

Effect of Cryoprotectants on the Eutectic Crystallization of NaCl in Frozen Solutions Studied by Differential Scanning Calorimetry (DSC) and Broad-Line Pulsed NMR

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The effects of cryoprotectants (sugars and polymers) on the eutectic crystallization of NaCl/water systems were studied by differential scanning calorimetry (DSC) and broad-line pulsed NMR. Thermal analysis of frozen NaCl solutions showed a melting peak of eutectic NaCl/water systems at around -21°C . The addition of various cryoprotectants inhibited the eutectic crystallization of the NaCl solution. The concentrations of polymers (dextran, polyvinylpyrrolidone and Ficoll) required to inhibit eutectic crystallization were slightly higher than those of sugars (glucose, sucrose, trehalose and lactose). The proportions of liquid-phase protons present in frozen H_2O and D_2O solutions, determined by broad-line pulsed NMR, showed that the addition of sugar inhibits eutectic crystallization of NaCl/water systems through the formation of an amorphous mixture containing NaCl, sugar and water between the ice crystals.

Key words cryoprotectant; eutectic crystallization; thermal analysis; broad-line pulsed NMR

The precipitation of a supercooled solute/water mixture in frozen aqueous solutions is known as eutectic crystallization. Sodium chloride hydrate ($\text{NaCl}\cdot 2\text{H}_2\text{O}$) is a typical eutectic crystal formed in frozen solutions.¹⁻³ The eutectic crystallization of solutes in frozen solutions is an important factor in formulation processes. It alters various physical properties of frozen solutions, and results in crystallized freeze-dried cakes.^{4,5}

Eutectic crystallization also affects the stability of biologicals in frozen solutions and during the freeze-drying process. The alteration of various physical properties causes freeze-thaw damage to cells and proteins. An osmolality change caused by the eutectic crystallization of sodium chloride (NaCl)/water systems decreases the viability of microorganisms by degrading their membranes.^{6,7} Changes in pH due to the eutectic crystallization of disodium hydrogenphosphate (Na_2HPO_4)/water systems can destabilize proteins.^{8,9}

Various polyols and amino acids can protect microorganisms and proteins during freezing and are called "cryoprotectants." The addition of cryoprotective compounds inhibits the eutectic crystallization of other solutes.^{3,6,7} For example, the addition of trehalose or sucrose prevents the eutectic crystallization of NaCl/water systems.^{7,10} This ability to prevent the eutectic crystallization of other solutes is thought to be one of the mechanisms underlying cryoprotection. The increasing use of biologicals for pharmaceutical purposes¹¹ indicates a need to elucidate the effects of various polyols on eutectic crystallization.

Thermal analysis and measurement of the electric resistance of frozen solutions has suggested that a mixture of NaCl, sugar and water forms a supercooled solution among ice crystals.^{1,12-14} Sucrose is thought to take the place of water as the solvent in the ternary NaCl/sucrose/water mixture.^{1,14} Since thermal analysis and electric resistance provide only limited information on the physical properties of frozen solutions, data obtained by other methods may help to evaluate this hypothesis.

In this study, the effects of sugars and polymers on the eutectic crystallization of NaCl/water systems was studied by differential scanning calorimetry (DSC) and broad-line pulsed NMR spectrometry. The number of liquid-phase protons present in the samples as determined by NMR provides information about the molecular motion of the components.¹⁵⁻¹⁷ The mechanism of inhibition of eutectic crystallization is discussed.

Materials and Methods

Materials Ficoll (approx. MW: 400000, type 400), polyvinylpyrrolidone (PVP, average MW: 40000) and dextran (from *Leuconostoc mesenteroides*, average MW: 11000) were purchased from Sigma Chemical Co. (St. Louis, MO). Deuterium oxide (100.00 atom % D) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Polyethylene glycols (PEGs) and other chemicals were of reagent quality and were purchased from Wako Pure Chemical Ind., Ltd. (Osaka). All chemicals were used without further purification.

Thermal Analysis of Frozen Solutions The effects of various cryoprotectants on the eutectic crystallization of a NaCl/water system were studied using a Shimadzu DSC-41M (Kyoto). Aliquots (25 μl) of solution in aluminum cells were cooled to -80°C , then scanned during rewarming at $2^{\circ}\text{C}/\text{min}$. Heat absorption by the melting of a eutectic NaCl/water system was obtained by the DSC study. Crystallinity of the eutectic system was described as the ratio (%) of the heat absorption relative to that of 100 mM NaCl solution. Glass transition temperature measurements for the frozen solutions were carried out in a TA Instruments DSC 2920. Samples (10 μl) in hermetic aluminum cells were cooled to -80°C , then scanned during rewarming at $2^{\circ}\text{C}/\text{min}$.

Measurement of Liquid-Phase Protons The number of liquid-phase protons present in each sample was measured using a broad-line pulsed NMR spectrometer with a Larmor frequency of 25 MHz (JNM-MU25, JEOL, Tokyo) with appropriate software. The sample temperature was controlled by a variable temperature unit (DVT-2, JEOL). An aliquot (1.0 ml) of sample solution in a glass tube (10 mm in diameter) was frozen by immersion in liquid nitrogen, and transferred to the NMR spectrometer. In experiments using deuterium oxide (D_2O) as the solvent, samples were analyzed after overnight storage at room temperature. The NMR signal was measured at every 2°C during rewarming of the samples from -60 to 20°C at $0.5^{\circ}\text{C}/\text{min}$. Signals indicating free induction decay (FID) were sampled every $0.2 \mu\text{s}$ after 90° pulses and used to construct an FID curve. This was resolved into two components indicating long and short relaxation times, which were considered to represent liquid and solid-state protons.^{15,16} The proportions of liquid-state protons in the samples were determined by extrapolating the portions of the FID

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curve between 40 and 120 μ s back to zero time. After these values had been corrected according to Curie's law, the number of liquid-phase protons was determined using a calibration curve obtained from measurements of the proton ratios in D₂O/H₂O solutions of varying concentrations at 25°C. The number of liquid-phase protons was expressed as the proportion (%) of the amount in H₂O at 25°C.

Results and Discussion

Thermal Analysis of Frozen Solutions Figure 1 shows the effect of sucrose on the rewarming DSC scans of frozen 100 mM NaCl solutions. An endothermic peak due to melting of the eutectic NaCl/water system^{1,10} was observed at around -21°C. This was reduced with each increase in sucrose concentration. The peak disappeared on the addition of 15 to 50 mg/ml sucrose, indicating the inhibition of eutectic crystallization.¹⁰

Table 1 shows the effects of various cryoprotectants on eutectic crystallization. The addition of sugars (glucose, sucrose, trehalose and lactose) and polymers (PEG 1000, dextran, Ficoll and PVP) decreased the area under the endothermic peak. The concentrations of polymers required for complete inhibition of the eutectic crystallization of NaCl/water systems were slightly higher than those of the sugars studied. PEG 3000 and PEG 20000 also

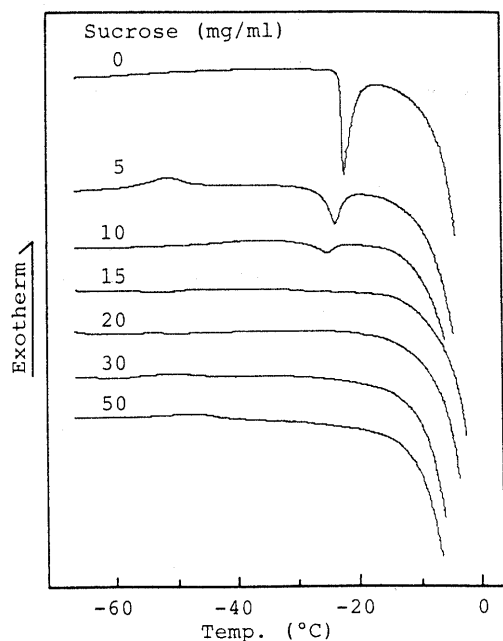


Fig. 1. Effect of Sucrose on the Thermal Behavior of Frozen 100 mM NaCl Solutions

Aliquots (25 μ l) of solutions containing 100 mM NaCl and 0 to 50 mg/ml sucrose in aluminum cells were heated from -60°C at 2°C/min.

inhibited the eutectic crystallization of NaCl/water systems at 20 mg/ml (data not shown). However, endothermic peaks due to the eutectic melting of PEG/water¹⁸ appeared in concentrated (30 or 50 mg/ml) solutions.

Measurement of Liquid-Phase Protons The molecular mobility of the components of the frozen solutions was also studied to elucidate the mechanism underlying the inhibition of eutectic crystallization. The FID curve obtained by broad-line pulsed NMR provides information on the number of liquid- and solid-state protons present.^{15,16} Figures 2A and 2B show the temperature dependence of the numbers of liquid-phase protons in frozen H₂O and D₂O solutions, respectively. The numbers of liquid-phase protons shown in Fig. 2A represent the sums of those in the mobile solute (sucrose) and in unfrozen water molecules. The numbers of liquid-phase protons observed in D₂O solutions (Fig. 2B) mainly reflect the protons of mobile sucrose molecules, with some water molecules produced by proton exchange between sucrose and D₂O. Most of the signals obtained from frozen D₂O solutions were due to sucrose molecules, since the majority of water molecules lose their mobility after crystallization.

Though the proportions of liquid-phase protons in H₂O solutions of sucrose and/or NaCl were similar at temperatures above 0°C, they differed significantly at temperatures below 0°C (Fig. 2A). Frozen pure H₂O showed very little molecular mobility. The proportion of liquid-phase protons at temperatures below -4°C was less than 0.1% of that at 25°C. By contrast, the proportions of liquid-phase protons in solutions containing sucrose and/or NaCl were much higher due to unfrozen water and mobile solute molecules.

The proportion of liquid-phase protons in a frozen 100 mM NaCl solution between -60 to -24°C was below 0.1% (Fig. 2A). It increased markedly at around -22°C, then increased gradually before the ice melted at around 0°C. A significant increase was observed at the eutectic temperature indicated by DSC (Fig. 1). This rapid increase in the proportion of liquid-phase protons may be ascribed to the melting of the eutectic NaCl/water system (NaCl(H₂O)₂). The water molecules in eutectic systems are assumed to become mobile "unfrozen water" at that temperature.

Figure 2A shows that the mobility of the molecules in the frozen sucrose solution also increased during the rewarming process. Since frozen sucrose solution forms an amorphous sucrose/water phase between ice crystals,¹² this indicates changes in the physical state of the amor-

Table 1. Effects of Cryoprotectants on Eutectic Crystallization in 100 mM NaCl Solutions

Cryoprotectant (mg/ml)	Crystallinity of eutectic NaCl/water systems (%)							
	Glucose	Sucrose	Trehalose	Lactose	PEG 1000	Dextran	PVP	Ficoll
5	43.9	53.6	54.1	56.5	57.3	67.6	66.0	68.3
10	1.4	13.9	16.3	24.2	31.0	47.2	48.9	51.0
15	0	0	0	0	0	3.8	11.6	21.8
20	0	0	0	0	0	0	0	0.7
30	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0

Crystallinity of a NaCl/water system is described as the ratio (%) relative to that of 100 mM NaCl solution. All values are expressed as the means of two experiments.

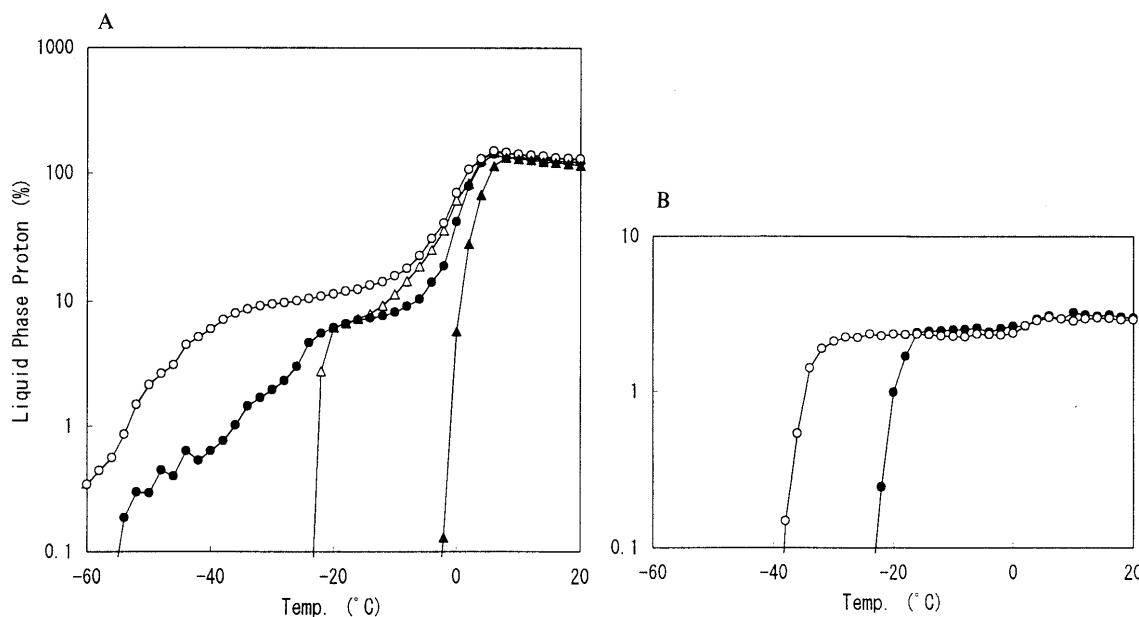


Fig. 2. Temperature Dependence of the Number of Liquid-Phase Protons in the Solutions

The number of liquid-phase protons in solutions in H₂O (A) or in D₂O (B) (▲, without solutes; △, 100 mM NaCl; ●, 20 mg/ml sucrose; ○, 100 mM NaCl and 20 mg/ml sucrose) was determined as described in the method, and expressed as the ratio (%) of that in H₂O at 25 °C.

phous phase. The glass transition temperature (T_g) of the frozen 20 mg/ml sucrose obtained by thermal analysis was around -36 °C (data not shown), which was consistent with that found in other studies.¹⁹⁾ Glass transition of the amorphous phase may be one of the causes underlying the increase in the proportion of liquid-phase protons.²⁰⁾

A frozen solution containing both NaCl and sucrose retained a greater number of liquid-phase protons than solutions containing each solute separately (Fig. 2A). The proportion increased during the rewarming process, but was smaller between -30 and -15 °C. The lack of any apparent change in the number of liquid-phase protons at around -21 °C indicates that no eutectic NaCl/water system was formed in the sample. This suggests the formation of an amorphous mixture containing NaCl, sucrose and water between ice crystals. The glass transition temperature of the frozen solution was around -54 °C. The decrease in T_g caused by adding inorganic salts has previously been reported.¹⁴⁾ The physical properties of the amorphous phase appeared to be somehow altered by sodium chloride.

The proportion of liquid-phase protons in a D₂O solution of sucrose increased markedly at around -22 °C (Fig. 2B), indicating an increase in the mobility of the sucrose molecules. A similar increase was observed at -36 °C in a D₂O solution containing both NaCl and sucrose. Melting of the frozen solution at around 0 °C had only a small effect on the number of liquid-phase protons. The T_g (-32 and -50 °C) of these solutions was a little higher than in H₂O solutions. The mobility of sucrose molecules appeared to increase at temperatures 10 to 15 °C higher than the glass transition temperature of the solutions. The proportions of liquid-phase protons in Figs. 2A and 2B indicate that the mobility of water and sugar molecules in the amorphous phase increases at different temperatures around the glass transition tem-

perature. It is suggested that water molecules gradually become mobile around the glass transition temperature, whereas mobilization of the sucrose molecule occurs above the glass transition temperature since it requires sufficient mobilization of surrounding water molecules.

The results of the NMR study support the observations obtained by thermal analysis and studies of the electric resistance of frozen solutions.^{1,12-14)} The present study suggests that microscopic amorphous phases containing NaCl, sucrose and unfrozen water are formed between ice crystals, and that these prevent the eutectic crystallization of NaCl/water systems.

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