

# Relationship between the Antifreeze Activities and the Chemical Structures of Oligo- and Poly(glutamic acid)s

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Antifreeze activities of various oligo- and poly(amino acid)s, more particularly oligo(glutamic acid)s (OGAs) and poly(glutamic acid)s (PGAs), were measured by differential scanning calorimetry (DSC). The polymers composed of acidic amino acids, such as PGA, had higher antifreeze activity than the other polymers. The antifreeze activities of the OGAs and PGAs tended to decrease as their molecular weights increased. However, their activities in the molecular weight range below 20 000 were higher than the antifreeze activity of glucose, which is known as a highly antifreeze substance. The antifreeze activities were only slightly affected by the optical isomerism and the peptide linkage type and decreased in the order Na salt = K salt  $\gg$  Ca salt  $\gg$  acidic form. It was considered that the high antifreeze activities of OGA and PGA are mainly caused by Coulomb's force of the dissociating counterions.

**Keywords:** Antifreeze activity; oligo(glutamic acid); poly(glutamic acid); molecular weight; counterion

## INTRODUCTION

It is well-known that freezing and thawing frequently cause undesirable deterioration in living cells, biologically active substances, and foods. To protect them from deterioration, methods to add cryoprotectants, methods to rapidly quench, and combinations of these methods have been widely used.

Saccharides are generally used as the cryoprotectant (Shikama and Yamazaki, 1961; Arai and Takashi, 1973; Whittam and Rosano, 1973; Arakawa and Timasheff, 1982). In addition, the cryoprotective effect of amino acids and their salts and inorganic salts are well recognized (Tamiya et al., 1985; Seguro et al., 1990; Arakawa, 1992; Seguro, 1992). Though there are many theories that explain why these solutes have such cryoprotective properties, one of the convincing mechanisms is that the depression of both the freezing point and the ice fraction of these frozen materials occurs when these solutes are added.

In this context, Uraji et al. (1996) compared the depression effect of various solutes on the freezing points of their solutions. Furthermore, it was reported that some antifreeze proteins, AFPs, have a depression several hundred fold greater than that predicted from colligative effects (Berman et al., 1980). Recently, we investigated the depression effects of various solutes on the ice fractions of the solutions (Mizuno et al., 1997). These results suggested that there were greater differences among the depression effects of solutes on the ice fraction, which are called antifreeze activities, than those among the depression effects of solutes on the freezing point, in the low concentration range (below 10%). It was also suggested that the height of the antifreeze activity of a solute was dependent on its immobilizing activity for water molecules rather than

on its molecular weight, which was thought to be correlated to the colligative effects. For example, the antifreeze activities of glucose and oligosaccharides consisting of glucose, salts with a high ionic charge, and amino acids without eutectic formation by freezing, such as proline, were higher than those of the others in the same type of substances.

Almost all of the highly antifreeze active substances also have cryoprotective effects (Arai and Takashi, 1973; Seguro, 1992). Therefore, it is important for effective cryoprotection to characterize the antifreeze activities of various substances. With only a few exceptions, there has been no study to characterize the activities of peptides and proteins; one is the study of Kuntz and Kauzmann (1974). We recently found that the sodium salts of peptides mainly consisting of acidic amino acids had the highest antifreeze activity in the enzymic digests of bovine hemoglobin (Mitsuiki et al., 1996). The sodium salts of acidic amino acids, which are the constituents of the peptides, are regarded as important cryoprotectants, because of their high cryoprotective effect on the freeze denaturation of lactate dehydrogenase (Seguro et al., 1990). According to these results, it should be expected that the sodium salts of oligomers and polymers composed of acidic amino acids have high antifreeze activities.

It is well-known that the sodium salts of acidic amino acids such as monosodium glutamate have a strong taste (Yamaguchi, 1987). This sensory property makes them not suitable for the cryoprotection of foods. Thus, we were interested in the antifreeze activities of the sodium salts of oligomers and polymers composed of the acidic amino acids, which have a weaker taste. Although it is recognized that the oligomer and the polymer composed of acidic amino acids have a high immobilizing activity for water molecules (Cooke and Kuntz, 1974), their antifreeze activities have not been widely studied. For instance, the effects of their chemi-

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cal structures such as molecular weight, counterions, optical isomerism (D or L), and peptide linkage type ( $\alpha$  or  $\gamma$ ) on the antifreeze activities are not yet completely understood.

Therefore, the objectives of this study were to characterize the antifreeze activities of various oligo- and poly(amino acid)s, most particularly, OGAs and PGAs, and to clarify the relationship between their antifreeze activities and chemical structures.

## MATERIALS AND METHODS

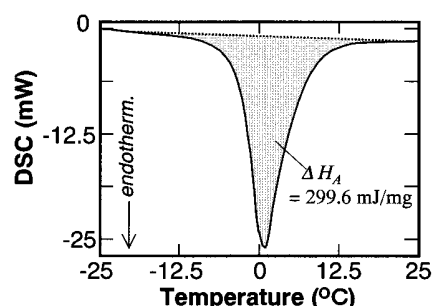
**Materials.** All materials used for measuring the antifreeze activities, except for  $\gamma$ -PGA, were G. R. grade reagents. Pyroglutamic acid (Aldrich Chemical Co., Milwaukee, WI), D- and L-glutamic acid (Sigma Chemical Co., St. Louis, MO),  $\alpha$ - and  $\gamma$ -L-glu-glu,  $\alpha$ - and  $\gamma$ -L-glu-glu-glu (BACHEM Feinchemikalien AG., Bubendorf, Switzerland), and  $\alpha$ -D- and  $\alpha$ -L-PGA (Sigma Chemical Co.) were purchased. Poly(amino acid) samples such as poly( $\alpha$ -L-aspartic acid), poly( $\alpha$ -L-alanine), poly( $\alpha$ -L-lysine), and poly( $\alpha$ -L-arginine), for the purpose of comparing their antifreeze activities with those of OGA and PGA, were also purchased from Sigma Chemical Co.

**$\gamma$ -PGA Preparation.**  $\gamma$ -PGA was prepared by the modified method of Murao et al. (1971) and Sawa et al. (1971). The method is as follows: the 5% precultured suspension of *Bacillus licheniformis* No. 138 separated from commercially available *natto*, which is a fermented soybean and a traditional Japanese food, was aerobically cultured in a medium containing L-glutamic acid (100 mg/mL), glucose (100 mg/mL),  $(\text{NH}_4)_2\text{SO}_4$  (15 mg/mL),  $\text{K}_2\text{HPO}_4$  (1.5 mg/mL),  $\text{MgSO}_4$  (0.35 mg/mL), and  $\text{MnSO}_4$  (0.05 mg/mL) at 30 °C for 49 h. After collection of the precipitate of the viscous cultured fluid at pH 1.9, it was neutralized and dissolved in a 0.1 N NaOH solution. After removal of the precipitate by centrifugal separation (10 000g  $\times$  20 min), the supernatant was adjusted to pH 2.0 using a 0.1 N HCl solution and then the same weight of ethanol was added to it. The precipitate at that time was suspended in distilled water at ca. 10 mg/mL. The suspension was dissolved and accurately adjusted to pH 7.0 with 0.1 N NaOH. The lyophilized powder of the solution was regarded as a purified  $\gamma$ -PGA sample.

An Asahipak GFA-7M HQ column (Showa Denko Co., Ltd., Tokyo, Japan) was used for high-performance liquid chromatography (HPLC). The chromatogram of the HPLC showed only  $\gamma$ -PGA. The glutamic acid content in the constituent amino acids of  $\gamma$ -PGA, which was analyzed with an L-8500 amino acid analyzer (Hitachi, Ltd., Tokyo, Japan) after hydrolysis with 6 N HCl containing 1% phenol at 150 °C for 1 h under reduced pressure, was over 99%. The D/L ratio in the constituent glutamic acids of  $\gamma$ -PGA, which was estimated using an L-glutamic acid assay kit (Yamasa Corp., Chiba, Japan), was 83/17. The sugar content in  $\gamma$ -PGA, which was determined by the phenol-sulfuric acid calorimetric method (Hodge and Hofreiter, 1962), was less than 0.1%.

The lower molecular weight  $\gamma$ -PGAs were prepared as follows:  $\gamma$ -PGA was hydrolyzed in the solution at pH 3.0 and 50 or 70 °C for an appropriate time; then the solution was accurately adjusted to pH 7.0 with 0.1 N NaOH and lyophilized.

**Various Salts Preparation.** For the purpose of investigating the effects of counterions on the antifreeze activities of OGA and PGA, their acidic samples and their various salts were prepared. At first, to prepare the acidic sample for each peptide, the pH of the solution (ca. 10 mg/mL) was adjusted to 2.0 with 0.1 N HCl, and then the desalting of it was performed at that pH. The desalting of a low-weight molecule (molecular weight, MW, <10 000) was carried out using a microaclyzer G1 (Asahi Chemical Industries Co., Ltd., Tokyo, Japan) with an AC 110-10 filter (Asahi Chemical Industries Co., Ltd.). On the other hand, the desalting of a high-weight molecule (MW >10 000) was carried out with dialysis against distilled water. The lyophilized powder of the desalted suspension was regarded as a dried acidic form sample.



**Figure 1.** DSC thermogram of a glucose solution. The weight fraction of glucose in the solution was 0.0506. The heat of fusion of the solution,  $\Delta H_A$ , which was obtained by the integration of an endothermic peak, was 299.6 mJ/mg.

The dried sample was suspended in distilled water at ca. 10 mg/mL. The suspension was dissolved and accurately adjusted to pH 7.0 with 0.1 N NaOH, KOH, and  $\text{Ca}(\text{OH})_2$ ; then the lyophilized powder of the solution was regarded as a dried Na, K, and Ca salt, respectively.

**Molecular Weight Measurement of PGAs.** The molecular weights of the  $\alpha$ - and  $\gamma$ -PGAs were measured by gel-permeation chromatography-low-angle laser light scattering (GPC-LALLS), based on the method developed by Ouano and Kaye (1974). The GPC-LALLS measurement was carried out on HPLC equipment consisting of the following components: an 880-PU pump (Japan Spectroscopic Co., Ltd., Tokyo, Japan), an 851-AS autosampler (Japan Spectroscopic Co., Ltd.), an Asahipak GFA-7M HQ column, an LS-8000 LALLS photometer (Tosoh Corp., Tokyo, Japan), and an RI-8011 differential refractometer (Tosoh Corp.). The column was thermostated at 40 °C and eluted with 10 mM Tris-HCl buffer (pH 8.6) containing 1% NaCl. Samples were dissolved in the same solvent at 2.0 mg/mL and then injected (200  $\mu$ L). Commercial pullulans (Asahi Chemical Industries Co., Ltd.) were used as the standards for the molecular weight measurement.

**Measurement of Antifreeze Activity.** Antifreeze activities were evaluated by their thermal behavior during the thawing of the frozen solutions of the materials, as measured by heat flux DSC. The DSC used was a DSC220C (Seiko Instruments Inc., Chiba, Japan) and calibrated with In, Sn, and Ga. An empty aluminum pan was used as the reference sample.

The ca. 5% (w/w) aqueous solutions of each material, of which the concentration was accurately known, and pure water were used for the measurements of the antifreeze activities. The solutions and pure water (ca. 15 mg) were placed in aluminum pans (15  $\mu$ L), which were then hermetically sealed and accurately weighed.

In the DSC, each pan was scanned from -22 to +30 °C at 3 °C/min, after cooling to -22 °C at 5 °C/min and holding at -22 °C for 10 min. As can be seen from Figure 1, an endothermic peak of the melting of frozen water in a sample solution and pure water was obtained by the scanning. The endothermic peak was integrated from -20 °C to the end temperature of melting in order to measure the heat of fusion of a sample solution,  $\Delta H_A$ , and pure water,  $\Delta H_{\text{water}}$ .  $\Delta H_{\text{water}}$  in this study was  $335.0 \pm 3.6$  mJ/mg (mean value  $\pm$  standard deviation of five experiments).  $\Delta H_A$  of glucose in this study was  $299.6 \pm 2.9$  mJ/mg (Figure 1).

The antifreeze activity (AF) of each sample was defined as the grams of unfrozen water in its solution per gram of the solid sample (Mitsuiki et al., 1996) and was calculated using eq 1, where  $w$  is the weight fraction of the sample in the

$$\begin{aligned} \text{AF} &= \{\text{unfrozen water}\} / \{\text{solid sample}\} \\ &= [\Delta H_{\text{water}} (1 - w) - \Delta H_A] (1 - w) / \\ &\quad \Delta H_{\text{water}} (1 - w) \} / \{w\} \\ &= [\Delta H_{\text{water}} (1 - w) - \Delta H_A] / \Delta H_{\text{water}} w \end{aligned} \quad (1)$$

**Table 1. Antifreeze Activities of Various Poly( $\alpha$ -L-amino acids) and Glucose**

	mol wt	antifreeze activity (g of H <sub>2</sub> O/g)
poly( $\alpha$ -L-aspartic acid) (Na salt)	23 400	1.18
poly( $\alpha$ -L-glutamic acid) (Na salt)	12 100	1.14
poly( $\alpha$ -L-arginine) (hydrochloride)	41 800	0.38
poly( $\alpha$ -L-lysine) (hydrochloride)	21 000	0.89
poly( $\alpha$ -L-alanine)	21 000	nd <sup>a</sup>
glucose	180	1.09

<sup>a</sup> Not detected.

solution. According to eq 1, the AF of glucose ( $w = 0.0506$ ) was 1.09 g of H<sub>2</sub>O/g.

The antifreeze activities of various poly(amino acids) in this study were the mean value of two experiments.

## RESULTS AND DISCUSSION

**Antifreeze Activities of Various Poly( $\alpha$ -L-amino acids).** Table 1 shows the antifreeze activities of various poly( $\alpha$ -L-amino acids) ranging in molecular weight from 10 000 to 40 000, and of glucose. Poly(amino acids), other than polyalanine, had high antifreeze activities. The activity of polyalanine was not detected because its solubility is fairly low; that is, the difference in the heat of fusion between pure water and its suspension was within the margin of error of the DSC analysis.

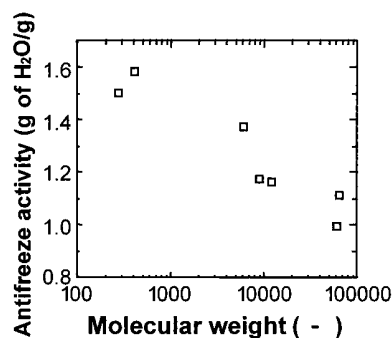
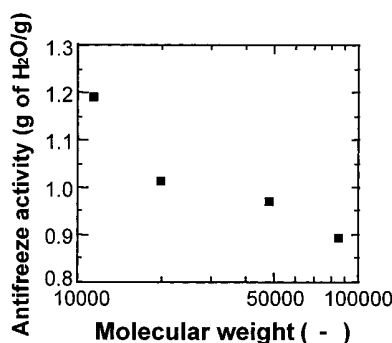
As Table 1 shows, the polypeptides consisting of acidic amino acids (poly(aspartic acid) and PGA) had higher activities than the others. Kuntz and Kauzmann (1974) reported that poly(glutamic acid) had the largest amount of bound water adsorbed at a given relative humidity in the proteins and polypeptides studied. Cooke and Kuntz (1974) reported that it had the highest immobilizing activity for water molecules in the polypeptides investigated. These results agree with our data concerning antifreeze activity (Table 1). It is, therefore, suggested that the height of antifreeze activity of a poly(amino acid) is dependent on its immobilizing activities for water molecules, as well as saccharides and inorganic salts (Mizuno et al., 1997).

It is noteworthy that the antifreeze activities of poly(aspartic acid) (1.18 g of H<sub>2</sub>O/g) and PGA (1.14 g of H<sub>2</sub>O/g), despite about 100 times higher molecular weight, were higher than that of glucose (1.09 g of H<sub>2</sub>O/g), which is known as a highly antifreeze active substance.

Since it was thought that there was no difference in antifreeze activity between poly(aspartic acid) and PGA (Table 1), the effects of their chemical structures on the antifreeze activities were hereinafter investigated only for OGA and PGA.

**Effect of Molecular Weight on Antifreeze Activity.** The effect of molecular weight on antifreeze activity for the Na salts of  $\alpha$ -L-OGA and PGA is shown in Figure 2. Their antifreeze activities tended to decrease as their molecular weights increased. Also, the antifreeze activities of the Na salts of  $\alpha$ -D-PGA tended to decrease as their molecular weights increased (Figure 3).

The hydrolysis conditions of  $\gamma$ -PGA to obtain the lower weight molecules and the effect of molecular weight on antifreeze activity of the various Na salts of  $\gamma$ -OGA and PGA are shown in Table 2. The molecular weight of  $\gamma$ -PGA, which was obtained by a fermentation method and purification, was 632 000. The lower weight molecules ranging in molecular weight from 18 000 to 250 000 were obtained by hydrolyzation at pH 3.0 and 50 or 70 °C for an appropriate time.

**Figure 2.** Relationship between the molecular weight and the antifreeze activity for Na salts of  $\alpha$ -L-OGA and PGA.**Figure 3.** Relationship between the molecular weight and the antifreeze activity for Na salts of  $\alpha$ -D-PGA.**Table 2. Hydrolysis Conditions of  $\gamma$ -PGA for Obtaining the Lower Weight Molecules and the Effect of the Molecular Weight on Antifreeze Activity of Various Na Salts of  $\gamma$ -OGA and PGA**

sample	hydrolysis condition			antifreeze activity (g of H <sub>2</sub> O/g)
	temp (°C)	time (h)	mol wt	
A <sup>a</sup>			632 000	0.85
B <sup>b</sup>	50	0.5	250 000	0.99
C <sup>b</sup>	50	1.0	247 000	0.96
D <sup>b</sup>	50	2.0	104 000	0.88
E <sup>b</sup>	70	0.5	65 000	0.87
F <sup>b</sup>	70	2.0	25 000	1.13
G <sup>b</sup>	70	6.0	18 000	1.00
H <sup>c</sup>			405	1.42
I <sup>d</sup>			276	1.41

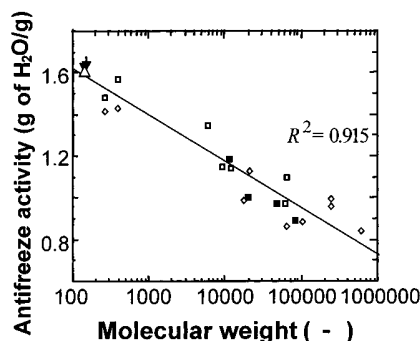
<sup>a</sup>  $\gamma$ -PGA, which was obtained by a fermentation method and purified. <sup>b</sup> Hydrolyzate of  $\gamma$ -PGA. <sup>c</sup>  $\gamma$ -Glu-glu-glu, purchased. <sup>d</sup>  $\gamma$ -Glu-glu, purchased.

As seen in Table 2, the antifreeze activities of  $\gamma$ -OGA and PGA tended to decrease as their molecular weights increased. This same sort of trend can be seen in the results with  $\alpha$ -OGA and PGA (Figures 2 and 3).

Figure 4 shows the relationship between the molecular weight and the antifreeze activity for the various Na salts of OGA and PGA. The activities of monomers such as pyroglutamic acid (1.65 g of H<sub>2</sub>O/g), and D- and L-glutamic acid (1.61 and 1.60 g of H<sub>2</sub>O/g, respectively) were also plotted in the figure.

As can be seen from this figure, the activities of  $\gamma$ -,  $\alpha$ -D-, and  $\alpha$ -L-OGA and PGA gradually decreased as their molecular weights increased in a similar manner. It is noteworthy that the plot of the log of molecular weight vs antifreeze activity for all OGAs, PGAs, and monomers gives a single straight line (eq 2) with good correlation ( $R^2 = 0.915$ ).

$$\text{AF} = 2.0611 - 0.22154 \log \text{MW} \quad (2)$$



**Figure 4.** Relationship between the molecular weight and the antifreeze activity for various Na salts of OGA, PGA, and acidic amino acid monomer: (□)  $\alpha$ -L-OGA and PGA; (■)  $\alpha$ -D-OGA and PGA; (◇)  $\gamma$ -OGA and PGA; (△) L-glutamic acid; (▼) D-glutamic acid; (+) pyroglutamic acid.

According to eq 2, it is also noteworthy that OGAs and PGAs with a molecular weight up to 20 000 had activities higher than that of glucose (1.09 g of H<sub>2</sub>O/g (Table 1)), which is known as a highly antifreeze active substance.

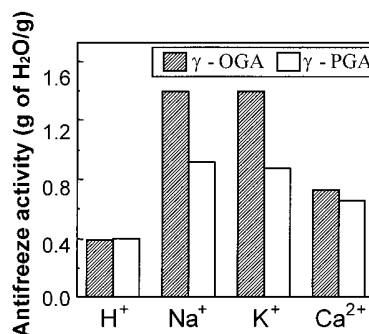
On the other hand, the antifreeze activities of glucose (MW = 180), maltose (MW = 342), and maltotriose (MW = 504), which are calculated from their heat of fusion shown in a previous paper (Mizuno et al., 1997), are 1.23, 0.82, and 0.42 g of H<sub>2</sub>O/g, respectively. These results show that the decrease in the antifreeze activity of saccharides consisting of glucose with increasing molecular weight is significant. On the other hand, the decrease in the antifreeze activities of OGAs and PGAs with increasing molecular weight was slower (Figure 4). Therefore, it seems that the antifreeze activities of OGA and PGA are caused by a certain mechanism in which there is a slight difference between the lower weight molecule and the higher weight molecule.

**Effect of the Optical Isomerism and the Peptide Linkage Type on the Antifreeze Activity.** As mentioned above, the antifreeze activities of various OGAs and PGAs decreased gradually as their molecular weight increased in a similar manner, regardless of their optical isomerism and their peptide linkage type (Figure 4). In other words, their antifreeze activities seem to be dependent on their molecular weight, but not on their optical isomerism and their peptide linkage type.

It is well-known that the antifreeze activities of saccharides (Mizuno et al., 1997) and AFPs (Wen and Laursen, 1993) are influenced by their configuration, while those of inorganic salts are not (Mizuno et al., 1997). Because the optical isomerism and the peptide linkage type, which relate to the configuration of OGA and PGA, did not influence the antifreeze activities (Figure 4), it can be presumed that the antifreeze activities are caused by the effect of their charged ions rather than by their configuration. Therefore, the effects of counterions on the antifreeze activities were hereinafter investigated.

**Effect of the Counterions on the Antifreeze Activity.**  $\gamma$ -Glu-glu as the representative of OGAs and hydrolyzed  $\gamma$ -PGA (MW = 250 000) as the representative of PGAs were used for studying the effect of the counterions on the antifreeze activities of OGA and PGA. The antifreeze activities of the acidic form and their various salts are shown in Figure 5.

As shown in the figure, the activity of both OGA and PGA decreased in the order Na salt = K salt  $\gg$  Ca salt



**Figure 5.** Effect of the counterions on the antifreeze activity of  $\gamma$ -OGA and  $\gamma$ -PGA. Hydrolyzed  $\gamma$ -PGA (molecular weight = 250 000) and  $\gamma$ -glu-glu were used as the  $\gamma$ -PGA and  $\gamma$ -OGA samples, respectively.

$\gg$  acidic form, suggesting that the charged univalent counterions are essential for the high antifreeze activities of OGA and PGA.

In synthetic water-soluble polymers, Maeda et al. (1993) evaluated the number of hydrogen bond defects introduced per a monomeric unit of polymer chain ( $N$ ) in the hydrogen-bonded network structure of water molecules by Raman spectroscopy. They revealed that the polymers having ionizable and polar groups (carboxylic groups) and counterions (Na<sup>+</sup>) have a higher  $N$  value than any other polymers analyzed. Moreover, Berthold et al. (1996) estimated the nonfreezing bound water absorbed by carboxylic groups and counterions from the data of both moisture sorption and thermal analysis for ligno-cellulosic materials. They revealed that the amount of nonfreezing bound water absorbed by the materials at a given relative humidity is determined by the sum of the smaller amount of water absorbed by carboxylic groups and the larger amount of water absorbed by the counterions.

These results are in fair agreement with the results concerning the antifreeze activity of OGA and PGA (Figure 5), though the analyzed polymer concentration range and their backbones are different from each other. Therefore, the high antifreeze activities of OGA and PGA also seem to be mainly caused by the breaking effect (Coulomb's force) of the charged counterions on the hydrogen-bonded network structure of water.

The antifreeze activity was cation dependent, and the antifreeze activity of both OGA and PGA decreased in the order Na<sup>+</sup> = K<sup>+</sup>  $\gg$  Ca<sup>2+</sup>  $\gg$  H<sup>+</sup> (Figure 5). This order disagrees with that for inorganic chlorides, which decreased in the order Ca<sup>2+</sup>  $\gg$  Na<sup>+</sup>  $\gg$  K<sup>+</sup> (Mizuno et al., 1997). It can be considered that the antifreeze activities of inorganic chlorides are based upon the immobilizing effect for the water molecules of each ion dissociating completely in the solution (Endom et al., 1967). On the other hand, it seems that the degrees of dissociation of the salts of a polymer such as OGA and PGA are fairly lower than that of inorganic chlorides and are different among the counterions.

According to the results in previous papers concerning the moisture sorption of polyanions with counterions, the differences in adsorption behavior seem to be explained by the degree of dissociation of the ion pair (Lowry and Mauritz, 1980). In the study of Berthold et al. (1996) described above, they revealed that the numbers of nonfreezing bound water molecules associated with a carboxylic acid decreased in the order Na<sup>+</sup>  $>$  Ca<sup>2+</sup>  $\gg$  H<sup>+</sup> at rather high relative humidity (above 92%) and are affected by the degree of dissociation of

the ion pair. Moreover, Yamamoto et al. (1994) reported that OGA and PGA have inhibitory activity against Ca phosphate insolubilization and high Ca binding capacity. From the results in the papers already described, it can be assumed that the degrees of dissociation of the Ca salt of OGA and PGA are lower than those of the K and Na salts; therefore, the antifreeze activity of the Ca salt is consequently lower than those of K and Na salts.

On the other hand, because the Ca ion is divalent, the molarity of the Ca ion in the Ca salts of OGA and PGA is lower than that of the K and Na ions in K and Na salts, respectively, in the same concentration at a pH value around neutral. Therefore, it is thought that the difference in molarity of a cation affects the antifreeze activity. Nevertheless, we assumed that the antifreeze activity is affected not only by the molarity but rather by the degree of dissociation of the ion pair, since the Ca ion has several times as high antifreeze activity as the K and Na ions for inorganic chloride solutions (Mizuno et al., 1997).

Because there was little difference in the activity between acidic OGA and acidic PGA (Figure 5), it is assumed that the effect of the molecular weight on the antifreeze activities of OGAs and PGAs (Figure 4) is caused by the difference in the degree of dissociation among them. However, further experiments are necessary to more directly clarify the relationship between the degree of their dissociation and molecular weight.

**Conclusions.** We have investigated the antifreeze activity of various OGAs and PGAs. The results suggest that the high antifreeze activities of OGA and PGA are mainly caused by Coulomb's force of the dissociating counterions and that OGAs and PGAs with molecular weights below 20 000 have an activity higher than that of glucose, which is known as a highly antifreeze active substance.

For cryoprotection, the depression of the ice fraction of the frozen materials by adding some highly antifreeze active substances is an important technique. We believe that OGA and PGA are effective cryoprotectants, especially for frozen foods, because they have a weaker taste than the lower weight molecule cryoprotectants such as saccharides, inorganic salts, and amino acids, and they can be added to foods in larger quantities without a serious change in the taste.

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