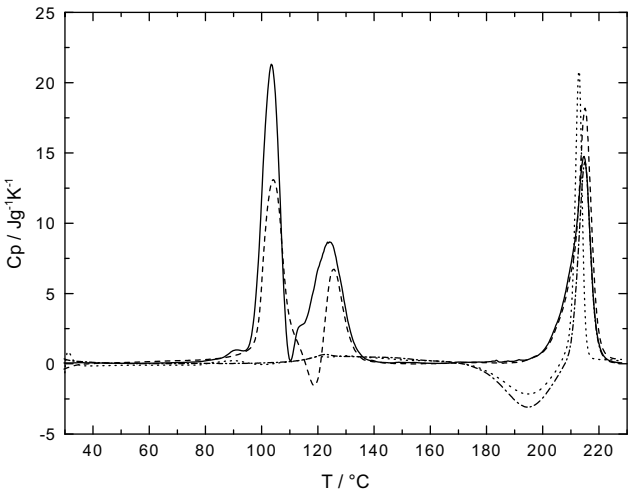

Figure 5. DSC heating thermograms of TRE-h conditioned in microwave oven at 350 W for 2 (full line), 8 (dash line), 14 (dash dot line) and 20 min (dot line). Scan rate 5 K min^{-1}.

Whether mild conditions (e.g., 20 min at 350 W) are able to remove completely the water molecules and induce the formation of some TRE-α is difficult to ascertain. The small peak at 125 °C cannot be unambiguously ascribed to melting of TRE-α since the larger jump of the glass transition is more evident. However, some anhydrous crystallites of TRE-α may have been produced under these conditions, and cold crystallization is induced after melting this crystalline form.

A comment is necessary on the heating processes of microwave treated samples in comparison with those heated in a conventional oven. The heat diffusion in a conventional oven provokes a negative temperature gradient from the surface to the core of crystal grains. Microwave power absorption heats up the interior of grains that are exposed to a lower external temperature; therefore, a positive temperature gradient is generated in the grains from the colder surface to the warmer core. Neatness and reproducibility of results obtained with microwave encourage an attempt of modeling the process in view of possible more general uses of this approach. Attention should also be given to different set-up, in particular to the set-up in which the power is continuously controlled without pulses, as in normal microwave ovens.

In a recent work by Seo et al.[35] sugar glasses were also prepared by microwave oven by using a quite different experimental formulation of the samples, as water was added to TRE-h. The authors found that the water loss in the sample after 150 s of heating was greater than the amount of added water, correctly attributing the difference in weight to a partial dehydration of crystalline water of TRE-h. The form TRE-γ, which was previously identified as a mixture of TRE-h and TRE-β,[22,24] has been also produced in this literature study.

3.2.6. Freeze-dry of TRE solution (sol–H_2O)

Freeze-dried samples are thought to preserve the native solution state of biological macromolecules since abrupt freezing of the solution can avoid conformational changes and biomolecule solute aggregation. However, while analyzing the properties of the freeze-dried product it has to be taken into account that residual

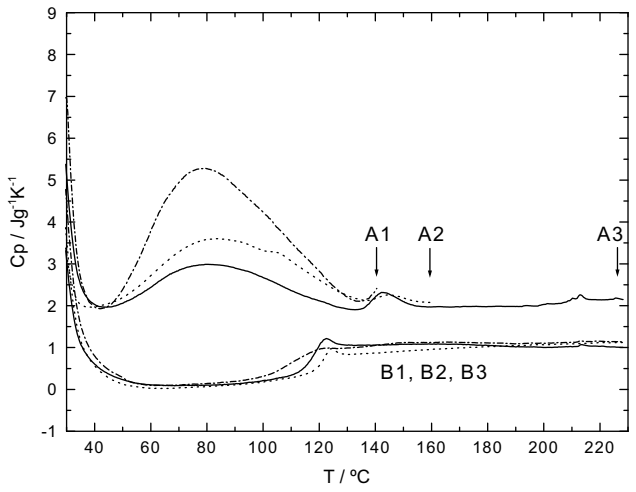

Figure 6. DSC heating thermograms of freeze-dried samples; scans were stopped at 140, 160, and 230 °C for samples A1, A2, and A3, respectively. Curves B1, B2, and B3 are recorded after cooling samples A1, A2 and A3, respectively.

hydration water molecules are retained also after freeze-drying and that diffusion dynamics of small molecular weight solutes cannot be completely quenched. Indeed, some routine protocols of freeze-drying include a thermal treatment to remove this residual water from the sample. The actual temperature and time of this additional treatment is very variable, for example, freeze-dry followed by treatments at 60 °C for 8 h is reported in Ref. 29 to produce samples with 1.4 ± 0.7%. Given the difference in the preparation procedure and the possibility that heat treatments induce structural modifications, the data of Ref. 29 are not included in Table 1, but will be discussed in relation to the general cold crystallization phenomenon.

Several samples have been prepared by freeze-drying trehalose solution following standard laboratory procedures (see experimental) without any further treatment at higher temperature. Then, an amount of residual water of about 2–5% has been found in the freeze-dried products associated to the carbohydrate powder. Indeed, all samples give DSC patterns with a broad evaporation band of the residual water in the range from 40 °C to 135 °C and a very small signal at 140 °C (Fig. 6). The freeze-dried samples, cooled after a heating up to 140, 160, and 230 °C, show a glass transition and no other phenomena of cold crystallization and melting (curves B1, B2, and B3 in Fig. 6).

3.2.7. Spray-drying or evaporation of a liquid solution

Spray-drying in the presence of sugars is used to protect proteins from conformational changes and chemical degradations arising from drying processes and storage conditions such as the humidity. A previous work on the use of trehalose in spray-dried formulations explicitly mentions the physical properties on the glassy state of trehalose in protein-free spray-dried systems.[30] The T_g of the preparation has been invariantly affected by the presence of residual moisture of 2.4% or more, giving a T_g of 84 °C or less, as evidently plasticized by water. No other assessments have been done about the nature of the powder that it is assumed to be amorphous (completely?) on the basis of wide-angle X-ray scattering diffractogram showing the characteristic amorphous halo. The properties of amorphous trehalose as a function of spray-drying concentration have been also investigated.[36] On the basis of these results, the statement has been made that trehalose can form different amorphous states, differing in the packing structure, as a function of the solution concentration being spray dried. Every batch of spray-dried samples retains a variable amount of water (ranging from 5.6 to 2.8) and once dried and then exposed to 75% RH, they invariably showed crystallization toward THE-h.

In our laboratory a few experiments have been carried out on trehalose solutions in order to investigate the effect of reduction of water activity by evaporation or by freezing. Upon drying (T = 35 °C), trehalose solutions have univocally generated a glassy state, that does not show cold crystallization. On the contrary, upon drying in the presence of salt (NaCl), the solutions give a slow but clear precipitation of TRE-h. These experiments may provide insight for the behavior of trehalose concentration in biological fluids, but do not add further information on the amorphous trehalose state.

3.2.8. Supercritical fluid extraction (SFE) of TRE-h

The supercritical fluid CO_2 extraction (SFE) process has been used by Akao et al.[32] to extract water from trehalose dihydrate TRE-h, under controlled extraction conditions of temperature, pressure, and entrainment. The SFE method under several conditions of pressure and temperature was shown to generate also variable amounts of the amorphous form together with anhydrous crystalline fractions of TRE-α, as revealed by FTIR spectroscopic analysis.

Unfortunately, no information has been given about the stability of the amorphous phase after heating the two different partially crystalline samples. The possibility of maintaining under control the thermodynamic parameters in the scaling-up the SFE process is undoubtedly appealing. Therefore, the method is an alternative to a simple heating of TRE-h under controlled scan rate and water effusion to produce reasonable quantities of the crystalline polymorph TRE-α.[20] Inasmuch the polymorph TRE-α is obtained, then the method should be classified as one able to give eventually a recrystallizable amorphous form.

4. Discussion

4.1. Rationalization of amorphous trehalose properties

The thesis underlying the analysis of the data on the stability of amorphous trehalose preparations is that recrystallization is a direct signature of molecular mobility, provided that no nuclei are left or formed after amorphization. The use of DSC as investigation method has been thought to be a simple and reproducible way for comparing trehalose polymorphs and transformations well characterized in previous work.

Summarizing the experimental results, two main groups can be envisaged according to the existence or not of a cold-crystallization phenomenon monitored when heating the amorphous above 130 °C, in relation to the method used for the preparation of amorphous TRE.

4.1.1. Group A, non-crystallizable amorphous phase (procedures 1, 3, 4, 6, 7)

Undercooled liquid obtained from melting of TRE-β and amorphous powders obtained from extensive milling, from freeze-dried or evaporated trehalose solutions appear unable to undergo cold-crystallization and are grouped here under the collective name TRE-am1. Under the same rationale, all the procedures identified to give either partially or totally TRE-β invariably fall in the scheme of producing TRE-am1. Dehydration of TRE-h carried out with rapid collapse also produces TRE-am1.

4.1.2. Group B: crystallizable amorphous phase (procedures 2, 3, 5, 8)

On the other side, melting of polymorph TRE-α produces an amorphous phase (hereafter named TRE-am2). Tre-am2 shows

the typical exothermic cold crystallization phenomenon either continuing in raising temperature after melting at ca 130 °C or after rapid cooling of the melt (but not if slowly cooled). A similar behavior (i.e., amorphous able to crystallize) is apparently offered by other experimental conditions that, however, under fine scrutiny reveal to produce a transient TRE-α form.

Among the routes of group B, let us specifically mention the procedure with the use of supercritical fluid CO_2 extraction (SFE) process to eliminate water from crystalline di-hydrate form TRE-h and the use of dosed energy by microwave. Furthermore, the procedure 3 (dehydration of TRE-h by heating scan) can be considered ambivalent. This procedure has been included in both groups, inasmuch it gives different products depending on the conditions of scan rate and cell environment; slow controlled dehydration always gives TRE-α, and therefore crystallizable amorphous form TRE-am2.

The following analysis can be made in order to rationalize the results here discussed. The evidence of recrystallization to form TRE-β in the temperature range between T_g and T_m (of that form) may have some very *naïve* interpretation: the amorphous preparations TRE-am2 may contain crystalline nuclei seeding the formation of TRE-β as the temperature increases above the T_g. Although this hypothesis cannot be completely excluded, it is not considered here in view of some additional evidence: (a) some relation exists between the crystallizable TRE-am2 form with the polymorph TRE-α; (b) time-resolved x-ray diffraction studies at synchrotron radiation of the thermal transition of polymorph TRE-α do not reveal the formation of TRE-β form; (c) crystallization occurs in TRE-am1 once it is not prepared to carefully exclude residual nuclei of TRE-β. A thoughtful analysis of these data necessary to validate the present hypothesis for the different behavior of TRE-am1 and TRE-am2 will be presented in a forthcoming paper. The hypothesis ascribes the cold-crystallization event to higher mobility of the amorphous preparation that derives from the polymorph TRE-α.

The apparent difference seems to originate from melting two different crystalline polymorphs. However, one would have expected that a real polymorphism be characterized by two different values of T_g, unless the unusual melting of TRE-α is so close to T_g that makes this event more like a solid-solid phase transition (as suggested by one of Reviewers) or that superheating occurs for the melting of TRE-α.[37] Indeed, a small but reproducible difference is revealed by careful inspection of the fictive temperatures of aged glasses formed by TRE-am1 and TRE-am2, respectively.[38] The above evidence for the existence of two amorphous trehalose forms can still be taken as indirect. On the other hand, methods to explore local distribution in the time and space domain have not been exploited for this problem. Glassy mobility and relaxation of the two amorphous forms, TRE-am1 and TRE-am2, need to be explored.

4.2. Trehalose polymorphism, mobility in the amorphous phase and effect of moisture

A detailed analysis of the transformation paths of the several trehalose forms has been carried out in the past years in many laboratories, including ours, and recently summarized in a review.[39] In the present work, after having scrutinized the several preparation methods and the occurrence of the phenomenology of cold crystallization for one particular form, it seems possible to get some further information about the recrystallizable amorphous form of trehalose and as a consequence to draw some conclusions on the differences between the two forms prepared. Although the appearance of a 'high' glass transition at about 70 °C has been quoted from previous studies,[40,41] this value has been shown to be incorrect by subsequent studies[22,42,43] and by all more recent

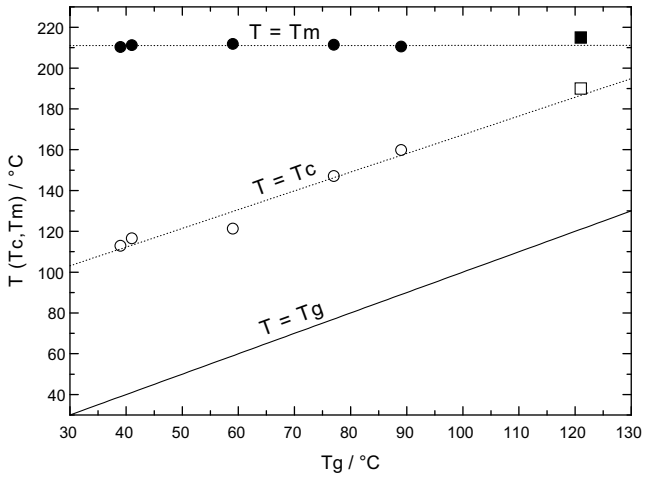

Figure 7. Dependence of the temperature of cold crystallization ($T = T_c$, open symbols) and of the temperature of melting ($T = T_m$, full symbols) on the temperature of glass transition (T_g) for amorphous trehalose plasticized with different amount of water (○,● data taken from Table 1, Ref. 29) and for pure amorphous crystallizable trehalose, TRE-am2. Scan rate 10 K min^{-1}.

reports. The possibility that the polymorph TRE-α is intrinsically characterized by low dimensionality that determine therefore a significant drop of melting enthalpy and temperature could be considered relevant, although the value of T_m does not depend on the degree of crystallinity of TRE-α preparations and may be consequence of the instability of the liquid below the T_g.

Given the relevance of the glass transition temperature in the delimitation of the lower temperature value for mobilizing amorphous structures, it is easy to speculate that recrystallization from the amorphous TRE to form TRE-β would only occur in the range of temperatures between T_g = 120 and T_m = 207. Within this scheme, it would be difficult to reconcile a melting point at 126 °C for TRE-α.

Indeed, taking the literature results on systematic determination of T_g, T_c, and T_m of moistened amorphous trehalose[29] it is possible to draw (Fig. 7) the dependence of the temperature T_c on the original value of T_g (while T_m is unchanged). In this plot the full line shows the lower limit for crystallization (i.e., $T = T_g$) and the dotted lines are the regression fit of T_c and T_m data. Two additional data points (square) taken from the thermal behavior of the amorphous trehalose TRE-am2 are added in the plot showing an excellent agreement with the other data. Therefore, a single mechanism for crystallization into TRE-β occurs either in the anhydrous or in the moistened TRE amorphous phase. As water molecules have the role of plasticizer and T_g depression is the evidence for increased mobility, amorphous recrystallizable trehalose (anhydrous) possesses a mobility that fit the behavior of plasticized phases. The fact that, contrary to polymer realm, the crystals of TRE-β obtained by cold crystallization at several temperatures always have constant melting temperatures indicates that the crystals dimension is always above the critical nano-size.[44]

4.3. Relevance to bioprotection

Even without claiming that an investigation on trehalose physical properties is 'per se' related to the issue of its bioprotection, it is clear that some results here presented need to be discussed within this matter. The very difference between the two categories apparently resides only in the ability of one form, called TRE-am2, to undergo cold crystallization while heating above the glass transition temperature. First of all, the appearance of two different

routes of producing amorphous trehalose undoubtedly asks for a fully assessment of whether trehalose exhibits polyamorphism or not. Some further evidence has been collected in favor of different mobilities of the two forms[38] and will be published in a subsequent paper. Still, one may wonder why these high temperature processes should be of interest for bioprotection. Indeed, the only reason lies on the fact that a non-crystalline trehalose phase is probably involved and that for non-crystalline states all transformations are subjected to the time-temperature-transition (TTT) principle. Roughly, this implies that transformation time scales measured at high temperature in a calorimeter have a counterpart in slower phenomena at nature temperatures. The need of slow transformations has already mentioned by many researchers and was specifically claimed by Sussich et al.[12] also for the reversible formation of TRE-α from the di-hydrate TRE-h and its rehydration back to TRE-h.[10,39,45]

Therefore, once the different mobility of the two forms, TRE-am1 and TRE-am2, is confirmed by other experimental results, the question returns about which of these forms (or both?) is the species involved in bioprotection of living organisms upon desiccation at desert temperature and low humidity.

5. Conclusions

By investigating all presently known procedures for amorphization, evidences have been presented showing a dual behavior of the undercooled liquid of trehalose, which depends on the way the amorphous/glassy state has been reached. This duality has been unequivocally ascribed to whether the glassy state is reached by undercooling the melt of the anhydrous crystalline form called TRE-α or by other routes that presumably would provide the form TRE-β. Although only indirectly, these findings seem to justify the claim for the existence of two different glassy phases of trehalose. The claim is being supported by other evidences currently collected in our laboratory on the physical aging of these glassy phases and on other structural features (paper to be submitted).

As a matter of fact, the scenario previously opened by identifying the several tracks for dehydrating trehalose TRE-h is now enriched by the possible existence of the two amorphous states. Therefore, not only the trehalose story appears more complex than originally thought, but also the existence of these polymorphs/polyamorphs which may interconvert to each other with a dynamic control seems to reinforce the interpretative key reported since our previous study.[22] Whether and how, among the various species reported here, the understanding of these transformations may have a role in the still unraveled mechanism of bioprotection is surely matter of further studies.

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