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Effects of preheating on ice growth in antifreeze polypeptides solutions in a narrow space

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Abstract. We conducted measurements on the unidirectional freezing of aqueous solutions of polypeptide or of winter flounder antifreeze protein. The polypeptide was based on a part of the antifreeze protein. We measured temperatures in the solutions and ice with a small thermocouple, and defined the interface temperature as the temperature at the tip of the serrated or pectinate interface. It was found that the interface temperature of these solutions was lower than that of pure water. To vary the activity of these solutes, we preheated the solutions and cooled them before conducting the measurements. We found that preheating for several hours caused further decreases in the interface temperature and a decrease in the interface velocity. In addition, the inclined interfaces became wider as a result of the preheating. Thus, the supercooled states in the solutions were enhanced by the preheating. To investigate the reasons for these changes, we measured the aggregates of the solutes in the solutions. These aggregates became larger as a result of preheating. It can therefore be concluded that these large aggregates attenuated the ice growth by their interaction with the ice surfaces.

1. Introduction
The control of ice crystal growth is an important issue in various fields, such as food preservation [1, 2]; the storage of cells, tissues and organs in medical science [1, 3]; cryosurgery [4]; and the control of the ice packing factor of ice slurry to be used in air conditioning systems [5]. One of the most promising methods of controlling the growth of ice crystals is to use additives which change the freezing point and interface shapes. Antifreeze protein (AFP) and antifreeze glycoprotein (AFGP) are appropriate additives [1–3, 5] for this purpose. This is because AFP and AFGP have the following advantages: (1) they decrease the freezing point while retaining the melting point in a thermal quasi-equilibrium condition and (2) they do not increase the osmotic pressure significantly. Note that these freezing and melting points were defined to be respectively the temperatures at which a small seed crystal started to grow and shrink in the solution in an osmometer [6].

When using AF(G)P, the following three points have to be considered: (1) the cooling rate or the rate of ice growth, (2) thermal denaturation, and (3) the enhancement of supercooled states. Concerning point (1), the absolute value of the cooling rate \( K \) and the rate of ice crystal growth \( R \) in the osmometers (e.g. \( K = 0.074 \) °C/min and \( R = 0.2 \) μm/s [6]) are much lower than those in cryosurgery \( K = 10 \) °C/min and \( R \approx 30–40 \) μm/s [4]). To understand the effects of these rates on the ice-crystal shape and temperature, results for unidirectional freezing experiments, in which these rates are much higher than...
those for the osmometers, are required. Thus, many experiments on unidirectional freezing have been carried out [7–11]. From these results, in the case of AF(G)P solutions, it was found from the results of these experiments that the decrease in the temperature at the most advanced point of the serrated interface was consistent with the freezing point depression in the case of osmometers, although the interface temperature depended on the interface velocity.

Concerning point (2) above, two types of thermal denaturation for the specific AFPs have been reported [12, 13]: reversible and irreversible. The reversible denaturation occurred when the AFP solutions were warmed gradually to a temperature in the range 10–50 °C. The helix content, which demonstrates the secondary structure of the AFPs, disappeared partially as a result of this warming. The helix content recovered, though, when the AFP solutions were cooled back down to 0 °C or 4 °C. In contrast, the irreversible denaturation occurred when the solution hyperactive AFP (molecular weight: 17 kDa), extracted from the plasma of winter flounder, was gradually heated up to 55 °C [12]. In this case, the helix content never recovered. Also, the irreversible denaturation occurred when the solutions of snailfish AFPs (molecular weight: 9.3–9.6 kDa) were gradually heated up to 70 °C [13]. It can be surmised from these results that the lower the molecular weight of AFP is, the higher the critical temperature of irreversible thermal denaturation is. Thus, low-molecular-weight AFPs or alternatives are effective for reducing the thermal denaturation. The denaturation may be predicted with a protein model [14]. Recently, Kun and Mastai [15] synthesized three polypeptides (molecular weight: 1.0–1.1 kDa) based on parts of winter flounder AFP (HPLC6, molecular weight: 3.24 kDa). Using an osmometer, they measured the non-colligative freezing point depression for a solution of one of these polypeptides. The present authors expect that denaturation of the polypeptides does not occur because the short helical structure of the polypeptide is maintained with strong hydrophobic interaction and hydrogen bonds.

Regarding point (3) above, the enhancement of supercooled states has not been discussed in detail except as a cooperative effect for the mixed solutions of AF(G)P and salts in the quasi-equilibrium condition [16, 17]. In Ref. [16], the freezing point depression for the mixed solution had the following three components: (i) a colligative portion produced by the salts; (ii) a portion produced by AF(G)P alone; and (iii) a synergetic portion produced by the interaction of the salts and AF(G)P. However, it is not always possible to control the salt concentration in the aforementioned applications. Thus, other stimuli or stresses on the solution of AFP or polypeptides derived from AFP are required to enhance the supercooled states.

In the present study, we experimentally investigate the following two factors for this polypeptide: the ice morphology and interface temperature in the unidirectional freezing of the solutions of HPLC6 (the major fraction of the winter flounder AFP) and the polypeptide (related to point (1) mentioned in the second paragraph of this section), and secondly whether the morphology and temperature change as a result of preheating these solutions (related to points (2) and (3)).

2. Apparatus

The apparatus was the same as that in our previous study [18]. It consisted of an inverted microscope (Nikon C2+), a monochrome CCD camera, a digital multi-meter (Yokogawa 7561) and a pulse generator. The light source was a halogen lamp. The apparatus was set up in a temperature-controlled room maintained at 8 °C.

Figure 1 shows the details of the cooling section mounted on the bench of the microscope. A dilute aqueous solution of HPLC6 or polypeptide was introduced into a narrow space of 11 mm$^3$ (25 mm × 22 mm × 0.02 mm) between two parallel cover glasses. The gap of 0.02 mm between the cover glasses was created by using a screen printed on the lower side of the upper cover glass. The screen controls the growth direction of the ice crystal. The lower cover glass was in contact with the edge of the copper plate. This plate was cooled by a Peltier device with a coolant flowing through the device. The cooling
rate of the device was controlled with a controller (Sensor Control Inc., FC3510). To obtain a low temperature gradient, we operated the controller of the Peltier device at its lowest temperature-drop rate of 1.0 °C/min. In addition, ice formed naturally from the cooler edge of the solution layer without any seed crystals.

3. Solutions

3.1. HPLC6
The primary structure and molecular weight of HPLC6 are shown in Table 1. The secondary structure of HPLC6 is an α-helix. We purchased synthetic HPLC6 from Life Technologies Corporation (Carlsbad CA, USA). The concentration of the HPLC6 solution was 1mg/mL.

3.2. Polypeptide
The primary structure and molecular weight of the polypeptide used in this study are also shown in Table 1. This primary structure is the same as a part of the primary structure of HPLC6. A helical secondary structure is expected from the discussion in Ref. [15]. We purchased the synthetic polypeptide from GenScript Inc. (Taito, Tokyo, Japan). The concentration of the polypeptide solution was the same as that for the HPLC6 solution.

3.3. Preheating of solutions
The solutions of HPLC6 and the polypeptide were preheated for a predetermined period of time and cooled in the temperature-controlled room before the measurements were taken. In the case of the experiments on unidirectional freezing, a plastic bottle containing the sample liquid (0.5 mL) was installed in a thermostatic bath (Yamato Scientific, BB301) in the preheating procedure. The temperature of the bath was set to 40, 60 and 80 °C. The concentration of preheated HPLC6 solution was the same as that for the unheated solution.

In the other experiments described in Sections 4.3–4.4, the sample liquid (0.1-0.5 mL) was preheated using a copper bath. The temperature and duration of preheating were the same as those for the experiments on unidirectional freezing.

![Figure 1](image_url). Details of cooling section. The solutions were stored in a space of 25 mm × 22 mm × 0.02 mm between parallel cover glasses.

| Material          | Primary structure                        | Molecular weight |
|-------------------|------------------------------------------|------------------|
| Winter flounder AFP | DTASDAAAALTAANAKAAELTAANAAAATAR          | 3243 Da          |
| Polypeptide       | DTASDAAAAL                                | 1046 Da          |

D: Aspartate, T: Threonine, A: Alanine, L: Leucine, N: Asparagine, K: Lysine, E: Glutamic acid, R: Arginine
Table 2. Image-capturing conditions.

| Magnification    | × 20  | Frame rate [frame/s] | 1 |
|------------------|-------|----------------------|---|
| Area size [µm²]  | 391.8 × 298.5 | Exposure time [s] | auto |
| Pixel numbers    | 336 × 256 | Depth [bit] | 8 |
| Pixel resolution [µm²] | 1.17 × 1.17 | Binning | 4 |

4. Measurement methods

We measured the configuration, velocity and temperature of interfaces. The measurement methods were the same as those used in our previous studies [11, 18]. We also measured the absorbance of ultraviolet light and dynamic light scattering for the preheated solutions.

4.1. Interface velocity

The movement of the interface was unidirectional with a margin of approximately 20° regardless of the solute and its concentration. The interface configuration did not remarkably change during the image-capturing period. Thus, the interface velocity was defined as the distance between the tip of the convex part of an irregular interface in one image and that in another image, divided by the time interval between the two captured images. The margin of error for the interface velocity was 1.7%. The image-capturing conditions are shown in Table 2.

4.2. Interface temperature

We used a K-type thermocouple (ANBE SMT Co. Japan, KFT-13) to measure the local temperature. The dimensions of the junction were approximately 60 μm × 40 μm × 10 μm. The thermocouple was inserted into the space between the cover glasses before the space was filled with liquid. The thermocouple was connected to the multi-meter, and the output from the multi-meter was recorded on a PC. The margin of error was 0.075 °C.

The interface temperature was defined as the measured temperature when the tip of the convex part of an irregular interface reached the center of the thermocouple junction. Rather than the interface temperature ($T_i$), we focused on the temperature difference $\Delta T = T_i - T_0$, where $T_0$ is the average interface temperature for pure water. Hereafter, this difference is called the decrease in the temperature at the interface.

4.3. Absorbance of ultraviolet and visible light

The absorbance of transmitted ultraviolet and visible light through the preheated solutions was measured using a spectrophotometer (Shimadzu, UV1650PC). The sample solution was stored in a quartz cuvette and the intensity of the light passing through the cuvette was measured with the detector. The absorbance $A(\lambda, T)$ is expressed as follows:

$$A(\lambda, T) = -\log_{10}[I_t(\lambda, T)/I_0(\lambda, T)],$$

where $\lambda$ is the wavelength of light, $T$ is the temperature, $I_t$ is the intensity of transmitted light, and $I_0$ is the intensity of incident light. The temperature was maintained at 20 °C throughout the absorbance measurement.

4.4. Dynamic light scattering

We carried out measurements of dynamic light scattering for the HPLC6 and polypeptide solutions using a particle size analyzer (Otsuka Electronics Co., Ltd., Japan, ELSZ-1000) to measure the size of aggregates in the solutions.
5. Results and discussion

5.1. Interface morphology

Figure 2 shows typical snapshots of the ice/liquid interfaces. The interface was nearly flat and perpendicular to the ice growth direction in the case of pure water (Figure 2(a)). The interface of the non-preheated HPLC6 solution, on the other hand, had a serrated shape (Figure 2(b)). The interface with a finite thickness is seen near the tip of the interface (see the local interface indicated with arrows). This shows the inclined interface to the Z axis.

For the preheated solutions of HPLC6, bigger ice crystals were sometimes observed (see Figures 2(c) – 2(e)). The liquid regions between the ice crystals were wider than those in the case of the non-preheated solution. On the other hand, thicker interfaces were observed in wider regions compared with the
interfaces for non-preheated solutions. These facts suggest that protein/ice interaction is more localized in specific regions and stronger in these regions than those in the case of the non-preheated solutions.

Pectinate shapes were observed at the interfaces of the ice/polypeptide solutions, regardless of the preheating time and concentration. The pitch of the teeth of the pectinate shapes in the lateral direction was shorter than that of the serrated shapes in the case of HPLC solutions. In addition, the liquid regions between the ice crystals were narrower than those in the case of HPLC6 solution. These facts indicate that the interaction of polypeptide with ice surfaces is weaker than that of HPLC6 with ice surfaces.

5.2. Interface velocity

Table 3 shows the interface velocity. The average interface velocity for the non-preheated HPLC6 solution is lower than that for pure water and for the polypeptide solution with the same concentration. It should be noted that the average interface velocities for the preheated HPLC6 solutions at 80 °C are nearly equal to or lower than that for the non-preheated HPLC6 solutions or the preheated polypeptide solutions. Thus, the preheating is effective for the inhibition of unidirectional ice growth in the AFP solution.

Table 3. Interface velocity [μm/s].

| Without preheating | Preheating |
|--------------------|------------|
|                     | Water HPLC6 | Polypeptide | HPLC6 40 °C/1 h | Polypeptide 80 °C/1 h | HPLC6 80 °C/3 h | Polypeptide 80 °C/5 h |
|                    | 12.6 (σ = 2.6) | 9.9 (σ = 2.4) | 8.0 (σ = 2.2) | 7.2 (σ = 2.9) | 6.8 (σ = 2.6) | 6.3 (σ = 1.4) | 7.0 (σ = 1.9) | 8.1 (σ = 2.8) |

The values in the brackets show the standard deviation.

Table 4. Decrease in the temperature at the tip of the ice/solution interface relative to the temperature at the ice/water interface [°C].

| Without preheating | Preheating |
|--------------------|------------|
|                     | HPLC6 Polypeptide | HPLC6 40 °C/1 h | Polypeptide 80 °C/1 h | HPLC6 80 °C/3 h | Polypeptide 80 °C/5 h |
|                    | -0.088 (σ = 0.037) | -0.054 (σ = 0.028) | -0.253 (σ = 0.061) | -0.219 (σ = 0.058) | -0.369 (σ = 0.101) | -0.085 (σ = 0.055) | -0.188 (σ = 0.074) | -0.169 (σ = 0.110) |

The values in the brackets show the standard deviation.

Figure 3. Effect of preheating period on the absorbance of ultraviolet and visible light; (a) preheated at 80 °C and (b) preheated at 40 °C.
Table 5. Average dimension of aggregates [nm].

| Without preheating | Preheating |
|--------------------|------------|
| HPLC6 Polypeptide  | HPLC6 Polypeptide |
| 40 °C/1 h          | 80 °C/1 h     |
| 64                       | 499                     |
| 80 °C/3 h          | 80 °C/1 h     |
| 618                     | 523                     |

5.3. Decreases of temperature at the interface
Table 4 shows the decrease of the temperature at the tip of the ice/solution interface relative to the temperature at the ice/water interface. It can be seen from this table that the decreases of the temperature at the interfaces became significant as a result of the preheating. In particular, one-hour preheating for the HPLC6 solution caused significant changes in the amount of decrease. Thus, the one-hour preheating for the HPLC6 solution is effective for maintaining the supercooled state of the solution. This maintenance of supercooling can produce less-strict controls for the temperature of refrigerators, and thus contribute significantly to the energy saving of the refrigerators. In addition, further inhibition of ice growth is expected by higher concentration of HPLC6 in the one-hour preheated solution.

5.4. Absorbance of light
Figure 3 shows the profiles of absorbance for the HPLC6 solutions as a function of wavelength in the range 230 – 320 nm. The absorbance is highest at the lowest wavelength, and noticeably decreases for all the solutions with an increasing wavelength. The longer the preheating time, the higher the absorbance in the low wavelength range is, particularly for the preheating at 80 °C. This shows that preheating has caused a change in the solute and that ultraviolet light in this wavelength range is more scattered by the modified solute. The generation of aggregates is most probably a modification of the HPLC6 as a result of preheating. Thus, the increase in the absorbance of ultraviolet light is caused by the enhancement of aggregate formation by preheating.

5.5. Dimension of aggregates
Table 5 shows the average diameters of aggregates obtained with the Dynamic light scattering measurement. It is clearly seen that the average diameters of aggregates of HPLC6 and the polypeptide in the case of one-hour preheating are larger than those for non-preheating. The average volumes of aggregates for the preheated solutions were approximately 3.2 times and 16 times larger than those for the non-preheated solutions of HPLC6 and the polypeptide, respectively. Thus, preheating enhanced the formation of large aggregates. The large-scale inclined interfaces seen in Figures 2(c) -2(f) are possibly due to the interaction between many large aggregates and the ice surfaces.

6. Conclusions
We have carried out experiments on the gradual unidirectional freezing of dilute solutions of winter flounder antifreeze protein and of a polypeptide, based on the protein, in a narrow gap between two cover glasses. In addition, we measured the absorbance of ultraviolet light and dynamic light scattering of the solutions with preheating and without it. The main conclusions are as follows:

(1) The interface velocity was decreased by the preheating of the protein solution and the polypeptide solution at 80 °C for several hours. The decrease of the temperature at the interface for these solutions also became significant as a result of the preheating for one hour. Thus, the supercooled state of the protein solution and the polypeptide solution was enhanced by the appropriate preheating.

(2) The aggregates of the protein and the polypeptide became large as a result of one-hour preheating. The large aggregates possibly contributed to the formation and maintenance of the wide regions of solutions and the inclined interfaces.
The interaction of these large aggregates with the ice/preheated-solution interface can be the main reason for the ice growth inhibition and the enhancement of the supercooled states of the solutions.

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References
[1] Fletcher G L, Goddard S V and Wu Y 1999 Antifreeze proteins and their genes: From basic research to business opportunity CHEMTECH 29 17.
[2] Li B and Sun D-W 2001 Novel methods for rapid freezing and thawing of foods – a review J. Food Eng. 54 175.
[3] Amir G et al. 2005 Improved viability and reduced apoptosis in sub-zero 21-hour preservation of transplanted rat hearts using anti-freeze proteins J. Heart and Lung Transplantation 24 1915.
[4] Shitzer A 2011 Cryosurgery: Analysis and experimentation of cryoprobes in phase changing media J. Heat Transfer, 133 011005.
[5] Inada T and Modak P R 2006 Growth control of ice crystals by poly(vinyl alcohol) and antifreeze protein in ice slurries Chemical Eng. Science 61 3149.
[6] Chao H, Houston Jr. M E, Hodges R S, Kay C M, Sykes B D, Loewen M C, Davies P L and Sönßichsen F D 1997 A diminished role for hydrogen bonds in antifreeze protein binding to ice Biochemistry 36 14652.
[7] Coger R, Rubinsky B and Fletcher G L 1994 Microscopic pattern of ice crystal growth in the presence of thermal hysteresis proteins J. Offshore Mechanics and Arctic Eng. 116 173.
[8] Furukawa Y, Inohara N and Yokoyama E 2005 Growth patterns and interfacial kinetic supercooling at ice/water interfaces at which anti-freeze glycoprotein molecules are adsorbed J. Crystal Growth 275 167.
[9] Butler M F 2002 Freeze concentration of solutes at the ice/solution interface studied by optical interferometry Crystal Growth and Design 2 541.
[10] Hagiwara Y and Yamamoto D 2012 Temperature distribution and local heat flux in the unidirectional freezing of antifreeze-protein solution Int. J. Heat Mass Transfer 55 2384.
[11] Hagiwara Y and Aomatsu H 2015 Supercooling enhancement by adding antifreeze protein and ions to water in a narrow space Int. J. Heat Mass Transfer 86 55.
[12] Marshall C B, Chakrabarty A and Davies P L 2005 Hyperactive antifreeze protein from winter flounder is a very long rod-like dimer of α-helices J. Biological Chemistry 280 17920.
[13] Evans R P and Fletcher G L 2001 Isolation and characterization of type I antifreeze proteins from Atlantic snailfish (Liparis atlanticus) and dusky snailfish (Liparis gibbus), Biochimica et Biophysica Acta 1547 235.
[14] Banach M, Prymula K, Jurkowski W, Konieczny L and Roterman I 2012 Fuzzy oil drop model to interpret the structure of antifreeze proteins and their mutants J Mol. Model. 18 229.
[15] Kun H and Mastai Y 2007 Activity of short segments of type I antifreeze protein Peptide Science 88 807.
[16] Evans R P, Hobbs R S, Goddard S V and Fletcher G L 2007 The importance of dissolved salts to the in vivo efficacy of antifreeze proteins Comp. Biochemistry and Physiology Part A 148 556.
[17] Kristiansen E, Pedersen S A and Zacharaisen K E 2008 Salt-induced enhancement of antifreeze protein activity: A salting-out effect Cryobiology 57 122.
[18] Nishi N, Waku T, Tanaka N and Hagiwara Y 2016 Ice growth inhibition in antifreeze polypeptide solution by short-time solution preheating under review.