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Dependence of freeze-concentration inhibition on antifreeze protein

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The ability for freeze-concentration inhibition (FCI) was examined for type I antifreeze protein (AFPI) and antifreeze glycoprotein (AFGP) through the observation of the condensation of red-colored ink in an ice block for various concentrations of the proteins. The thermal hysteresis (TH), which indicates the ice growth inhibition strength, and ice-shaping ability were also examined for the two samples. The amount of AFPI and AFGP necessary for FCI was determined to be 0.1 mg/ml and 0.25 mg/ml, respectively. There was no significant difference in the TH of both samples. AFPI and AFGP shaped the ice crystals into hexagonal trapezohedrons and hexagonal bipyramids, respectively, where the former was thinner than the latter. These results suggest that a principle determinant of FCI is the ice-shaping ability, rather than the ice growth inhibition of AFPs.

Key words : antifreeze protein, freeze-concentration inhibition, ice-shaping, thermal hysteresis.

In general, numerous ice nuclei are created in undercooled water at the moment it is frozen (Akyurt et al. 2002). The ice nuclei bind to one another with time to form a multicrystalline state, which is known as the ice recrystallization process. During this process, most substances other than water are excluded from the ice phase because they cannot be incorporated into the network of water molecules forming the ice lattice (Viskanta et al. 1997). The excluded substances are inevitably concentrated into an unfrozen portion, a process that is referred to as the "freeze-concentration phenomenon," and has been one of the problems associated with the quality preservation of any water-containing material such as tissues, foods, and drags (Pegg, 2007). Antifreeze protein (AFP) inhibits the ice recrystallization (Griffith and Ewart, 1995); however, its effect on the freeze concentration is not well understood.

AFP was first discovered as a fish serum protein in 1969 and has been identified from various species of fish, insects, plants, and fungi, which live in ice-laden environments (Fletcher et al. 2001). AFP has been thought to facilitate their cold survival by eliminating the growth of the ice created in their tissues. AFPs have the common function of specifically binding onto a set of water molecules in an ice crystal in order to inhibit its growth (Graether and Sykes, 2004). The typical method for evaluating the strength of ice growth inhibition of an AFP is to measure the nonequilibrium freezing temperature and melting temperature (T_f and T_m, respectively) of an ice crystal prepared in the AFP solution. The difference between the two temperatures is called thermal hysteresis (TH) (Yeh and Feeney, 1996). AFP also shows ice-shaping

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ability; it modifies the ice crystal into a unique shape such as hexagonal bipyramid through adsorption onto the surface of the crystal in the TH temperature range.

Freeze concentration is a macroscopic phenomenon that can be observed in a cup or an industrial tank and thus, an industrial amount of fish AFP is desired for application in this field. However, until recently, it has only been obtained in milligram quantities as natural fish AFP; its scarcity was due to the need to purify the protein from the blood serum of arctic and antarctic fish. A newly developed technique for purifying grams to kilogram quantities of fish AFP, which is based on the discovery that many midlatitude fish also contain AFP in their muscles (Nishimiya et al. 2008), has overcome this problem. At present, the commercial manufacturing of AFP has led to its wide availability and enabled the analysis of its functions on a larger scale.

Here we report on the ability of freeze-concentration inhibition (FCI) for two species of AFP purified from fish muscle, type I AFP (AFPI) and antifreeze glycoprotein (AFGP). A simple device was prepared that allows the observation of the condensation of a red-colored ink in an ice plate in order to evaluate the FCI ability of the samples. Notably, AFPI exhibited FCI at a much lower concentration than AFGP. On the basis of the additional measurements of the ice-shaping ability and TH values, a mechanism for FCI by AFP is proposed.

Materials and methods

The AFPI and AFGP samples came from the Japanese fish *Pleuronectiformes* and *Gadiformes*, respectively, and were provided by Nichirei Foods Inc. (9-Shinminato, Mihama-ku, Chiba-shi, Chiba 261-8545, Japan) and used without further purification. These two AFPs were obtained from the muscle homogenate, not the blood serum, of these fish. The measurement of the FCI ability of the AFPs is described in the Results section. The TH was determined with the accuracy of 0.01°C as described by Takamichi et al. (2007) using an inhouse system with a Leica DMLB 100 photomicroscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a Linkam LK600 temperature controller (Linkam, Surrey, UK). Briefly, a solution of AFP was momentarily frozen by lowering its temperature to −25°C, and then warmed up close to 0 °C on the sample stage in order to create a single ice crystal in the solution. The solution was then cooled down or warmed up slightly, which enabled the observation of the morphological changes of the single crystal (i.e., the ice-shaping ability of AFP) and also the determination of its growth initiation and/or melting temperatures. The two temperatures are defined as the nonequilibrium T_f and T_m, respectively, thus giving the TH value (i.e., TH $= |T_f - T_m|$). All photomicroscope images and movies were recorded using a color video 3CCD camera (Sony, Tokyo, Japan).

Results

A preliminary test for FCI was initially performed using 1 mg/ml solution each of AFPI and AFGP (Fig. 1). Each AFP sample was dissolved in 60 ml of Milli Q water with 20 μl of red-colored ink (Pilot Co. Ltd) in a vessel, which was then stored overnight in a home freezer where the temperature was set to −18°C. As shown in Fig. 1a-c, a condensed ink portion was created in the frozen vessel when the solution contained no protein, suggesting that freeze concentration occurred in the vessel. An observed slight increase in the water volume was attributed to the fact that water uniquely becomes less dense when it freezes. In contrast, the inks were dispersed throughout the entire vessel in the presence of AFPI (Fig. 1d-f), leading to the creation of uniformly red-colored ice in the vessel. Such a colored ice was similarly created when the AFGP sample was used (data not shown). These results indicated that AFPI and AFGP are capable of performing FCI against the red ink in the vessel.

To examine the FCI ability of AFPs more closely, an acrylic box (1×8×10 cm) containing 60 ml of the MilliQ water and 20 μl of red ink (Fig. 2) was used. The box was placed inside a heat insulating material, and then stored in the same freezer to induce one-directional freezing from the top to bottom direction. As expected, a condensed red-ink portion was created in the absence of the AFPs (Fig. 2b), whereas the addition of the proteins prevented the condensation of the inks as a consequence of the FCI ability of AFPs (Fig. 2d). The photographs of the ice block at various protein concentrations were taken, and the brightness of the ice from the top to bottom was traced using the "Image J" software (http://rsbweb.nih.gov/ij/). The

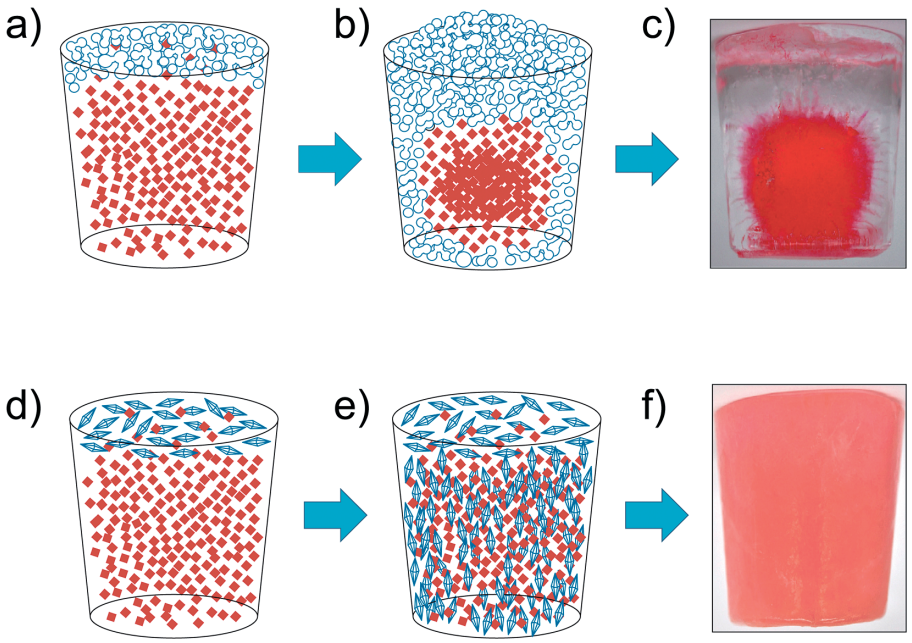

Figure 1：Freeze concentration observed for an ordinary solution (a-c) and freeze-concentration inhibition (FCI) observed for the solutions containing AFPI (d-f). (a) Numerous seed ice crystals are created in the water solution at the moment it is frozen. The freezing is initiated near the top of the solution in this case. (b) Ice crystals bind to one another with time to form a multicrystalline state, which excludes the ink particles and concentrates them into an unfrozen portion of the vessel. (c) A snapshot of the entirely frozen ink solution without an AFP. The ink particles are trapped/concentrated in the middle of the ice block. (d) The seed ice crystals are created near the top of the water, while their growth and mutual binding are inhibited by AFPI. (e) The ice crystals assemble to form a slurry state, which cannot exclude the ink particles, leading to the formation of a frozen mixture of the ink and ice crystals. (f) A snapshot of the entirely frozen ink solution containing AFPI showing a uniformly colored ice block.

tracing was performed for five different positions of the ice, and the averaged value (i.e., condensation) was plotted as a function of the distance between the two ice termini. The curved profile with a peak illustrated in Fig. 2c indicates that freeze concentration occurred at the peak position, whereas the flat profile without a peak shown in Fig. 2d indicates the disappearance of condensation as a consequence of the FCI ability of the AFP.

The condensation of the red-colored ink in the ice block at various concentrations of AFPI and AFGP are plotted in Figs. 2f and 2g, respectively. For both AFP samples, the height of the condensation peak was lowered and eventually became flat with increasing protein concentration. These observations indicate that both AFPI and AFGP possess an ability to cause FCI. The amount of AFPI and AFGP samples necessary for the FCI ability was determined to be 0.1 mg/ml and 0.25 mg/ml, respectively.

The TH value was further examined for the AFPI and AFGP samples as a function of the protein concentration between 0 and 1.2 mg/ml which was found to be the range suitable for the FCI observation. Significantly, nearly identical profiles for the TH with a maximum TH value of approximately 0.1 ℃ were obtained for both protein samples (Fig. 3a). Note that 1 ℃ of TH was typically obtained for purified samples of AFPI and AFGP (Fletcher et al. 2001). These results indicate that there is no significant difference in their strength for ice growth inhibition.

The ice-shaping ability was photomicroscopically observed during the TH measurement. Figures 3b and 3c show the ice crystals created for approximately 0.6 mg/ml of the AFP1 and AFGP samples, respectively. As shown in the figure, a hexagonal trapezohedron ice crystal with two flat basal planes was observed for the AFPI solution (Fig. 3b). In contrast, a hexagonal ice crystal plate with two basal planes was observed for the AFGP solution (Fig. 3c). Importantly, the radius of the ice crystal observed for AFPI (Fig. 3b) was narrower than that for AFGP (Fig. 3c).

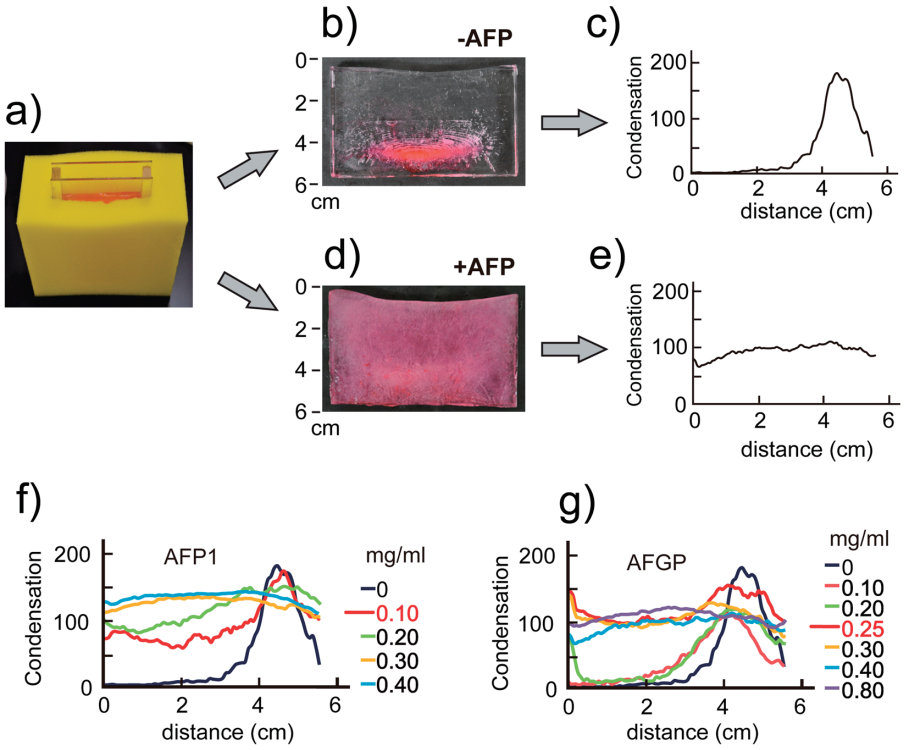

Figure 2：Measurement of the FCI ability of AFPs. (a) An acrylic box (1×8×10 cm) containing the sample solution placed in a heat insulating material. One-directional freezing progresses from the opened top to bottom. (b) An ice block showing the condensed ink portion after overnight freezing of the sample without AFP. (c) The ink condensation plot created by a brightness tracing for (b). (d) An ice block showing a dispersion of the ink in the frozen sample containing AFPI. (e) The ink condensation plot created by a brightness tracing for (d). (f) Ink condensation profiles examined for 0–0.40 mg/ml solutions of AFPI. (g) Ink condensation profiles examined for 0–0.80 mg/ml solutions of AFGP.

Discussion

The ability for FCI of a red-colored ink was examined for the two AFP samples, AFPI and AFGP, which were mass purified from the muscle homogenate of midlatitude fish. The particles of red-colored ink were concentrated in a vessel during freezing with the AFPs, whereas this concentration was prevented by the addition of the AFPs. A proposed mechanism for each result is illustrated in Fig. 1. When a water solution without AFP was frozen at −18°C, the seed ice crystals, or ice nuclei, underwent recrystallization to form a multicrystalline state. The water freezing progressed from the outer surface to the inner center of the vessel (Fig. 1a). The red ink, whose molecular structure is different from that of water, was barely incorporated into the ice lattice, and thus could not participate in the ice recrystallization process. As a result, the ink was excluded from the ice phase and concentrated in the unfrozen center portion of the vessel (Fig. 1b-c).

When AFPI was dissolved in the water at a concentration of 1 mg/ml, it accumulated on the surface of the seed ice crystals. Because the surfaces of the ice crystals were covered with AFPs, they could barely bind to one another. Consequently, the seed ice crystals assembled to form a slurry state but not an ice block (Fig. 1d-e). Note that the ice crystal slurry became solid/immobile as the temperature decreased. Each ice crystal was assumed to form a bipyramid, or a derivative morphology, according to the type of accumulated AFP and several experimental parameters such as pH, viscosity, ionic strength, and pressure (Griffith and Ewart, 1995). The ice slurry could not exert the power to exclude the ink particles and thus, a frozen ice-ink mixture was formed in the vessel (Fig. 1e-f).

To examine the FCI ability of the AFPs more closely, a simple device was prepared to monitor the freeze concentration during one-directional freezing (Fig. 2). It provided two different ink condensation profiles along with the freezing direction. One profile contained a peak corresponding to the condensed ink portion arising from the freeze concentration (Fig.

2c), whereas the other showed a flat profile without a peak, implying that the AFPs exerted an FCI ability (Fig. 2e). Significantly, only 0.1 mg/ml of AFPI was sufficient; however, 0.25 mg/ml of AFGP was necessary to inhibit the freeze concentration. These results imply that the FCI ability of AFPI is 2.5-fold higher than that of AFGP. It can be assumed that this difference is due to a difference in the strength of the ice growth inhibition, or the TH activity, of AFPI and AFGP. However, this idea lacks support because both AFPs exhibited nearly identical TH values (Fig. 3a).

AFPI shaped the ice crystal into a hexagonal trapezohedron (Fig. 3b); whereas AFGP created a hexagonal bipyramid, although the two tip portions were truncated and became flat hexagonal surfaces (Fig. 3c). These observations are consistent with previous observations (Evans and Fletcher, 2001; Hachisu et al. 2009). Interestingly, the radius (r) of the bipyramidal crystal of Fig. 3b was shorter than that of Fig. 3c; the former was thinner than the latter. It has been shown that the spaces between growth-terminated ice crystals formed in the presence of an AFP can trap a solute such as NaCl (Lin et al. 1976). On the basis of these observations, it was hypothesized that a larger space was created between the grain boundaries of the thinner ice crystals (Fig. 4a) compared with the thicker ones (Fig. 4b). That is, AFPI was assumed to create a slurry composed of thinner ice crystals in order to provide a larger space for the ink particles, leading to easier formation of the ice-ink mixture in the frozen state. However, the thicker ice crystals created by AFGP allow less space between the grain boundaries, and freeze concentration is barely prevented. The radius of the ice bipyramid presumably decreases with increasing AFP concentration. Hence, we assumed that the effective AFPI exhibited FCI with a quantity less than that required for AFGP. While more experiments are necessary to confirm this conclusion, our assumption is consistent with the previous indications for the AFPs discovered from plants. They have commonly exhibited a very small TH value, yet effectively caused recrystallization inhibition with small ice crystals (Griffith and Evart, 1995).

Currently, freeze-concentration inhibition (FCI) of water-containing material is achieved using cryotechniques such as −80°C deep freezing or the use of −196°C liquid nitrogen because quick/flash freezing utilizing such a low temperature prevents freeze concentration. However, this technique requires high energy consumption. AFP does not use high energy to perform FCI; therefore, it is an ideal substance for the quality preservation of many water-containing materials.

In summary, the FCI ability, TH, and ice-shaping behavior of AFPI and AFGP were examined. AFPI exhibited FCI with a quantity less than AFGP. The

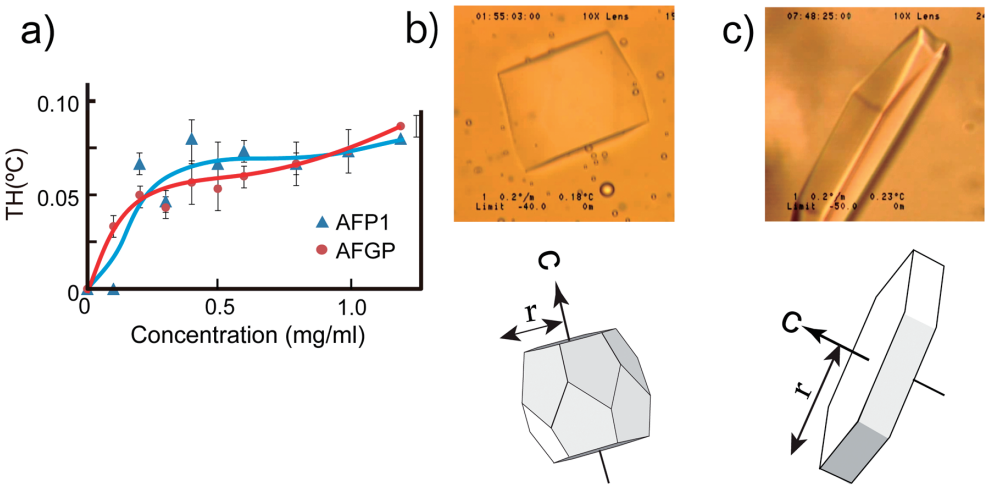

Figure 3：Measurement of the TH and ice-shaping ability of the AFPs. (a) TH values plotted as a function of the concentration of AFPI (triangles) and AFGP (circles). For clarity, a smooth curve is drawn to fit each set of data. (b) Photomicrograph and illustration of an ice crystal created in a 0.6 mg/ml AFPI solution, showing a hexagonal trapezohedron shaped crystal with two flat basal planes. (c) Photomicrograph and illustration of an ice crystal created in a 0.6 mg/ml AFGP solution. This hexagonal plate is one of the derivatives of the hexagonal bipyramid as described in Griffith & Ewart (1995). The radius (r) in (b) is shorter than that in (c).

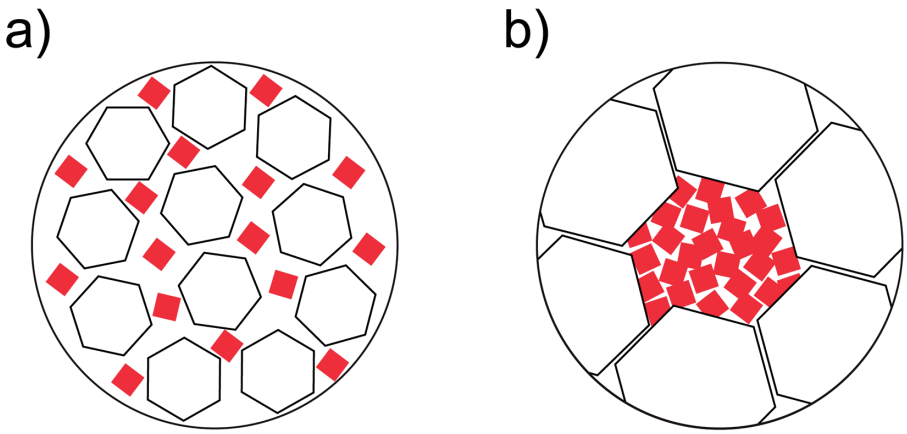

Figure 4：A proposed mechanism for the FCI ability of the AFPs. (a) Illustration showing the spaces created between the growth-terminated ice crystals formed in the presence of AFPI, which should be capable of trapping the solute. AFPI creates thin ice crystals; thus, a smaller amount might be able to exhibit the FCI ability. (b) Illustration showing the creation of smaller spaces between the thick ice crystals, which tends to cause freeze concentration against the solute. Therefore, a larger amount of AFGP might be required to make the ice crystals smaller to provide the FCI ability.

two proteins showed no significant difference in the strength of ice growth inhibition; however, the ice crystal shaped with AFPI was thinner than that with AFGP. These results suggest that the FCI efficiency is determined by the ice-shaping ability of AFPs, rather than their ice growth inhibition.

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