LOW RESOLUTION ^{1}H-PULSED NMR FOR SUGAR CRYSTALLIZATION STUDIES

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Abstract- **The stability of food and other labile biological systems containing sugars has been related to the amorphous characteristics of the matrices which they formed, being inversely related to the degree of crystallization. It is thus necessary to explore the applicability of simple, non-destructive and reliable methods to analyze sugar crystallization. Solid fat content is a well-stablished AOCS method to study solid content in lipid systems. However, there are no literature reports on the use of this method to analyze sugar crystallization. Crystallization kinetics of concentrated trehalose and trehalose/salts solutions was followed by proton pulse nuclear magnetic resonance (^{1}Hp -NMR). Three different concen-trations (X_T= 0.60, 0.63 and 0.66) of trehalose solutions were crystallized to 25, 20, 15, 10 and 5°C and the degree of crystallization was investigated by following the index of solids with time. Crystallization rate was determined by a combined effect of supercooling and molecular diffusion. By analyzing trehalose systems by ^{1}H p-NMR, it was possible to confirm the effect of divalent cations on retarding sugar crystallization.**

Keywords- **NMR, Crystallization, Trehalose Solutions, Divalent cations**

I. INTRODUCTION

Sugars have frequently been used in food and pharmaceutical fields for protecting both proteins and cells during freezing and drying due to their ability to form glasses, to mimic the hydrogen bonding character of water, to increase the surface tension of the bulk solvent, to prevent thermotropic phase separations in lipid bilayers, and to prevent the fusion of membranes (Conrad and de Pablo, 1999). Trehalose is a nonreducing disaccharide of glucose. It occurs naturally in many organisms which suffer dehydration stresses. Trehalose´s success as a cryoprotectant has been attributed to its strong interaction with water and lipid membranes, its unique chemical stability, and its glass-forming behavior when amorphous (Crowe *et al*., 1996). The stabilizing effect of sugars has been inversely related to the degree of crystallization (Suzuki *et al*., 1997; Cardona *et al*., 1997). Divalent cations were reported to delay crystallization kinetics of sugars (Mazzobre and Buera, 1999).

The crystallization of sugars is also important in the food industry as evidenced by the many processes where the degree of crystallinity of sugars is critical to acceptance of the final product, i.e., storage stability and quality of milk powders are significantly affected by the physical state of lactose, one of the main components in regular milk powders (Jouppila and Roos, 1994). Crystallization of lactose in ice-cream, condensed milk and milk powders is considered undesirable, while in products such as milk chocolate lactose crystallization is desirable. Likewise sucrose crystallization evidenced by graining in boiled sweets is considered to be a defect whereas the fine crystals present in fondant icing are desirable because they help enable the icing to retain its shape in confectionery products (Hartel and Shastry, 1991; Hartel, 1992).

The control of lactose and sucrose crystallization during processing is important for a wide variety of products and there is a need for a suitable method to determine the degree of crystallization which would occur under specific processing conditions (Kedward *et al*., 1998). Nuclear Magnetic Resonance (NMR) is a noninvasive technique often used for the examination of biological systems. This technique allows the determination of relaxation effects of protons containing in different states. Low resolution ^{1}H- pNMR is widely employed to determine the solid/liquid ratio in fats (AOCS official method). However, no previous references have been found for the application of this technique to determine the degree of crystallization in sugar solutions. The most common techniques in sugar studies are the evaluation of spin-spin and spin-lattice relaxation times. The experiments involve the study of a one phase systems and are usually performed in dehydrated systems (Cornillon, 2000) or vitreous systems (Rubin *et al*., 1990). Diffusion of sugars in aqueous solutions was also investigated performing spin echo and spin-spin relaxation experiments (Martin *et al*., 1999) and the determination of soluble solids content in fruits (Cho *et al*., 1993; Keener *et al*., 1997) by NMR was also reported.

The aim of the present work was to analyze the applicability of low resolution proton pulse nuclear magnetic resonance (^{1}H p-NMR) to describe crystallization behavior of sugars. Isothermal

crystallization behaviour of trehalose solutions with time was analyzed and the effect of salts of divalent cations such as $ZnCl_2.4H_2O$, $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ on crystallization kinetics was also investigated.

II. METHODS

A. Sample preparation

α,α-Trehalose dihydrate, (α-D-Glucopyranosyl-1-1-α-D-glucopyranoside) from *Saccharomyces cerevisiae*, 99% was used without any further purification. HPLC water was used for all experimental work. $CaCl_2.2H_2O$, $ZnCl_2.4H_2O$ and $MgCl_2.6H_2O$ were analytical grade. Concentrated trehalose solutions (mass fraction of sugar (X_T) of 0.60, 0.63 and 0.66) were prepared by weighting carefully cold water and trehalose. Salts were added to the X_T= 0.60 solution in a molar ratio trehalose/salt 5:1. The solutions were heated at 70°C with stirring for 1 h, then the NMR tubes were filled with the samples and placed into a bath at crystallization temperature. Selected crystallization temperatures were 5, 10, 15, 20 and 25°C.

B. Measurement of solid content

Solids contents of the samples were measured by ^1H pulsed nuclear magnetic resonance (^1H-pNMR) in a Minispec PC/120 series NMR analyzer (Bruker, Karlsruhe, Germany). A 20 MHz pulse of radio frequency radiation, lasting only 2 microseconds at a 90° between incident pulse and magnetic field vector (AOCS official method), was used. This pulse excited the hydrogen nuclei in all phases, and when the pulse was switched off, the nuclei returned to their original state, emitting an NMR signal as they did so. The initial amplitude of the signal is proportional to the total number of hydrogen nuclei in the sample. The signals due to nuclei in different physical phases decay at different rates. The number of hydrogen nuclei in the liquid phase and the total number of hydrogen nuclei in liquid and solid were measured. The solids fraction is given by the following equation:

$$X_S = \frac{(SA_1 - SA_2)\,F}{(SA_1 - SA_2)\,F + SA_2} \qquad (1)$$

where SA_1 is the signal amplitude proportional to the total number of hydrogen nuclei, SA_2 is the signal amplitude proportional to the hydrogen nuclei in the liquid phase, and F is a factor to correct the dead-time of the receiver since it is not possible to measure the samples at time zero. F was previously determined by using certified standards of plastic in oil where the actual solid content is known exactly. The relaxation properties of standards and any other systems such as fats, polymers and food emulsions follow proportionality. F' factor of proportionality can be determined by measuring the solid fraction by the indirect method Cd 16-81.

$$X_S = \frac{\text{signal amplitude at 70°C (70μs)} - \text{signal amplitude at } T_C \text{ (70μs)}}{\text{signal amplitude at 70°C (70μs)}} \qquad (2)$$

The ratio between this value and the one obtained by the direct method Cd 16b-93 is the F' factor. Different F' factors can be obtained for different fats, different polymorphic forms, emulsions and polymers. In the polymer industry, the solid ratio experiments becomes very important since it is possible to measure the degree of polymerization in actual chemical processes by measuring the increasing amount of solid in the reaction as it proceeds. With the same methodology, F' factors can be determined for sugars. The F' factor can also be determined by comparing the NMR direct method solid content value to the solid content value obtained by other method such as DSC. Trehalose is a particularly simple case because it has just one crystalline form when it crystallizes from water at the conditions selected for this study. The F' factor for trehalose was 1.05. It is valid to assume that the hydrogen nuclei contents and relaxation properties of the trehalose and the standards are similar. Samples were run in quadruplicate and the values were averaged. Solids fraction was measured as a function of time.

C. Differential Scanning Calorimetry (DSC).

The thermal transitions of the trehalose systems with and without $MgCl_2$ were measured by differential scanning calorimetry (DSC) (Mettler DSC30, TA4000, graphware TA72). Hermetic aluminum pans were employed, an empty aluminum pan was used as reference, and the samples were scanned at a heating rate of 10°C/min. The instrument was calibrated with indium and T_g was calculated as the onset temperature of the transition.

D. Light polarized microscopy

To check the behavior found by NMR, crystallization was observed by polarized light microscopy. A Leitz microscope model Ortholux II (Leitz Co., Wetzlar, Germany) with a controlled temperature platform was used to follow crystallization. In the crystallization tests, for microscopic observation the samples were melted with stirring at 70°C and held at this temperature for 1 h. Samples were then placed on a concave slide at the selected crystallization temperature and covered with a cover slip. The platform temperature was controlled by a Lauda TUK cryostat (Werklauda, Königshofen, Germany), which was filled with a mixture of ethylene glycol/water 3:1 (v/v). Photographs of the crystals were taken with a Leitz-Vario-Orthomat camera under polarized light during crystallization with time. Magnification was 25X. Scale for all photographs: 1cm=137.5 μm. The selected example to show the

morphology was the trehalose solution of X_T= 0.60 and this solution with the addition of salts crystallized on a microscope slide at 20°C. It is very difficult to quantify crystallization kinetics with microscopy at the sugar concentrations used in this study because many fields are empty, very different crystal sizes are present at the same time and they are sometimes aggregated. In addition, to obtain results with statistical significance, at least 200 crystals must be analyzed for their area. For these samples, more than 20 repetitions must be done to reach that crystal number. However, qualitative results can be obtained. Crystallization studies were also performed at 10 and 15°C.

III. RESULTS AND DISCUSSION

A. Crystallization of Trehalose Solutions

Table 1 shows the supersaturation (β) and the difference between crystallization and glass transition temperatures ($T-T_g$) for the three trehalose solutions (X_T= 0.60, 0.63, 0.66) at different temperatures. Supersaturation is defined as $\beta=X_T-X_{SL}$, where X_{SL} is the amount of trehalose soluble at saturation. Solubility at different temperatures were reported by Miller *et al*., (1997). As evident from Table 1, supersaturation decreases when temperature increases. In addition, ($T-T_g$) values are an indication of the degree of plasticization of the sugar molecule by water. At low ($T-T_g$) values, although supersaturation is high, sugar molecule have low molecular mobility since water plasticization is poor.

Table 1: Characteristics of the systems at the conditions selected for the crystallization studies.

T, °C	X_T	β	($T-T_g$), °C
5	0.60	0.260	85
	0.63	0.290	80
	0.66	0.320	76
10	0.60	0.242	90
	0.63	0.272	85
	0.66	0.302	81
15	0.60	0.230	95
	0.63	0.260	90
	0.66	0.290	86
20	0.60	0.194	100
	0.63	0.224	95
	0.66	0.254	91
25	0.60	0.170	105
	0.63	0.200	100
	0.66	0.230	96

Figure 1 shows the solid or crystalline fraction (X_s) as a function of time for the trehalose solutions incubated at 25°C. The curves had sigmoidal shape, there was an induction time for sugar crystallization, followed by a period of increasing crystallization rate and a last period of decreasing crystallization rate. At 25°C trehalose crystallization was faster in concentrated solutions (X_T= 0.63 and 0.66). Probably at this temperature no molecular diffusion limitations take place, being sugar crystallyzation mainly governed by supersaturation.

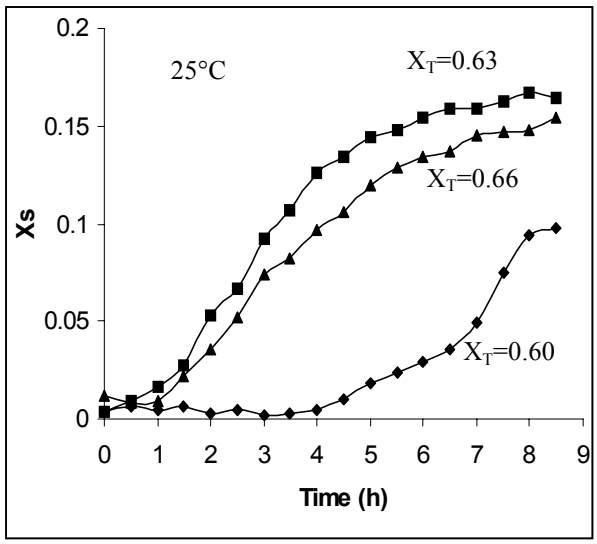


Figure 1. Change in the solid fraction (Xs) for solutions of initial mass fraction of trehalose (X_T) 0. 60, 0.63 and 0.66 during storage at 25°C.

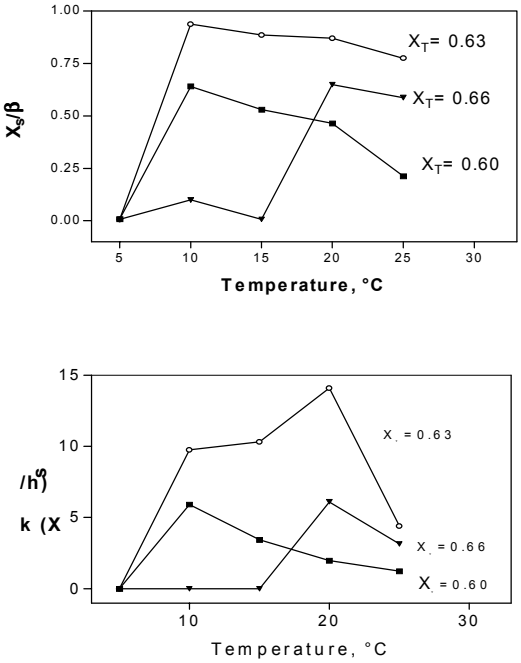


Figure 2. Effect of supersaturation and temperature on the maximum crystalline fraction (related to the super-saturation, β) (a) and to the crystallization rate (b) of trehalose solutions at several temperatures.

Figure 2a, shows the maximum level of crystallized sugar (related to the supersaturation value) after 9 h of storage at the selected temperatures. The slope of the linear portion of the curves X_s vs. time (k) (as indicated in Figure 1 for 25°C) was calculated as an indication of

the crystallization rate and plotted as a function of temperature (Figure 2b). As shown in Figure 2 (a,b), crystallization at 5°C did not occur after 9 h of storage, although β was high. Crystal growth drop off at high supersaturations or supercoolings due to the increased influence of mass transfer inhibition (Hartel 1992). As the driving force (β) increases, the crystal growth rate is expected to increase. However, at extremely high driving forces (very low temperatures or very high concentration), crystal growth goes to zero due to inhibition of molecular mobility. Systems that do not undergo glass transition such as edible fats and oils show higher crystallization rates for higher termodynamic driving force, that is higher supercoolings. In the case of sugar solutions, although high supercooling values thermodynamically make the crystallization process favorable, they are kinetically inhibited due to the low molecular mobility in the solution phase. It was reported that sugar solutions showed this behavior (Hartel 1992) but it was not quantified previously by NMR. Both, the maximum level of crystallized sugar and the rate of crystallization (Figure 2 a,b) were higher for the intermediate concentration solution (X_T= 0.63) and at temperatures between 10 and 20°C, diminishing with further increasing temperature. Solid fraction in the solution X_T= 0.63 reached almost the maximum solid fraction expected (related to the supersaturation value) at 10°C (Figure 2a). However, for solutions X_T= 0.60 and 0.66, the maximum solid fraction attain was lower than the expected on the basis of β. Although supersaturation is higher for the solution X_T=0.66, it became very viscous and therefore no crystallization occurred at temperatures lower than 20°C due to molecular diffusion limitations.

B. Effect of salts on crystallization kinetics

The modification of crystallization kinetics because of the addition of salts to the X_T= 0.60 trehalose solution is shown in Figure 3. As in all the systems the anion (Cl^-) was kept constant, the differential behaviour observed for the salts could be attributed to the cations. Among the selected chlorides, $CaCl_2$ was very efficient in delaying crystallization. After 6.5h solid fraction still remained lower than 0.01. $MgCl_2$ efficiency was intermediate between $CaCl_2$ and $ZnCl_2$. The three salts diminished crystallization rate and the maximum solid fraction (Table 2). However, the curves showed a similar shape than the one exhibited by the trehalose solution alone. Divalent cations were reported to have a greater effect than monovalent cations on stabilizing proteins (Carpenter *et al*., 1987). It was expected that the effect of divalent cations on crystallization rate followed the order $Mg^{2+} > Zn^{2+} > Ca^{2+}$ in agreement with their polarizing character. That is, the ratio charge/radius. $CaCl_2$ showed an unexpected behavior that may be due to solvation of Ca^{2+} ions with trehalose molecules instead of water.

Low resolution ^{1}H-NMR was presented as a suitable method to follow sugar crystallization degree along time, by determining the solid fraction. Parallel qualitatively experiments by two others methods, differential scanning calorimetry (DSC) and light polarized microscopy (LPM), confirmed the results obtained by ^{1}H-p-NMR.

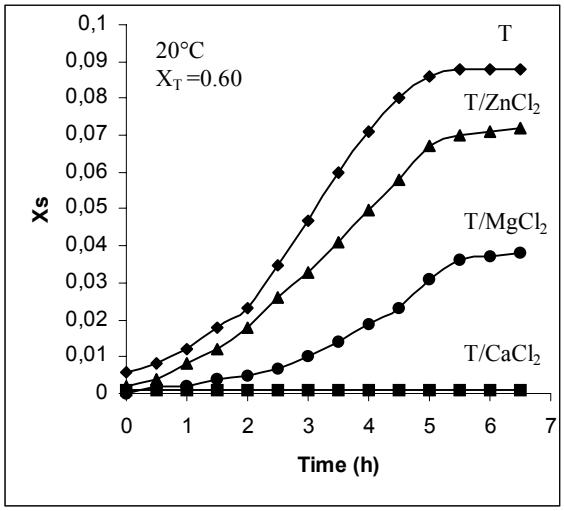


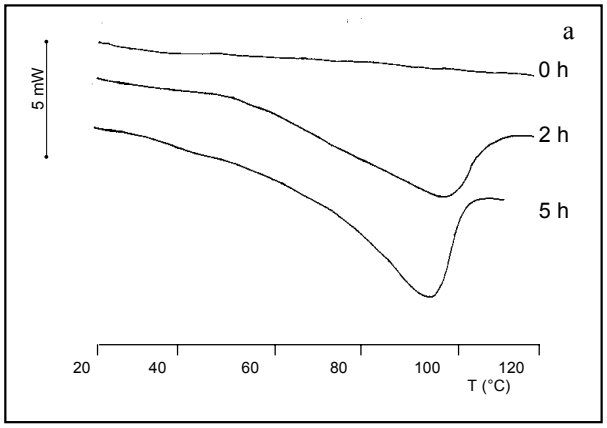
Figure 3 Change in solid fraction with time at different temperatures for the trehalose solution and with the addition of $ZnCl_2$, $MgCl_2$ and $CaCl_2$ in molar ratios trehalose/salt 5:1.

Table 2. Maximum solid fraction (X_s) and rate of crystallization (k) for trehalose and trehalose-salt systems of total mass fraction of solids X_T=0.60

System	Temperature (°C)	X_s	k (X_s/h)
Trehalose	10	0.16	7.7
	15	0.11	4.18
	20	0.08	2.1
Trehalose/$ZnCl_2$	10	0.12	3.9
	15	0.08	2.7
	20	0.07	1.6
Trehalose/$MgCl_2$	10	0.06	2.2
	15	0.04	1.2
	20	0.03	1.4
Trehalose/$CaCl_2$	10	<0.002	ND
	15	<0.001	ND
	20	<0.001	ND

Figure 4 shows the DSC thermograms obtained for trehalose and trehalose/$MgCl_2$ solutions after storage at 25°C. The endotherms indicate the solubilization of sugar which crystallized during the previous storage. The area of the endothermal peaks are thus related to the amount of crystalline sugar formed. In solutions containing only trehalose, the sugar crystallized after 2h at 25°C (Figure 4a). However, the absence of the endothermal peak in the trehalose/$MgCl_2$ thermogram

solution up to 23hs of storage indicate that trehalose crystallization was delayed in presence of $MgCl_2$.



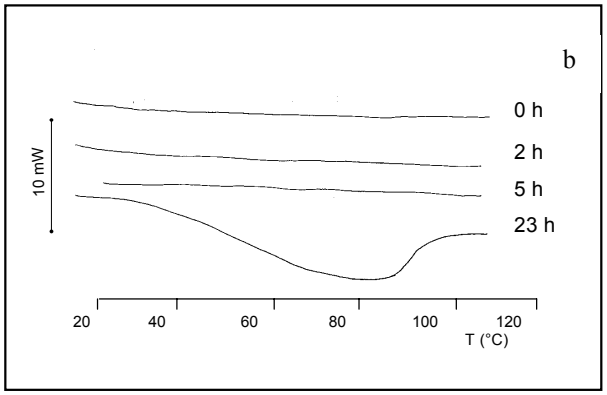
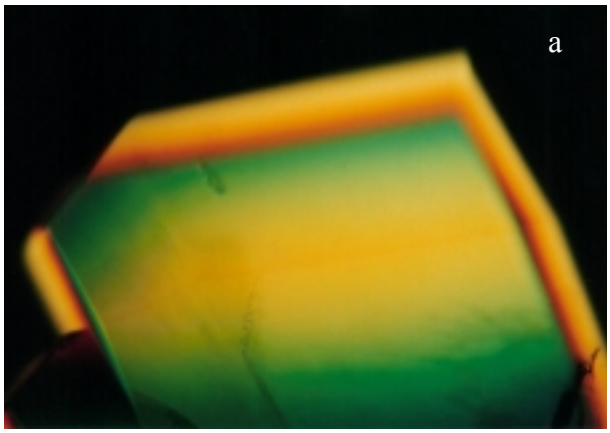


Figure 4. DSC thermograms for supersaturated solutions of trehalose (a) or trehalose-$MgCl_2$ (b) of mass fraction of solids (x_T) 0.60 after 0, 2, 5 and 23 h at 10°C.

C. Confirmation of salts effect

Spot observations of trehalose and trehalose/salt solutions were done by LPM. Trehalose solutions of X_T= 0.60 with and without salts were crystallized on a microscope slide at 10, 15 and 20°C. For the trehalose solution crystallized at 20°C, small crystals were observed after 1 h. Figure 5a shows the morphology of trehalose crystals after 2 h 30 min. Trehalose solution X_T= 0.60 in presence of $CaCl_2$ did not show crystals during the first 48 h.. In the presence of $ZnCl_2$ and $MgCl_2$ crystallization was not observed during the first 2 h. The crystals were smaller than those of trehalose alone and showed a different morphology as shown for trehalose/$MgCl_2$ in Figure 5b. As observed in solid systems (Longinotti *et al*., 2001), salts constrain the dimensionality of sugar crystal growth. The obtained qualitative results were in agreement with the order of crystallization delay observed by NMR for the three crystallization temperatures.



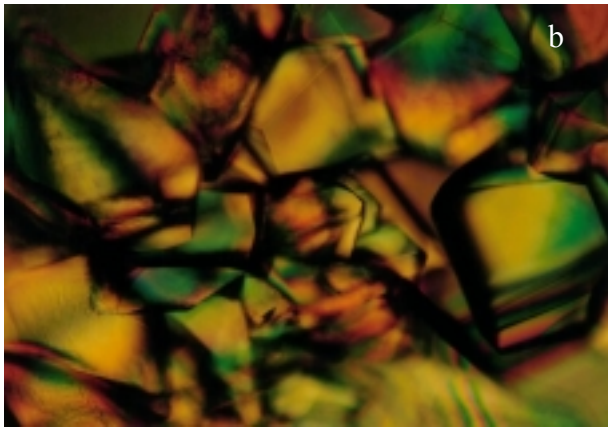


Figure 5. Images of crystals obtained by light polarized microscopy after 2h 30 min at 20°C. a) trehalose solution (X_T = 0.60), b) trehalose/$MgCl_2.6H_2O$ solution (X_T=0.60).

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