Antifreeze Activities of Various Food Components

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The antifreeze activities of various food components were measured by differential scanning calorimetry (DSC), and their immobilizing activities for water molecules were measured by nuclear magnetic resonance (NMR). The antifreeze activities of saccharides that consisted of glucose were higher than those of the others. In salts, those which possessed high ionic charge had high antifreeze activities. The DSC results were consistent with the NMR observations, that is, food components which had high antifreeze activity also had high immobilizing activity for water molecules. In water-soluble amino acids, a few amino acids which formed no eutectic mixture above −20 °C had especially high antifreeze activities.

Keywords: *Antifreeze activity; saccharide; salt; amino acid*

INTRODUCTION

Additives, usually sugars or polyols, are widely used to prevent deterioration of frozen foods or to stabilize cryo-preserved biomaterials. The cryoprotective effect of these additives on a food system has been shown to be related to their freezing point depression properties (Kato, 1985). Recently, many studies have focused on antifreeze polypeptides (AFP) or glycoprotein (AFGP) which have high antifreezing activity (Feeney and Yeh, 1993; Hew et al., 1988). The freezing point depression produced by these materials is several hundred-fold greater than expected from colligative effects (Berman et al., 1980). However, these materials are not yet used as food additives mainly because of their high costs.

Concerning antifreezing of foods, the most common approach in previous studies was quantifying the amount of unfreezable water in heterogeneous food samples by various methods, e.g., differential scanning calorimeter (DSC) (Parducci, 1972; Wakamatsu et al., 1979), nuclear magnetic resonance (NMR) (Toledo et al., 1968; Suzuki et al., 1982), water sorption data (Sato et al., 1993), and ultrasonic interferometer (Shiio, 1957). However, from these data, we can evaluate only the amounts of unfreezable water in foods and cannot evaluate antifreeze activities of each component in the foods. In the development of frozen foods, it is very useful to characterize antifreeze activities of each food component. However, there are few studies of characterization of antifreeze activities of various food components.

Generally, unfreezable water molecules in aqueous solution are immobilized translationally or rotationally by solutes. However, there have been few studies concerning how unfreezable water molecules are immobilized or how many water molecules are immobilized. To prevent freezing effectively, such kinds of information are also important including antifreeze activities of food components.

Therefore, the objective of this study was to characterize antifreeze activities of various food components measured by DSC. In addition, we compared the antifreeze activities with dynamic data by NMR to obtain information about the immobilizing mechanism of water molecules in the unfreezable state.

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MATERIALS AND METHODS

Materials. Materials that were used for measuring antifreeze activities were saccharides, salts, and amino acids. Ten monosaccharides, fourteen disaccharides, five trisaccharides, one tetrasaccharide, and eight sugar alcohols were used as saccharide samples. Seven chlorides, three carbonates, two hydrogencarbonates, and three sulfates were used as salt samples. Eight water-soluble amino acids were used as amino acid samples. The materials were G.R. grade chemicals.

Measurement of Antifreeze Activity. Antifreeze activities were evaluated from the thermal behavior during thawing of frozen solutions of the materials as measured by heat flux DSC. The DSC used was DSC220C (Seiko Instruments Inc.) and was calibrated with In, Sn, and Ga. As the result of estimation of the accuracy of the instrument by measuring the heat of fusion of pure water, the error was about 2%. An empty aluminum pan was used as a reference sample.

The 2.5%, 5%, and 10% aqueous solutions of each material were prepared for the DSC measurements. Sample solutions (about 10 mg) were placed in aluminum pans (15 μL), and the pans were hermetically sealed and weighed.

After cooling the sample down to −20 °C at 5 °C/min and holding at that temperature for 10 min, samples were scanned from −20 to 20 °C at 3 °C/min. After the scanning, an endothermic peak of melting of a sample was obtained. The onset temperature of melting was defined as the melting point (T_m), and the difference between T_m and the melting point of pure ice (0 °C) was defined as the melting point depression (ΔT_m). Moreover, the melting curve of a sample was integrated from −20 to 0 °C to determine the heat of fusion of the sample (ΔH).

ΔT_m and ΔH of a sample solution were used as the indication of antifreeze activity of the solute.

Estimation of Water Molecular Mobility. The water molecular mobility was evaluated from the relaxation times of proton in aqueous solutions as measured by proton NMR. All NMR experiments were performed using a broad-line pulsed proton NMR spectrometer (Minispec pc120, Bruker) operating at 20 MHz. The spin−lattice relaxation times (T_1) of proton were measured by using the inversion recovery sequence (180°−90°).

The 1−20% aqueous solutions of each material were prepared for the NMR experiments. The sample solutions were placed in a 7-mm o.d. tube (about 2 mL), respectively, and degassed for 5 min by an aspirator. The experiments were performed at 25 °C. The samples were equilibrated at the

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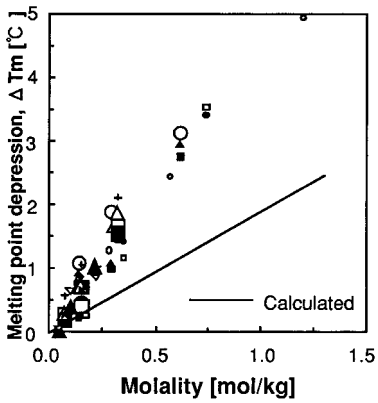

Figure 1. Relationship between the melting point depression and molality of various saccharide solutions. (○) Glycerol; (●) xylose; (□) arabinose; (■) sorbose; (△) galactose; (▲) fructose; (○) glucose; (+) sucrose; (●) treharose; (large open square) maltose; (◇) lactulose; (◆) melibiose; (large solid square) laminaribiose; (large open triangle) nigerose; (×) turanose; (large closed triangle) maltotriose; (▽) melezitose; (large up-side-down open triangle) cellotriose.

given temperature for about 5 min, and the measurement was performed after that.

The obtained T_1 value was used for estimation of water molecular mobility by the method shown below.

The values of T_1^0/T_1 for aqueous solutions, which indicate the amount of water molecules immobilized, are adequately represented by the empirical equation of the form (Uedaira et al., 1989)

$$T_1^0/T_1 = 1 + Bm + Cm^2 \quad (1)$$

where T_1^0 is the spin−lattice relaxation time of proton in pure water and m is the molality of the solute. We can calibrate the constants B and C from the T_1^0/T_1 vs m plot by the least-squares method.

In a dilute aqueous solution, eq 1 can be rewritten as

$$T_1^0/T_1 = 1 + Bm \quad (2)$$

We adopted B as an indicator of the immobilizing activity of solutes for water molecules.

RESULTS AND DISCUSSION

Saccharides. In Figure 1, ice melting point depressions, ΔT_m, of various saccharide solutions are plotted against their concentrations (molality). In a dilute aqueous solution, ΔT_m can be theoretically approximated to

$$\Delta T_m = Km \quad (3)$$

where K (=1.855) is the molal freezing point depression constant (Moore, 1972). The solid line shown in Figure 1 was calculated ΔT_m from eq 3.

The measured ΔT_m values were almost proportional to the molality, independently of the kind of saccharides. This shows that the melting points of various saccharide solutions were controlled by colligative properties in the range of the measured concentration. The magnitude of the discrepancy between the measured and the calculated ΔT_m values became greater with increasing concentration. This indicates deviation from ideal solution, and it might be caused by the interaction among solutes.

Figure 2 shows the heat of fusion, ΔH, of various saccharide solutions. In contrast with ΔT_m, there is a difference in the relation between molality and ΔH

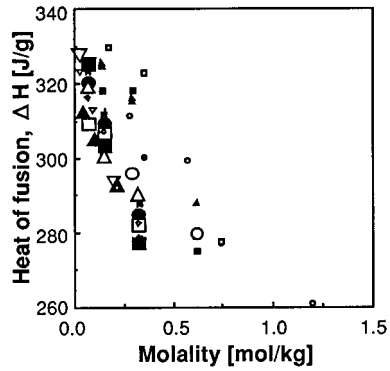

Figure 2. Relationship between heat of fusion and molality of various saccharide solutions. (○) Glycerol; (●) xylose; (□) arabinose; (■) sorbose; (△) galactose; (▲) fructose; (○) *glucose*; (+) sucrose; (●) *treharose*; (large open square) *maltose*; (◇) lactulose; (◆) melibiose; (large solid square) *laminaribiose*; (large open triangle) *nigerose*; (×) turanose; (large closed triangle) *maltotriose*; (▽) melezitose; (large up-side-down open triangle) *cellotriose*. (The larger symbols with the italic sugars represent the data from saccharides that consisted of glucose.)

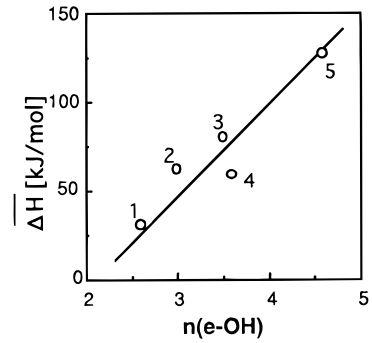

Figure 3. Relationship between the magnitude of the decrease in heat of fusion of monosaccharide solutions per 1 mol/kg and the mean number of the equatorial OH groups per molecule of saccharide. 1, Arabinose; 2, fructose; 3, xylose; 4, galactose; 5, glucose.

among solutes, even if we consider the accuracy of the DSC measurements. Recently, Uraji et al. (1996) measured the freezing point depressions of various saccharide solutions at high concentration (over 2 M), and there was a difference between concentration and freezing point depression among saccharides. This means that ΔT_m can be an indicator of the antifreeze activity at high saccharide concentration. However, it was suggested that ΔH indicated antifreeze activities of saccharides more sensitively than ΔT_m, in the low concentration range which we measured.

The larger symbols in Figure 2 represent the data of saccharides consisted of glucose (italic in the legend). The ΔH values of these saccharides were lower than those of other saccharides, indicating that saccharides consisted of glucose had higher antifreeze activities than other saccharides.

Figure 3 shows the relationship between the magnitude of the decrease in heat of fusion of monosaccharide solutions per 1 mol/kg, $\overline{\Delta H}$, and the mean number of the equatorial OH groups per molecule of saccharide, n(e-OH), which is obtained from the literature (Uedaira et al., 1990). Each $\overline{\Delta H}$ was calculated from eq 4

$$\overline{\Delta H} = (\Delta H_{water} - \Delta H_{5\%})/m_{5\%} \quad (4)$$

where ΔH_{water} is the heat of fusion of water (335 kJ/

Table 1. Relationship between the Immobilizing Activity, *B*, and Antifreeze Activity, $\overline{\Delta H}$, of Monosaccharides

	B^a	$\overline{\Delta H}$ (kJ/mol)^b
glucose	0.329	127.4
fructose	0.307	62.3
sorbose	0.231	33.2
galactose	0.204	58.9
arabinose	0.189	30.5

[a] From NMR data. [b] From DSC data.

kg), $\Delta H_{5\%}$ is the heat of fusion of a 5% (w/w) solution, and $m_{5\%}$ is the molality of a 5% (w/w) solution. Therefore, if the $\overline{\Delta H}$ value of a solution is high, it implies that the solute has high antifreeze activity. As can be seen from Figure 3, there was a linear correlation between $\overline{\Delta H}$ and *n*(e-OH), indicating that the antifreeze activity of monosaccharides increased with increasing number of equatorial OH groups.

Kabayama and Patterson (1958) thermodynamically indicated that hydration of saccharides was related to the conformation, that is, the e-OH groups are able to interact with water in a manner which forms a long-lived hydration structure, since e-OH groups on pyranose sugars match the unperturbed water lattice. Tait et al. (1972) examined the relaxation of ^{17}O-enriched water in monosaccharide solutions by NMR and indicated that the hydration of monosaccharides was partially explained with the model proposed by Kabayama and Patterson (1958).

The linear correlation between $\overline{\Delta H}$ and *n*(e-OH) in Figure 3 indicates that the magnitude of hydration of monosaccharides by DSC, which was obtained as an antifreeze activity, are also related to the conformation of monosaccharides.

We show in Table 1 the values of B and $\overline{\Delta H}$ for each monosaccharide solution. The value of B for glucose was the highest in the saccharides; that is to say, the mobility of water molecules around glucose was most strongly immobilized of all. This tendency obtained by ^{1}H-NMR agreed with the result obtained by ^{17}O-NMR (Uedaira et al., 1989). In addition, comparing each value of *B* and $\overline{\Delta H}$, the higher the *B* value of a saccharide was, the higher the $\overline{\Delta H}$ value of the saccharide was. This result suggests that in saccharides the molecular property of hydration obtained by NMR agree with the thermal property of hydration obtained by DSC, which is worthy of notice.

Salts. In Figure 4, ΔT_m of aqueous solutions of salts are plotted against their molalities. The dotted line is the regression curve calculated from data of saccharides shown in Figure 1. In contrast with saccharides, there is a small difference between molality and ΔT_m among solutes. However, ΔT_m of salt solutions were approximately proportional to the molality. This shows that ΔT_m of salt solutions are controlled by colligative properties in the measured concentration range, in the same manner as saccharides.

There was a difference between the data of salts and saccharides, which might be due to the difference of the extent of the electrolytic dissociation. In an actual solution of a salt, it is supposed that the molality of the salt become high by the electrolytic dissociation, therefore, the difference may become smaller by considering the degree of dissociation.

Figure 5 shows the heat of fusion, ΔH, of various chloride solutions. It can be seen from this figure that

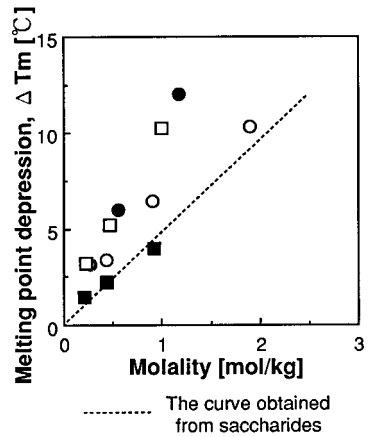

Figure 4. Relationship between melting point depression and molality of salt solutions. (○) NaCl; (●) $MgCl_2$; (□) $CaCl_2$; (■) $MgSO_4$.

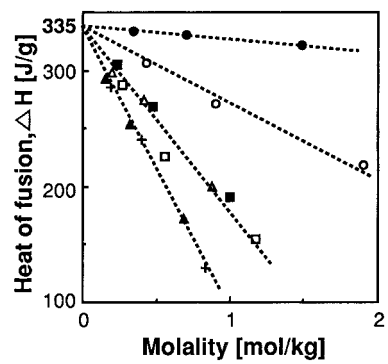

Figure 5. Relationship between heat of fusion and molality of salt solutions. (○) NaCl; (●) KCl; (□) $MgCl_2$; (■) $CaCl_2$; (△) $FeCl_2$; (▲) $FeCl_3$; (+) $AlCl_3$.

Table 2. Relationship between the Immobilizing Activity, *B*, and Antifreeze Activity, $\overline{\Delta H}$, of Salts

	B^a	$\overline{\Delta H}$ (kJ/mol)^b
$CaCl_2$	0.143	142.8
NaCl	0.048	64.3
KCl	−0.040	8.6

[a] From NMR data. [b] From DSC data.

the greater the valent number of cation was, the lower ΔH became. The result indicates that the salts which possess high ionic charge have high antifreeze activity. Thus, it is thought that most of antifreeze activity of salts are caused by their Coulomb's force.

On the other hand, KCl apparently had little antifreeze activity. The KCl solution formed eutectic mixture in addition to ice at temperatures of 0 to −20 °C (the eutectic point is −11.1 °C (Franks, 1985)). Because of the formation of the eutectic mixture, the apparent heat of fusion of KCl solution might be high.

We show in Table 2 the values of *B* and $\overline{\Delta H}$ for each salt solution. Concerning *B* values, the value for $CaCl_2$ was the greatest of all, indicating that $CaCl_2$ had the highest activity for immobilizing water molecules in a solution. Moreover, the values for $CaCl_2$ and NaCl were positive, while the value for KCl was negative. The result shows that $CaCl_2$ and NaCl make a "structure" with liquid water around them by immobilizing the mobility, while KCl breaks the structure. This result agreed with the result in previous papers (Jones et al., 1964; Endom et al., 1967). Concerning $\overline{\Delta H}$ values, the values increase in the order KCl, NaCl, $CaCl_2$. This tendency was agreeable to that of *B*, which indicated

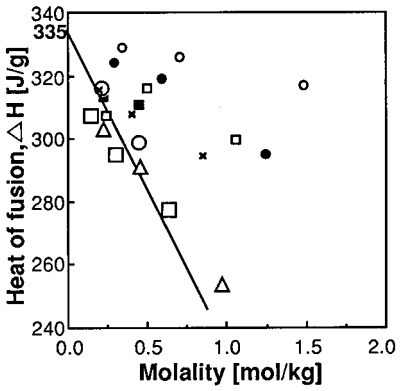

Figure 6. Relationship between heat of fusion and molality of amino acid solutions. (○) Glycine; (●) alanine; (□) serine; (■) valine; (◯) threonine; (large open square) arginine; (large open triangle) proline; (×) hydroxyproline.

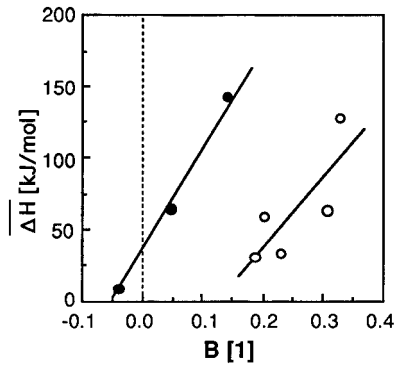

Figure 7. Relationship between the antifreezing activity and the immobilizing activity for water molecules. (○) Saccharides; (●) salts.

that not only in saccharides but also in salts, the molecular property of hydration obtained by NMR agreed with the thermal property obtained by DSC. Since there have been few studies about the comparison of thermal data with dynamic hydration data, this result of salts is worthy of notice.

Amino Acids. Figure 6 shows the heat of fusion, ΔH, of various water-soluble amino acid solutions. The values of three amino acids (threonine, arginine, and proline), represented with larger symbols in the figure, were lower than those of other amino acids, indicating that these three amino acids had higher antifreeze activity. On the other hand, the aqueous solutions of amino acids, except the above three amino acids, formed eutectic mixtures at temperatures of 0 to −20 °C. In consequence, the apparent heat of fusion might become high, same as the case for KCl. Therefore, threonine, arginine and proline were considered useful amino acids for antifreezing of frozen foods.

In previous papers, there were a lot of studies about the hydrophobicity of amino acids by the thermodynamic method (Nozaki et al., 1971; Eagland, 1975). The antifreeze activities of amino acids we obtained seemed not to be correlated to their hydrophobicity.

Antifreeze Activity and Molecular Mobility. To clarify the relationship between the antifreeze activity of materials and the immobilizing activity for water molecules, $\overline{\Delta H}$ and B values for the saccharide and salt samples are shown in Figure 7. In each case of saccharides and salts, there is a good correlation between $\overline{\Delta H}$ and B. These data show that high antifreeze activity is caused by high immobilizing activity for water molecules. However, there was a remarkable difference in the correlation between saccharides and salts. It is generally thought that in an aqueous solution of saccharides and salts, the water molecule is almost immobilized by hydrogen bond and Coulomb's force, respectively. It was considered that differences in the immobilizing mechanism between saccharides and salts caused differences in the correlation.

The B value of salts indicate the apparent immobilizing activity for water molecules, which is total activity of cations and anions. In salts, to examine in detail the relationship between antifreezing and the immobilizing activity for water molecules, it is necessary to obtain the B values of the individual ions.

Conclusions. These results about antifreeze activities seem to be useful in the development of frozen foods, because there were few studies about characterization of antifreeze activities of various food components.

Moreover, in the three sample types (saccharides, salts, amino acids), there was correlation between data by DSC and data by NMR, showing that high antifreeze activity was caused by high immobilizing activity of a sample for water molecules.

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