Effects of a type I antifreeze protein (AFP) on the melting of frozen AFP and AFP+solute aqueous solutions studied by NMR microimaging experiment

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Abstract The effects of a type I AFP on the bulk melting of frozen AFP solutions and frozen AFP+solute solutions were studied through an NMR microimaging experiment. The solutes studied include sodium chloride and glucose and the amino acids alanine, threonine, arginine, and aspartic acid. We found that the AFP is able to induce the bulk melting of the frozen AFP solutions at temperatures lower than 0 °C and can also keep the ice melted at higher temperatures in the AFP+solute solutions than those in the corresponding solute solutions. The latter shows that the ice phases were in super-heated states in the frozen AFP+solute solutions. We have tried to understand the first experimental phenomenon via the recent theoretical prediction that type I AFP can induce the local melting of ice upon adsorption to ice surfaces. The latter experimental phenomenon was explained with the hypothesis that the adsorption of AFP to ice surfaces introduces a less hydrophilic water-AFP-ice interfacial region, which repels the ionic/hydrophilic solutes. Thus, this interfacial region formed an intermediate chemical potential layer between the water phase and the ice phase, which prevented the transfer of water from the ice phase to the water phase. We have also attempted to understand the significance of the observed melting phenomena to the survival of organisms that express AFPs over cold winters.

Keywords Antifreeze protein · Nuclear magnetic resonance microimaging · Melting point lowering · Super-heated ice · Antifreeze protein activity

1 Introduction

Antifreeze proteins (AFPs), also referred to as ice binding proteins (IBPs), are found in certain organisms, including fish, insects, and plants, where they act to protect living cells from freezing damage in subzero environments [1–15]. The mechanism of action is

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attributed to their ability to bind to specific ice surfaces, thereby inhibiting the growth of seed-ice crystals. By the same mechanism, IBPs can also inhibit the recrystallization of ice, which can create large, tissue-damaging ice crystals [16]. AFPs can lower the freezing points of their aqueous solutions far below those predicted by their colligative effects on freezing point depression. This finding was based on the experiment on AFP's antifreeze activity [17–23] in which individual ice crystals (on the order of $\sim 50\text{ }\mu\text{m}$ in size) with confined shapes (mostly bipyramids) could be obtained in super-cooled AFP aqueous solutions. These ice crystals could remain in the solutions in certain temperature ranges, depending on the types and concentrations of the AFPs [24]. The freezing points of the super-cooled solutions were defined as the temperatures at which the ice crystals grew abruptly into the solutions, while the melting points of the ice crystals were defined as the temperatures at which the ice crystals shrank at a considerable rate. The difference between the so-defined freezing points and melting points was referred to as the thermal hysteresis. This activity description focuses on the changes of the individual ice crystals in super-cooled AFP solutions and thus describes how the freezing of an AFP solution is kinetically slowed down through inhibiting the growth of seed-ice crystals. To extensively understand AFP functions, we also need to know how AFPs influence the bulk melting of frozen AFP solutions. To study this, we have applied a nuclear magnetic resonance (NMR) microimaging technique to see the melting procedures of frozen AFP solutions. This technique can detect the spatial distribution of mobile water as positive proton signal intensities during the melting process of frozen AFP solutions, while solid ice shows no signal [25]. The HPLC6 isoform of type I AFP from the blood serum of winter flounder [26–28] was used in the experiments. Considering the existence of salts in any physiological fluids, we performed the experiments for both the frozen AFP and AFP+NaCl solutions. To see the generality of the AFP's effect on the melting of the frozen AFP+NaCl solutions, we also carried out the experiments on four amino acid +AFP solutions and one glucose+AFP solution.

2 Materials and methods

The type I AFP used in this experiment is the HPLC6 isoform from the blood serum of winter flounder [26–28]. The AFP consists of the following sequence: Asp-Thr-Ala-Ser-Asp-Ala-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asn-Ala-Lys-Ala-Ala-Ala-Glu-Leu-Thr-Ala-Ala-Asn-Ala-Ala-Ala-Ala-Ala-Ala-Ala-Thr-Ala-Arg. It has three 11-residue repeat units commencing with the threonine residues. Its secondary structure is α -helical. The type I AFP was supplied by A/F Protein, Inc. (Waltham MA, USA) (99.0 %). NaCl; amino acids, including alanine (Ala), threonine (Thr), arginine (Arg), and aspartic acid (Asp), and glucose were supplied by Sigma-Aldrich (reagent grade). The solutions were prepared using distilled water. The concentrations of the solutions are listed in Table 1. A half milliliter of each of the solutions was held in a glass tube with ID = 7.9 mm. All the solutions were frozen with liquid nitrogen and then placed in a $-20\text{ }^{\circ}\text{C}$ freezer overnight for the NMR microimaging experiments. About 20–30 s were needed to freeze 0.5 ml of the solutions. This rate would cause the non-equilibrium freezing of the solution. For the NaCl, amino acids, and glucose-water systems, heterogeneous systems with mixed ice phases, and highly concentrated solute water phases, could be initially created below $0\text{ }^{\circ}\text{C}$. Eutectic solute phases mixed with polycrystalline ice grains could be made when the temperatures were further lowered to 77 K. For the AFP+NaCl, AFP+amino acids,

Table 1 Concentrations of AFP, solutes, and AFP+solute aqueous solutions

Substances	Concentrations	Concentrations	Concentrations
AFP	1.0 mg/ml	2.0 mg/ml	5.0 mg/ml
AFP+NaCl	1.0 mg/ml + 1.0 mg/ml	2.0 mg/ml + 5.0 mg/ml	
NaCl	1.0 mg/ml	5.0 mg/ml	
AFP+Ala	2.0 mg/ml + 2.5 mg/ml		
Ala	2.5 mg/ml		
AFP+Thr	2.0 mg/ml + 3.34 mg/ml		
Thr	3.34 mg/ml		
AFP+Arg	2.0 mg/ml + 4.97 mg/ml		
Arg	4.97 mg/ml		
AFP+Asp	2.0 mg/ml + 3.6 mg/ml		
Asp	3.6 mg/ml		
AFP+glucose	2.0 mg/ml + 5.13 mg/ml		
Glucose	5.13 mg/ml		

and AFP+glucose water systems, AFPs could initially remain in the solute water phases, and many of them could also bind to the boundary ice surfaces between the ice phase and the solute water phase. Eventually, AFP could mix with the eutectic solute phases at a lower temperature. During the annealing procedure at -20°C overnight, any glassy ice structure and disordered ice (if any were created due to freezing with liquid nitrogen) would crystallize to ice I_h , and AFPs would relax in order to find the best match to the ice surfaces. The so-produced heterogeneous systems provided large areas of ice surfaces to allow the observation of the effect of the AFP on the melting of the frozen AFP and AFP+solute solutions.

NMR microimaging experiments were carried out with a Bruker microimaging system attached to a Bruker Avance™ 600 WB NMR spectrometer. A variable temperature-controlled Micro2.5 probe with a 30 mm ^1H birdcage resonator was used. After the temperature of the cooling channel was lowered to -30°C , the sample tubes holding the frozen aqueous solutions were quickly transferred inside the resonator. A multislice multi-spin-echo sequence (Bruker protocol MSME_bas) was used to image the mobile water in the samples. Multislices with a slice thickness of 1.0 mm, an interslice distance of 1.0 mm, and a slice orientation across the axial direction in a field of view of 3.5 cm were used. Other parameters included echo time = 10.567 ms, repetition time = 1000.000 ms, and number of averages = 4. The temperature was controlled by heater-regulated cold nitrogen gas. The images of the frozen samples were acquired at fixed temperatures, which were increased from a lower temperature (-30°C) to a higher temperature stepwise in order to observe the melting processes of the frozen solutions. The samples were stabilized at each temperature for ~ 15 min before acquiring the imaging signals. The accuracy of the temperature could be controlled within $\pm 0.025^{\circ}\text{C}$. Mobile water can be observed as positive proton signal intensity (or brightness), while solid ice gives zero proton signal intensity (or darkness) in the images using the given NMR microimaging protocol. Sixteen slices of images were acquired for each sample. A slice from the middle of each of the samples was presented in this manuscript. All other slices show similar images except a couple of the slices at each of the two ends of the samples due to the arched end surfaces.

Optical observation of the ice crystals in the AFP solutions was carried out with a set of equipment that included a custom-built Otago nanoliter osmometer, a thermoelectric temperature controlling device with a temperature-controlled cooling stage, an Olympus BX 51 microscope (maximum magnification 800 $\times$ with a resolution of 1 micron), and a

RETIGA 2000R Color Video Camera. The accuracy of the temperature can be controlled to within ± 0.02 °C.

3 Results

Figure 1 shows the images of the frozen AFP aqueous solutions with AFP concentrations of 0, 1.0, 2.0, and 5.0 mg/ml at various temperatures. With the increase in temperature, water signals of the frozen AFP solutions began to appear at -1.00 °C, where more mobile water could be observed in the 5.0 mg/ml AFP solution than in the 2.0 mg/ml AFP solution. The water signal of the frozen 1.0 mg/ml AFP solution started appearing more clearly at -0.80 °C. With further increases in temperature, larger volumes of ice began to melt for all of the AFP samples. The frozen solutions with higher AFP concentrations showed lower temperatures at which water began to appear and also showed larger volumes of melted ice at these temperatures. It was noticed that the melting started primarily from the interfaces of the ice and the inner glass walls to the internal bodies of the frozen solutions, while the internal melting was not significant. Most of the ice melted at -0.60 °C for the frozen 5.0 mg/ml AFP solution, and this occurred at -0.40 °C for the frozen 2.0 mg/ml AFP solution and at 0.00 °C for the frozen 1.0 mg/ml AFP sample. We also observed that once the samples were controlled at fixed temperatures, the amounts of liquid water and ice were quite stable for the entire experimental time (up to a couple of hours as tested).

Now, let us see the effect of the AFP on the melting of the frozen AFP+NaCl solutions. The first four samples that were tested included (a) 1.0 mg/ml NaCl, (b) 1.0 mg/ml AFP + 1.0 mg/ml NaCl, (c) 1.0 mg/ml AFP, and (d) water. The results are shown in Fig. 2a.

Fig. 1 Variable temperature-dependent NMR microimaging results showing the melting of the frozen AFP aqueous solutions, with AFP concentrations of 5.0, 2.0, 1.0, and 0.0 mg/ml as indicated in the lower-right frame

(a)

(b)

Fig. 2 Variable temperature-dependent NMR microimaging results showing the melting of the frozen AFP/NaCl solutions containing **a** 1.0 mg/ml NaCl, 1.0 mg/ml NaCl + 1.0 mg/ml AFP, 1.0 ml/ml AFP and distilled water as indicated in the lower-right frame; and **b** 5.0 mg/ml NaCl, 5.0 mg/ml NaCl + 2.0 mg/ml AFP, 2.0 mg/ml AFP and distilled water as indicated in the lower-right frame

(a)

(b)

Fig. 3 Variable temperature-dependent NMR microimaging results showing the melting of the frozen AFP and AFP + amino acid solutions containing **a** 2.0 mg/ml AFP + 2.5 mg/ml Ala and 2.5 mg/ml Ala; **b** 2.0 mg/ml AFP + 3.34 mg/ml Thr solution and 3.34 mg/ml Thr solution; **c** 2.0 mg/ml AFP + 4.97 mg/ml Arg and 4.97 mg/ml Arg, and **d** 2.0 mg/ml AFP + 3.6 mg/ml Asp and 3.6 mg/ml Asp

(c)

(d)

Fig. 3 (continued)

After the temperature was increased to -3.0°C , weak liquid water signals were detected in both the frozen NaCl solution and the frozen AFP+NaCl solution. To our surprise, the water signals in the frozen AFP+NaCl solution were much weaker than those in the frozen salt solution. A large amount of the ice in the frozen salt solution melted at -0.3°C , while there was still a significant amount of ice remaining in the frozen AFP+NaCl solution at this temperature. To further verify the observed phenomena, we conducted the experiment on a second set of four samples, including (a) 5.0 mg/ml NaCl, (b) 2.0 mg/ml AFP + 5.0 mg/ml NaCl, (c) 2.0 ml/ml AFP, and (d) water. The results are shown in Fig. 2b. Similar phenomena were observed from these frozen AFP/NaCl solutions but with lowered melting temperatures due to the increased salt concentrations. It is interesting to note that the melting pattern primarily displays mixed areas of brightness and darkness across each of the whole disks of the images, which indicates the mixed saline and salt+AFP water phases with ice phases, which is in contrast to those of the frozen AFP solutions and water. The latter show that the melting started from the interfaces of the inner glass walls and the frozen solution. To determine whether the melting phenomenon occurs only for the AFP+NaCl salt solutions or also for solutions of the AFP with other kinds of solutes, we carried out the NMR microimaging experiments for four amino acid solutions and one glucose solution. The results for the frozen Ala and AFP+Ala, Thr and AFP+Thr, Arg and AFP+Arg, and Asp and AFP+Asp solutions are shown in Fig. 3a, b, c, and d, respectively, and those of glucose and AFP+glucose solutions are shown in Fig. 4. All these results show that the frozen AFP+amino acid solutions and the frozen AFP+glucose solutions melted at higher temperatures than their corresponding amino acid and glucose solutions and that the melting patterns are also similar to those of the AFP+NaCl and NaCl solutions.

Fig. 4 Variable temperature-dependent NMR microimaging results showing the melting of the frozen AFP and AFP + sugar solutions containing 2.0 mg/ml AFP + 5.13 mg/ml glucose and 5.13 mg/ml glucose

4 Discussion

The theoretical water-ice equilibrium temperatures arising from colligative effect for the 1.0, 2.0, and 5.0 mg/ml AFP solutions were estimated to be -0.0006°C , -0.0012°C , and -0.0029°C , respectively. The equation $\Delta T_f = K_f b_B$ was used for the calculations, where $K_f = 1.86\text{ K kg mol}^{-1}$ is the cryoscopic constant of water and b_B is the molarity of the solute [29]. Obviously, the AFP has lowered the bulk melting points of the frozen AFP solutions (Fig. 1) beyond what was predicted by their colligative effects. Thus, the liquid water phases must be in super-cooled states, which are metastable in terms of thermodynamics. Super-cooled liquid water [30] at temperatures below 0°C can be created during a freezing process in which the freezing of water is kinetically delayed due to the lack of seed crystals [31, 32]. It is unusual that super-cooled liquid water could be created during a heating procedure. AFP must have played an unusual functional role to cause the ice to begin to melt at temperatures below 0°C . Now, we have realized that in addition to the already-known function of AFPs to inhibit the growth of seed-ice crystals [6–9, 22, 33] and restrain ice recrystallization [16], type I AFP can also induce the melting of its frozen aqueous solution at a temperature much lower than that predicted by its colligative effect.

It is a challenge to understand the mechanism that has induced the earlier melting of ice in the frozen AFP solutions. In the present experiment, the AFP samples were frozen quickly using liquid nitrogen, which should have resulted in the AFP molecules being frozen in the ice matrices, where the AFP molecules could be embedded in the boundary regions between the polycrystalline ice grains or could be frozen in the ice crystals as structural defects. The distribution of the AFP molecules in the ice matrices should be homogeneous due to the ice-binding property of the AFP and the quick freezing process using liquid nitrogen. We have known that, from our previous NMR relaxation study, water in the ice-AFP interface possessed dynamic orientations and that water in the AFP-water interface was much more mobile, even at quite low temperatures, in the frozen AFP solutions [34–36]. A recent molecular dynamics simulation study indicates that the adsorption of type I AFP can induce the melting of the local ice surface [37]. We will try to use this phenomenon to understand our experimental results. In our NMR microimaging experiment, the initiation of the local melting started from the interface of the glass wall and ice because this is where the heat exchange between the nitrogen gas and the frozen AFP solution occurred. The heat transferred to the frozen AFP solutions supplied kinetic energy for the diffusion of AFP to the newly generated ice surfaces, which could cause further melting of the ice surfaces. Through this process, AFP molecules could have induced the melting of the ice surface layer by layer and left super-cooled liquid water behind. Via this process, kinetic energy is transferred to the system in contrast to the freezing process. The velocities and amounts of ice melted depend on the rate of kinetic energy to be transferred to the frozen solutions. Higher concentrations of AFP solutions would induce the ice melting at lower temperatures because more AFP molecules would induce the ice melting in local areas.

Recall that the thermal hysteresis was defined as the difference between the temperatures at which the micro-sized ice crystal grows and shrinks abruptly in an AFP solution. The freezing point of the AFP solution defined by the thermal hysteresis experiment accurately describes the bulk freezing point of the solution. However, the so-defined melting point is not for the bulk melting of the frozen solution, but for the melting of a micro-sized single ice crystal in the super-cooled solution. What we have observed using the NMR microimaging technique is the bulk melting of the frozen AFP solutions. We were not able to determine if there were small amounts of individual ice crystals on the order of tens of micrometers

remaining in the AFP solutions after the samples appeared to have completely melted. This was due to the limited resolution of the NMR microimaging experiment. If we still adopt the freezing point of the AFP solution defined by the thermal hysteresis experiment, but define the melting point as the temperature at which all the frozen AFP solution appeared to have completely melted, as observed in our NMR microimaging experiments, we find that no significant difference between the freezing point and the melting point could be seen. This is demonstrated by the results of our thermal hysteresis experiment, in which the thermal hystereses are given as a function of AFP concentration, as shown in Fig. 5. In this figure, the thermal hystereses are shown as the AFP antifreeze activity. This result is quite consistent with what has been published earlier by others [4, 23, 38]. Figure 5 shows that the AFP antifreeze activity for the 5.0 mg/ml AFP is 0.56 and that, for the 2.0 mg/ml AFP, it is 0.38 °C, which corresponds to the melting temperatures, as shown in Fig. 1. We conclude that the bulk freezing point and the bulk melting point for the AFP solutions are actually similar. Although the previously introduced concept, “thermal hysteresis,” does describe the antifreeze activity of AFP, the so-defined melting point could be misinterpreted as the bulk melting of the frozen solution. Thus, we suggest using “AFP antifreeze activity” instead of “thermal hysteresis” to describe AFP’s ability to inhibit the growth of seed-ice crystals.

We will now discuss the results of AFP+solute solutions. We think that the reason for the mixed liquid water phases and ice phases across the whole samples during the melting of the frozen solute solutions and the frozen AFP+solute solutions (Figs. 2–4) is due to the much higher local solute concentrations (or eutectic solid phases at much lower temperatures) in the boundary areas surrounded by the polycrystalline ice grains, which caused local colligative melting at much lower temperatures.

In the following, we will discuss the physical meaning of the phenomenon that the frozen AFP+solute solutions melted at higher temperatures than their corresponding solute solutions (Fig. 2 to Fig. 4). 1.0 mg/ml NaCl and 5.0 mg/ml NaCl solutions have theoretical colligative ice-water equilibrium temperatures of -0.06324 °C and -0.3162 °C, respectively, and that for the amino acid solutions and the glucose solution is -0.05208 °C. AFP’s contribution to the bulk colligative effect is insignificant (-0.0012 °C) due to its

Fig. 5 Antifreeze activities of type I AFP versus the AFP concentrations

much lower molarity. Compared with the theoretical water-ice equilibrium temperatures, the frozen solutions began to melt at much lower temperatures. We believe this is due to the strong local colligative effects, starting from the eutectic solute solid phases. Because the amounts of melted ice in the AFP+NaCl samples were less than those in the corresponding NaCl samples at the same temperatures, the concentrations of the salt in the liquid phases of the AFP+NaCl samples should be higher than those in the corresponding NaCl solutions. Ideally, the chemical potentials of water in the NaCl water phases were the same as those of the corresponding ice phases for the NaCl samples at the given temperatures. Therefore, the chemical potentials of water in the AFP+NaCl water phases were lower than those of the corresponding ice phases in the AFP+NaCl samples. In other words, lower temperatures are needed to allow the same amounts of ice in the frozen NaCl samples to melt than in the corresponding AFP+NaCl samples. The same argument is also applicable for the AFP+amino acid and AFP+glucose solutions. Therefore, the theoretical equilibrium temperatures for the coexistences of the ice phases and the water phases in the AFP+solute samples should be lower than the experimental temperatures. This indicates that the ice phases in the AFP+solute solutions were in super-heated states.

We think that the phenomenon of the superheated ice in the NaCl+AFP solutions arose from the much-increased AFP concentrations in the water-AFP-ice (WAI) interfacial regions as compared to the AFP concentrations in the coexisting bulk AFP+NaCl water phases [39, 40]. The type I AFP consists of a large portion of hydrophobic side chains (largely due to the high proportion of Ala residues), which in turn made the WAI interfacial regions more hydrophobic than the liquid water phase. Thus, the Na^+ and Cl^- ions spontaneously disfavored the WAI interfacial regions as compared to the water phases. The total colligative effects of the WAI interfacial regions (due to the total molar concentrations of the AFP and the Na^+ and Cl^- ions) were less than those in the Na^+ and Cl^- ion-rich liquid water phases. The chemical potentials of water increased from the water phase to the WAI interfacial region and then perhaps to the ice phases. As shown in Figs. 3 and 4, the super-heated ices were also observed in the frozen AFP + amino acid and the AFP + sugar solutions. The same explanation can also be applied to these AFP+solute solutions because these solutes are either ionic or hydrophilic. The amine groups and the carboxylate groups of all the amino acids are partially positively ($-\text{NH}_3^+$) and negatively ($-\text{COO}^-$) charged. The Ala and Thr amino acids consist of hydrophobic side chains, the Arg amino acid consists of a partially positively charged side chain, and the Asp amino acid consists of a partially negatively charged side chain. Glucose consists of hydroxyl groups. Although the structures of these solutes are different, they all commonly dissolve in water and favor hydrophilic media. We believe that the bulk concentrations of these solutes in the water phases were higher than those in the WAI interfacial regions. The experimental results in this study indicate that AFP is able to inhibit the melting of ice in conditions in which there are water soluble solutes in the liquid water phase.

To this point, we have seen that type I AFP plays the role of a double-edged sword in the melting of frozen AFP aqueous solutions, depending on whether there are water soluble solutes in the water phase. AFP can lower the bulk melting point of the frozen AFP aqueous solution (Fig. 1) and can also keep the ice phase in a super-heated state or inhibit the melting of the ice (Figs. 2–4) of the frozen AFP+solute solution. Although we have only tested type I AFP, this phenomenon could also be true for other types of AFPs because the common structural feature of all kinds of AFPs found so far is the possession of relatively hydrophobic surfaces that interact with ice surfaces.

Super-heated ice in AFP solutions was first proposed by Knight and DeVries in a study to observe the melting pattern of a hole drilled in a single ice crystal filled with an antifreeze glycopeptide solution [41]. Celik et al. published a paper recently that used a fluorescence microscope and a nanoliter osmometer to observe super-heated ice in hyperactive GFP-*Mp*AFP and other moderately active AFP solutions [24]. The maximum super-heating was observed to be 0.44 °C for the hyperactive AFPs, while that for type I AFP is only 0.011 °C at a very high concentration (12 mM). The difference between their experiment and ours is that single micro-sized ice crystals in the AFP solutions were the center of focus of their experiment, while we observed the bulk melting behaviors of the frozen AFP+solute solutions. The super-heated ice phases observed in our NMR microimaging experiments appeared quite stable at fixed temperatures below 0 °C, while the super-heated ice observed by Celik et al. eventually disappeared over durations of up to ~2 min above 0 °C. The latter appeared to be more kinetically controlled. Owing to the lower accuracy of the NMR microimaging temperature controller and the lower resolution of the present NMR microimaging system, we were unable to determine whether there were super-heated micro-sized ice crystals in the AFP and AFP+solute solutions after the samples were heated above their colligative melting points.

The effects of AFP on the melting of the frozen AFP and AFP+solute solutions observed in this study, together with the already-known functions of AFPs to inhibit the growth of seed-ice crystals and restrain the recrystallization of ice crystals, may play concurrent roles in the survival of organisms that express AFPs in sub-zero winters. In the plant freezing damage studies, it was found that water is drawn away from cells by the formation of extracellular ice crystals and that freezing-induced cellular dehydration is the widespread cause of freezing injuries of plants [32, 42]. In a review of the mechanisms of tissue injury in cryosurgery, it was summarized that an optimized freeze–thaw cycle that can cause tissue injury includes: (1) a fast cooling rate, which can induce intracellular ice crystals and extracellular ice crystals, which cause mechanical damage to the cell membranes; (2) a prolonged freezing time, which increases tissue destruction; (3) as slow as possible thawing rate, which is a prime destructive factor due to the recrystallization of ice crystals; (4) repetition of the freeze–thaw cycle because the cells are subjected to the additional deleterious physicochemical changes via passing again through damaging thermal conditions. It has been shown that AFPs can spare organisms from freezing damage due to their ability to inhibit the growth of seed-ice crystals and to restrain the recrystallization of ice crystals. With regard to the phenomena observed in this study, we think that the earlier melting of the frozen AFP solution would shorten the freezing length of organisms through the wintertime and therefore help to prevent tissue destruction. However, when considering the existence of electrolytes and other water soluble substances in the body fluids of organisms, the circumstance looks different because AFPs could inhibit the melting of ice. How could this help to increase the survival rate? As we have just reviewed above, the repetition of the freeze–thaw cycle can cause more damage to tissues. It is also known that to prevent freezing injury in winter, people should avoid refreezing (i.e., freeze-thaw-freeze processes) as it can lead to “disastrous results,” such as amputation or disfigurement because after thawing, freezing-damaged areas are especially vulnerable to being frozen again. Because we found that the AFP is able to inhibit the thawing of frozen body fluids, the melting of frozen body fluids in early spring can be prevented. The fluctuation of the temperature in this season can otherwise cause more tissue damage due to the cycles of refreezing. Therefore, AFPs may increase the survival rate of AFP-producing organisms over subzero winters by reducing the number of freeze-thaw-freeze cycles.

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