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Characterizations of Functions of Biological Materials Having Controlling-Ability Against Ice Crystal Growth

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<http://dx.doi.org/10.5772/54535>

1. Introduction

Subzero winter temperatures pose a significant challenge to the survival of organisms in temperate and polar regions. Many organisms living in these areas have evolved a number of strategies for surviving in extreme environments such as subzero temperature [1-5]. Some strategies in a given organism use a mechanism based on freezing point depression through accumulation of cryoprotectants such as sugars and polyhydric alcohol [6]. Other strategies use a mechanism based in physical damage avoidance through production of antifreeze material and ice nucleators [7-9]. Overwintering strategies based in freeze tolerance and freeze avoidance play an important role in adaptation promoting cold hardiness. Freeze-tolerant organisms survive the formation of extracellular ice but typically do not survive intracellular freezing [3]. In contrast, freeze-avoiding organisms must avoid freezing or death will result. These two alternative overwintering strategies share many of the same physiological adaptations, such as the accumulation of polyhydric alcohols, antifreeze protein and/or glycoprotein during cold acclimation [3, 10].

In subzero conditions, all organisms are exposed to conditions that necessitate the partial removal of water from the intracellular space in order to maintain the structure and function of the cell. Any significant deviation in the accessibility of water due to dehydration, desiccation or alteration of water's physical state, that is, from the aqueous phase to an ice crystal, will pose a severe threat to the normal function and survival of an organism [11]. Some bacteria among various organisms can counteract or minimize the deleterious effect of ice crystal formation in the intracellular and extracellular spaces [12]. As shown in Figure 1 [12], ice crystal- controlling proteins and other materials were related to the phenomenon of three steps in the formation and growth of ice. Ice nuclei can be formed by homogeneous (no particle present) or heterogeneous (particle-induced) nucleation in the first step. The formation

of ice nuclei through heterogeneous ice nucleation is promoted by foreign particles that act as ice nucleation activator. Various types of ice nucleation activators of biogenic origin are known to exist in plant bacteria, fungi, insects, plants and lichens. Inhibitors of heterogeneous ice nucleation, which can favour supercooling, have been found in various organisms. These inhibitors can minimize the threats of intra- and extracellular ice formation. These inhibitors are known to exist in the xylem parenchyma cells of Katsura trees (*Cercidiphyllum japonicum*) [13]. Other ice crystal-controlling materials, which can play a crucial role in the second step of ice formation, are antifreeze proteins, antifreeze glycoproteins and antifreeze glycolipids. The function of AFP is to inhibit ice formation and ice crystal growth by suppressing the binding of water molecules to the ice crystal surface [14].

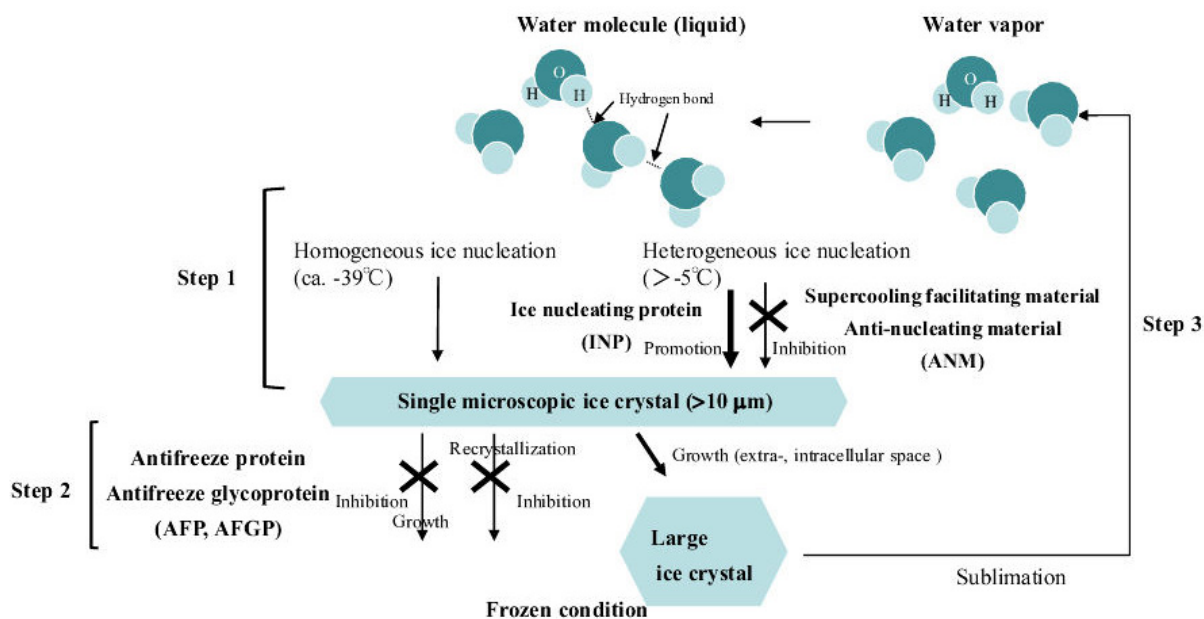


Figure 1. The representative functions on various ice crystal-controlling materials

In this chapter, we pay particular attention to the steps of ice crystal formation and growth along with the biogenic ice crystal- controlling materials. Among biogenic ice crystal-controlling materials, ice nucleation protein having the ability to promote ice nuclei formation, supercooling-facilitating materials having the ability to inhibit ice nucleation, and antifreeze materials having the ability to inhibit ice crystal growth and ice recrystallization are each explained as their structures, functions, and applications. Also, we mention the assay systems for each activity to seek these materials from various organisms and food wastes.

2. The mechanism of ice crystal formation

When pure liquid water is cooled at atmospheric pressure, it does not freeze spontaneously at 0°C. Due to density fluctuations in liquid water, water molecules form clusters that have the same water molecular arrangement (Figure 2) as ice crystals but remain in a liquid state

due to the fluctuation of energy. This state is called supercooling. A drop of pure water without perfectly foreign particles can display a supercooling temperature or freezing temperature at -39°C [15]. This process has been called 'homogeneous ice nucleation' (Figure 1, Step 1). However, impurities or foreign particles present in water can attach water molecules onto their surfaces. As water molecules may be oriented in a way such as to resemble an ice nucleus, these become compatible with the critical dimension of ice nucleation. Franks reported that the deciding factors for the formation of ice nuclei by materials included the following three conditions: similarity to the crystal lattice, paucity of surface charge, and high hydrophobicity of the ice nuclei [16]. This process is called 'heterogeneous ice nucleation', and occurred at a temperature between -2°C and -15°C . The formed ice crystal nuclei may become 'ice crystals' by starting crystal growth (Figure 1, Step 2). This type of ice crystal growth exhibits three different mechanisms [17]. The first mechanism of ice crystal growth is growth from a perfect crystal side, and the growth rate at the interface of an ice crystal serves as the controlled surface nucleation rate. The second mechanism of ice crystal growth is growth by screw dislocation. The ice crystal growth rate is related to the degree of interface supercooling. The third mechanism of ice crystal growth is called continuous growth with large driving energy of crystal growth. In this case, the nucleation obstacle, which should be overcome in the case of crystal growth, does not exist, but the crystal growth rate is proportional to the degree of interface supercooling. This growth rate is affected by freezing temperatures. As shown in Figure 3, the maximum ice crystal generation temperature region is from 0°C to -7°C . This temperature region is important for ice crystal structure formation. When the time to pass through this temperature region is short, a detailed ice crystal is formed, and when the time is long, a large and rough ice crystal is formed. Difference in the shape of this formed ice crystal could affect the nature of the physical damage to the cells and organs during freezing. The differences in how quickly this temperature region is passed through influences the survival rate of cells and organisms after freezing and thawing. In the case of this passage time with one of late and slow freezing in the realm of nature, all organisms have acquired high freezing tolerance through production of various ice crystal-controlling materials.

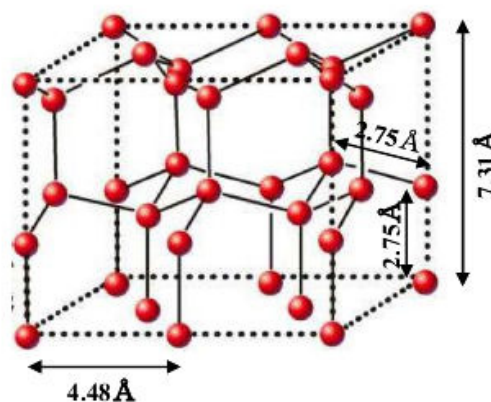


Figure 2. The structure of Ice crystal Ih.

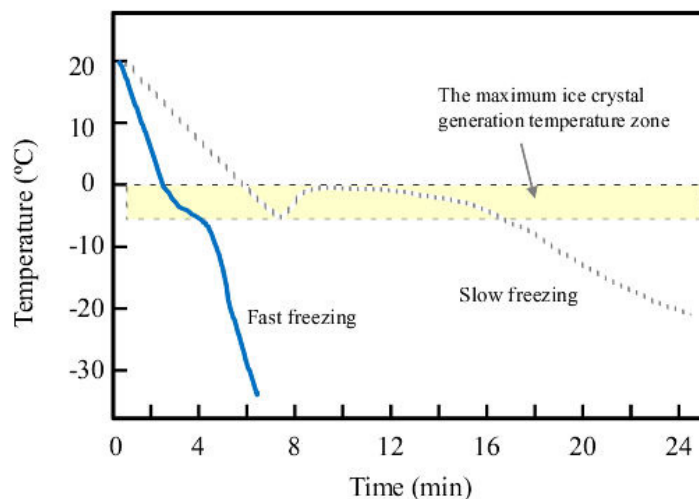


Figure 3. The maximum ice crystal generation temperature zone.

3. Structure and function of ice nucleation proteins from various organisms

As shown in Figure 1 Step1, the process called heterogeneous ice nucleation always occurs at a temperature higher than homogeneous ice nucleation. Ice nucleation proteins (INP) are integral components of various types of ice nucleation activators (INA) of biogenic origin. INAs are present in a variety of plant bacteria [18], insects [19], intertidal invertebrates [10], plants [20], and lichen [21-23]. The INA found in a species of frost-resistant frog, *Rana sylvatica*, has also been shown to be composed of proteins [24]. This protein was present in *Rana sylvatica* plasma collected in the autumn and spring.

Various Gram-negative epiphytic bacteria, which have been called ice-nucleating bacteria, have been known to produce INA at temperatures higher than -3°C . These bacteria belong to genera *Pseudomonas*, *Erwinia*, *Pantoea* and *Xanthomonas*. Six species of ice-nucleating bacteria have been found and various INPs from these bacteria have been analyzed to determine their amino acid sequences [25-30]. Also, some strains of *Fusarium acuminatum* and *F. avenaceum* are active in ice nucleation at a temperature of -2.5°C [31]. These substances have different properties when compared to those of bacterial and fungal INPs. These differences might be caused by the different components of each ice nucleation material which contain ice nucleation protein as the active center. Extracellular ice-nucleating material secreted into the culture broth and localized on the surface of cell wall was found to be composed of lipid, protein, saccharide, and polyamine as the minor component [32, 33]. This localization was caused by the formation of large homoaggregates on the surface of the outer membrane. Genes conferring ice-nucleating activity have encoded INPs (120- 150 kDa) with similar primary structures. All INPs are composed of a highly repetitive central domain flanked by nonrepetitive N- and C-terminal domains (Figure 4). The tandem consensus octapeptide,

Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr, of the central domain is hypothesized to form a β -helical fold secondary structure. This structure can bind water molecules in a configuration similar to an ice lattice [34]. This β -helical fold's secondary structure plays an important role in structures resembling ice lattices. Furthermore, the conserved glycine residues involved in chain bending are located at every turn of the proposed R-domain structure while the high Ser and Tyr residues are only present in the middle of β -strands, allowing them to act as an ice-like template. They are involved in the aggregation of each INP, thereby increasing the INP's hydrophobicity [35]. Based on their ice-binding abilities, it was suggested that INPs may have a similar β -helical fold and may interact with water through a repetitive TXT motif [36] (Figure 4). Large INPs having a molecular mass of 120-150 kDa could express high supercooling temperatures through both different tertiary structures of the R-domain. The N-domain is at least responsible for the binding of phosphatidylinositol as a lipid, saccharide (mannan) and INP [37]. Also, the C-terminal domain is rich in basic amino acid residues and is very hydrophilic. Among C-terminal amino acid residues, Tyr27 in this domain is important for ice nucleation, although not exclusively required, since nucleation was lost to a great extent when this residue was replaced by Gly or Ala but to a much lesser extent when it was replaced by Leu [38]. These results point to the important of the secondary and/or tertiary structure of the C-domain region for the ice nucleation with the hydroxyl group in the surface of its protein, which may interact with water molecule. Based on the structure and component of ice nucleation materials, we could predict that each domain has the following important role for the nucleation: N-domain through association with lipid and saccharide thereby increasing hydrophobicity; R-domain through structuralization of ice lattice-resembling protein; and C-domain through stabilization of tertiary structure of the complex.

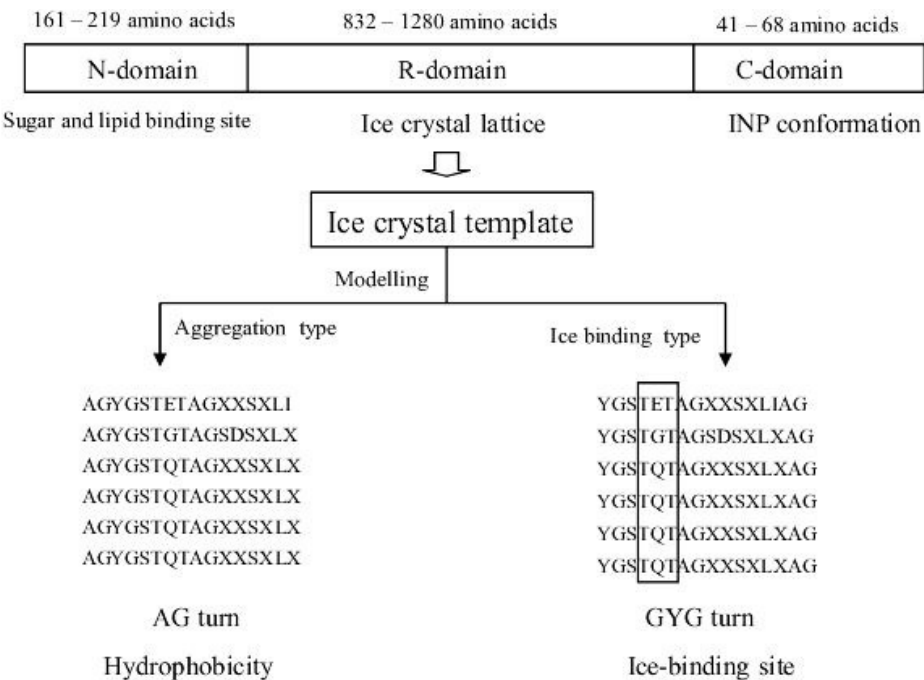


Figure 4. The structure of ice-nucleating protein and both models of different properties.

The wood frog (*Rana sylvatica*) is able to tolerate freezing of its body tissue. This tolerance is promoted by the initiation of ice formation at high subzero temperatures which allows ice to form gradually [39]. Also, some ice-nucleating bacteria, including *Pseudomonas putida*, *P. fluorescens*, and *Pantoea* (*Enterobacter*) *agglomerans* were isolated from the gut of frogs collected in the field. The maximum nucleation temperature of an aqueous suspension of *P. putida* cells ranged from -1.6 to -3.0°C [40]. These ice-nucleating bacteria may play a role in enhancing winter survival by promoting ice nucleation at high subzero temperature.

Frost-sensitive plant species have a limited ability to tolerate ice formation in their tissues [41]. Alternatively, some plants can supercool to some extent below 0°C and avoid damaging ice formation [42]. The temperature to which a given plant can supercool varies by plant species and is influenced by the presence of ice-nucleating agents that may be of plant origin [43]. Ice nuclei active at approximately -2°C and intrinsic to woody tissues of *Prunus* sp. were shown to have properties distinct from bacterial ice nuclei [20]. Development of ice nuclei in immature peach buds and sweet cherry stems did not occur until midsummer and their formation was essentially complete by late seasonal changes in growth. The apparent physiological function of the ice nuclei in promoting cold hardiness of woody plants illustrates the importance of supercooling and endogenously-controlled ice nucleation during dormancy and deacclimation [20].

Then, how is this ice nucleation activity measured? Ice-nucleating activity of bacterial cells was measured with a freezing nucleus spectrometer (thermoelectric plate, Mitsuwa model K-1), as described by Vali [44]. Thirty drops, 10 µl each, were placed on a controlled-temperature surface and the temperature was slowly lowered from ambient to -20°C at a rate of 1°C per min. The ice-nucleating spectra were obtained by the droplet-freezing method as modified by Lindow et al. [45]. After examining the shapes of these cumulative spectra, it was suggested that the sample nuclei could be separated into three classes: type I, II and III, with respective threshold temperature ranges of -5°C or warmer, -5°C to -8°C, and -10°C or colder [46]. Another simple procedure is to measure the highest threshold temperature of the INA in the sample using a glass capillary [47]. However, this method does not assay for less active nucleators and is best suited for cases where INA does not exhibit activity for screening of the ice nucleator.

The most representative application of INP is its use as the template of artificial snow. The sterilized and freeze-dried cell powder of the ice-active bacterium, *Pseudomonas syringae*, was used for the Calgary Winter Olympics in 1988 as an artificial snow agent. However, these highly active ice-nucleating bacteria were almost epiphytic bacteria causing frost damage. *Xanthomonas campestris*, which are known as a species of xanthum gum-producing bacterium, was isolated from frost-damaged tea leaves [48]. This strain, INXC-1, can be easily sterilized by a high-pressure treatment at low temperature without decreasing ice nucleation activity [49]. This cell preparation has been used for various processed foods, such as freeze concentration. Watanabe et al. have succeeded in applying freeze concentration to soy sauce, removing about half of the salt as eutectic crystals and leaving behind the flavor substances [50]. Soy sauce was frozen in the presence of this cell preparation at -25°C. After removing the ice and eutectic crystals of salt and water, the

product retained well its original aroma and taste substances at 1.6 times concentration. However, this pressurized-cell preparation has not yet been permitted for food use by the Japanese Ministry of Health and Welfare.

4. Structure and function of supercooling-facilitating material (anti nucleating material) in various organisms and chemicals

Ice-nucleating inhibitors have the ability to lower the supercooling point of water. This activity is termed either 'supercooling-facilitating activity' or 'anti-nucleating activity'. An enzyme-modified gelatin (EMG-12) has been reported as an ice-nucleating inhibitor of silver iodine, AgI, a well-known ice-nucleating agent [51]. Also, there are some reports regarding anti-ice nucleation substances that enhance the supercooling of water as shown in Table 1. Antifreeze proteins from insects [52], antifreeze proteins and antifreeze glycoproteins from fish [53], anti-nucleating proteins from bacteria [54], and polysaccharides from bacteria [55] all exhibit anti-ice nucleation activity toward water droplets. As substances originating from plants, hinokitiol from the leaves of Taiwan yellow cypress [56] and eugenol from cloves both reduce the ice-nucleation activity of water [57]. Crude extracts from the seeds of woody plants and supernatant liquids from germinating legume seeds exhibit very high anti-ice nucleation activity toward water droplets, although the causative substances for supercooling in these plant extracts were not identified [58]. As chemical substances, polyvinyl alcohol and polyglycerol enhance supercooling of aqueous solutions [59, 60]. Recently, it was reported that deep supercooling xylem parenchyma cells (XPCs) of the katsura tree (*Cercidiphyllum japonicum*) contain four kinds of flavonol glycosides with high anti-nucleating activities. These flavonol glycosides have very similar structures, but their activities are very different [61]. It was clear that the combination of the position of attachment of the glycosyl moiety, the kind of attached glycosyl moiety and the structure of aglycone determined the magnitude of this activity [62] (Figure 5). We have also purified an anti-nucleating protein from *Acinetobacter calcoaceticus* KINI-1, which was isolated from the camphor leaf [54]. This anti-nucleating protein has a molecular mass of 550 kDa. It exhibits a broad specificity with the capacity to lower the nucleating activity of a wide range of ice nucleators, including some bacterial components and AgI (Table 1). However, the expression mechanism of its anti-nucleating activity remains unknown. Also, the xylem extract of the katsura tree exhibited anti-nucleating activity against a wide range of ice nucleators. The anti-nucleating activities (°C) of this extract at the same concentration against cell suspensions of *P. fluorescens*, *E. ananas* and *X. campestris* were found to be 0.7, 1.9, and 1.3, respectively. This activity against AgI was 1.8. After isolating each active compounds, the main active compounds in the xylem extract found to be four flavonol glycosides; kaempferol 7-O- β -D-glucopyranoside, kaempferol 3-O- β -D-glucopyranoside, 8-methoxykaempferol 3-O- β -D-glucopyranoside, and quercetin 3-O- β -D-glucopyranoside [61]. It is clear that the activity of the flavonol glycosides are controlled by a combination of the position of attachment of the glycosyl moiety, the kind of attached glycosyl moiety and the structure of aglycone. Although the features of the structures in flavonol glycosides that clearly affect this activity were not found, judging

from the active low molecular compounds in regards to the flavonol related compound, hinokitiol [56] and eugenol [57], the functional group in polyphenol may interact with the active site Tyr residue in the C-domain of ice nucleation protein.

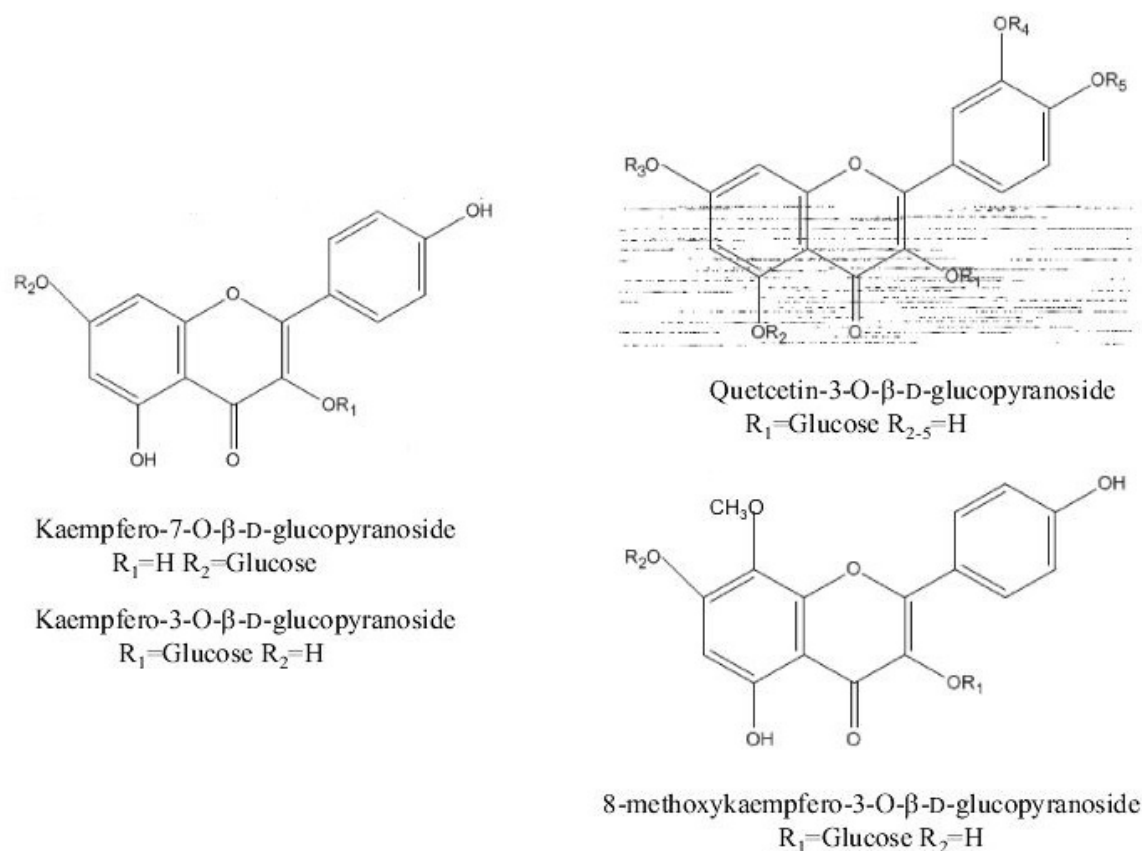


Figure 5. The structures of five flavonol glycosides having anti-nucleating activity.

Then, how is this anti nucleation activity measured? The measurement modified method that was used previously for the ice-nucleating activity [43] was used. Briefly, the anti-nucleating activity was measured as follows. A sample solution (270 μl) and a suspension (30 μl) containing lyophilized cells of various ice-nucleating bacteria in a potassium phosphate buffer to an absorbance at 0.1 of 660 nm (50 mM, pH 7.0) were mixed and incubated in ice for 10 min. The ice-nucleating temperature of this mixture solution was measured. A mixture solution including 270 μl of 50 mM potassium phosphate buffer (pH 7.0) was measured as a control. Also, a mixture solution of the sample solution (270 μl) and the AgI (1mg/ml) suspension (30 μl) was examined. The difference between the ice-nucleating temperature of the sample and the control was defined as the anti-nucleating activity or supercooling-facilitating activity ($^{\circ}\text{C}$).

Materials	Concentration	Ice nucleator	Anti ice nucleating activity (°C)	Condition
Xylem extract				
<i>Castanea crenata</i>	100 mosmol/kg	<i>Erwinia ananas</i>	1.2	1
<i>Fagus crenata</i>	100 mosmol/kg	<i>E. ananas</i>	1.3	1
<i>Cercidiphyllum japonicum</i>	100 mosmol/kg	<i>E. ananas</i>	1.9	1
	100 mosmol/kg	<i>Pseudomonas syringae</i>	0.7	1
	100 mosmol/kg	Silver iodide	1.8	1
55-kDa protein				
<i>A. calcoaceticus</i>	10 µg/ml	<i>Erwinia uredovora</i>	2.2	2
130-kDa polysaccharide				
<i>B. thuringiensis</i>	50 µg/ml	<i>Pntoea ananas</i>	2.3	2
	50 µg/ml	Silver iodide	4.2	2
Hinokitiol	10 mM	<i>P. fluorescens</i>	2.1	2
Eugenol	1 mg/ml	<i>P. fluorescens</i>	1.9	2

*This list was modified table 1 in reference [61]

Condition 1: Volume of droplets was 2 µl and cooling rate was 0.2 °C/min

Condition 2: Volume of droplets was 10 µl and cooling rate was 1.0 °C/min

Table 1. A list of anti-nucleating materials*

Only a few studies have been performed on the practical application of supercooling facilitating material. Organ cryopreservation is hindered by ice-inflicted damages and non-freezing preservation of livers at subzero temperature over -5°C might offer advantages over the current method of preservation. A solution containing bacterial anti-nucleating protein (20 µg/ml) [52] and ascorbic acid 2-glucoside (100 µg/ml) as an antioxidant was used as a subzero non-freezing storage method (SNZF) for rat liver graft [63]. When liver grafts were kept for 24 h at SNZF storage (-3.0°C), apoptotic cells were greatly diminished. Also, ATP concentrations in grafted liver tissues preserved with SNZF were significantly higher than those that underwent normal storage at 4°C for 24 h. In the case of flavonol glycoside, the supplemental addition of kaempferol 7-O-β-D-glucopyranoside to diluted vitrification solution, which consists of 2.0 M glycerol, 0.4 M sucrose and 4% dimethylsulfoxide (Me₂SO, w/v) in basal culture medium was examined [64]. The addition of 0.5 mg/ml kaempferol 7-O-β-D-glucopyranoside to the diluted plant vitrification solution 2, which consists of 30% glycerol (w/v), 15% ethylene glycol (w/v) and 15%Me₂SO (w/v) in basal culture medium containing 0.4 M sucrose (pH 5.2). resulted in significantly higher regrowth rates after cryopreservation.

5. Structure and function of antifreeze protein (AFP) and AFP related material from various organisms

In the late 1960s, DeVries and Wohlschlag reported that a carbohydrate-containing protein (antifreeze glycoprotein; AFGP) that was isolated from the blood plasma of an Antarctic notothenioid fish accounted for a freezing point depression of -1.31°C [65]. This discovery provided a biophysical explanation for how such organisms escape lethal freezing events despite continual contact with -1.9°C sea water. Many mechanisms containing the production of AFP have been utilized by various species. Other than these adaptive mechanisms, other mechanisms include seasonal migration, hibernation, supercooling, synthesis of small cryoprotectant molecules such as glycerol, trehalose, mannitol and others. Almost all AFPs identified in various organisms were orders of magnitude more active than that which could be explained by colligative properties. AFPs excepting some AFP-related materials, had thermal hysteresis (TH) activity without change in the melting point and recrystallization inhibition (RI) activity [66]. Ice can exist in several crystalline polymorphic structures and also in an amorphous or vitreous state of rather uncertain structure. Of these, only ordinary or hexagonal ice (I_h) is stable under normal pressure at 0°C (Figure 2). This ice structure, I_h , grows along the a and c axis (Figure 6 a). The plane growing along the a axis is called the prism face, and the plane growing along the c axis is called the basal face.

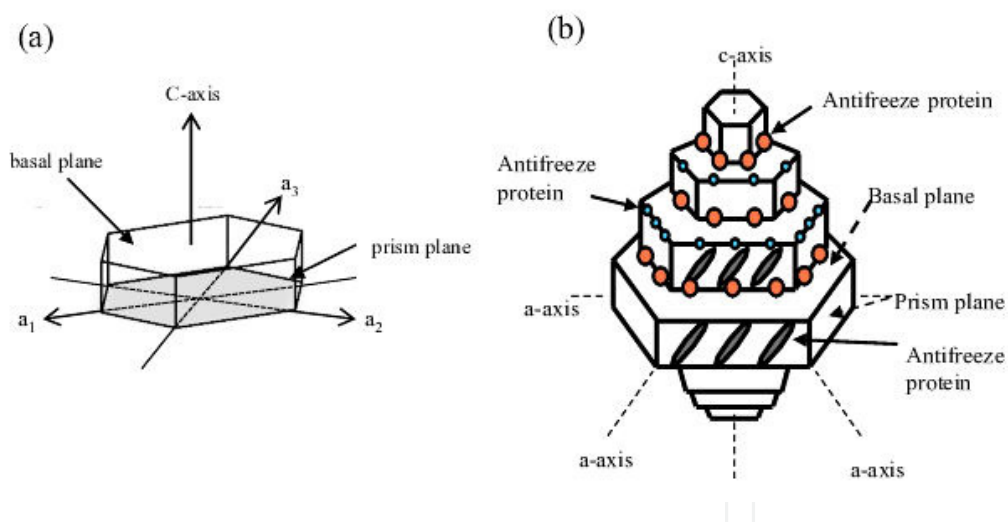


Figure 6. The structure of hexagonal ice crystal and its binding sites of antifreeze proteins. (a) Hexagonal ice crystal (b) Various binding sites of antifreeze protein

Flat AFP peptides and the flat sides of AFP tertiary structure contact sides could bind to ice lattices (Figure 6) and interfere with crystal growth along the a -axis by making it thermodynamically unfavorable for water molecules to join the ice surface [67]. Therefore, AFPs appeared to inhibit the normal growth direction of ice by preferentially adsorbing to the prism faces of ice crystals, thereby forming needle-shape crystals (Figure 7 (a)). Several models have been proposed to describe how molecular binding between the peptide and ice occurs

[68], but the specific nature of this interaction is still not well-understood. Not all AFPs have an effect on freezing point depression, that is, Thermal hysteresis activity (TH activity), and have only recrystallization inhibiting activity (RI activity) [69]. Those ice-binding proteins having ice affinity are often referred to as ice-active or ice-structuring proteins [70] and ice recrystallization inhibiting proteins. Various AFPs have been isolated from fishes, plants, insects, fungi, and bacteria [71]. Among AFPs from various organisms, each AFP of different origins could be divided into groups based on its structure. As shown in Table 2, fish AFPs and AFGP could be divided into five groups. Four groups of fish AFP having different structures and molecular weights each had different TH activity. For instance, type I AFPs were defined as small (3 ~ 4 kDa), Ala-rich (~60% Ala) α -helices [72]. These AFPs were isolated from three taxonomic orders; pleuronectiforme such as the winter flounder [73], scorpaeniforme such as sculpins [74] and perciforme such as cunner [75]. Typically, type I AFPs form amphipathic helices with a well-conserved Ala-rich surface opposite a less conserved, more hydrophilic helix side [76]. Although AFP types I, II, III and IV, as well as AFGP, produce ~1°C of thermal hysteresis at high concentrations (10 ~ 40 mg/ml), hyperactive AFP type I, which provides ~1.1°C of TH activity at a concentration of 0.1 mg/ml, was isolated from winter flounder [77]. The structure of hyperactive AFP type I was two extended 195-amino acid α -helices forming an amphipathic homodimer with a series of linked Ala- and Thr-rich patches on the surface of the dimer [78]. As with the discovery of hyperactive AFP, further study may be in progress to find and characterize new type of fish AFPs.

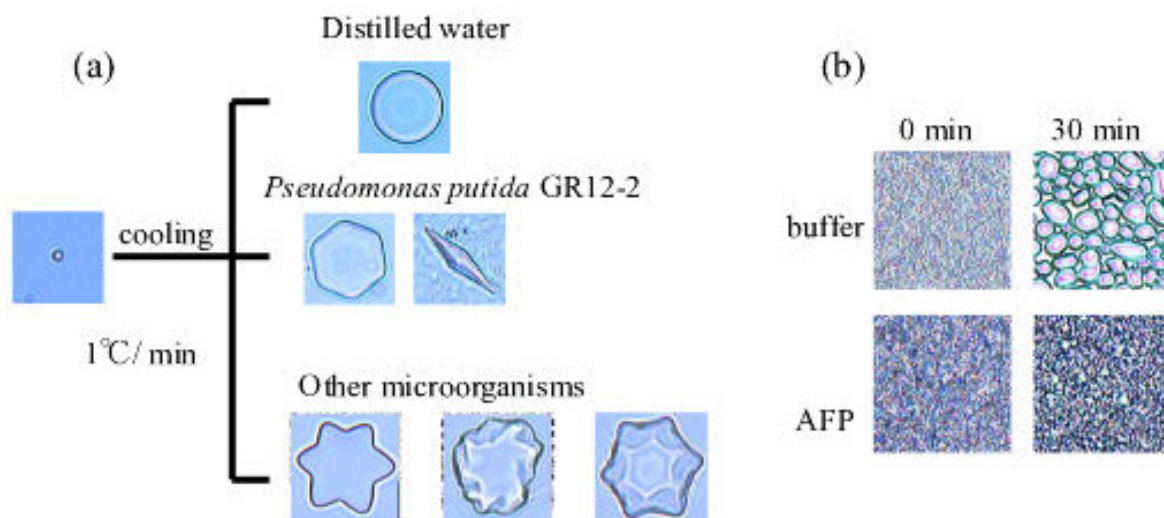


Figure 7. Ice crystal regulation by some antifreeze inhibition. (a) Ice crystal morphology (b) Ice crystal recrystallization inhibition.

Animal AFPs exhibit significant differences in the levels of TH, ranging from 1 to 2°C in fishes and 5 to 10°C in insects [79]. In contrast, plant AFPs, which characteristically have low levels of TH activity (0.1 ~ 0.6°C) [80], were divided into two groups based on structure. In winter rye, six AFPs ranging in size from 15 to 35 kDa have been identified from the apo-

plastic fraction. These AFPs are similar to pathogenesis-related proteins containing chitinase, β -glucanase and thomathin-like proteins [81]. An AFP with higher RI activity and lower TH activity compared with other AFPs was isolated from the perennial ryegrass *Lolium perenne* [82]. In carrots, an LT-up-regulated AFP shows a significant similarity (50-65%) to the polygalacturonase inhibitor family of plant leucine-rich repeat (LRR) proteins [83].











Characteristics	AFGP	Type I AFP	Type II AFP	Type III AFP	Type IV AFP
Mass (Da)	2600-33000	3300-4500	11000-24000	6500	12000
Key Properties	AAT repeat Disaccharide	Alanine-rich α -helix	Disulfide bonded	β -sandwich	Alanine rich herical bundle
Representative Structure					
Natural Sources	Antarctic- notothenioids	Right-eyed, flouders	Sea raven, Smelt, herring	Ocean pout, Wolfish, eel pout	Longhorn, sculpin
					

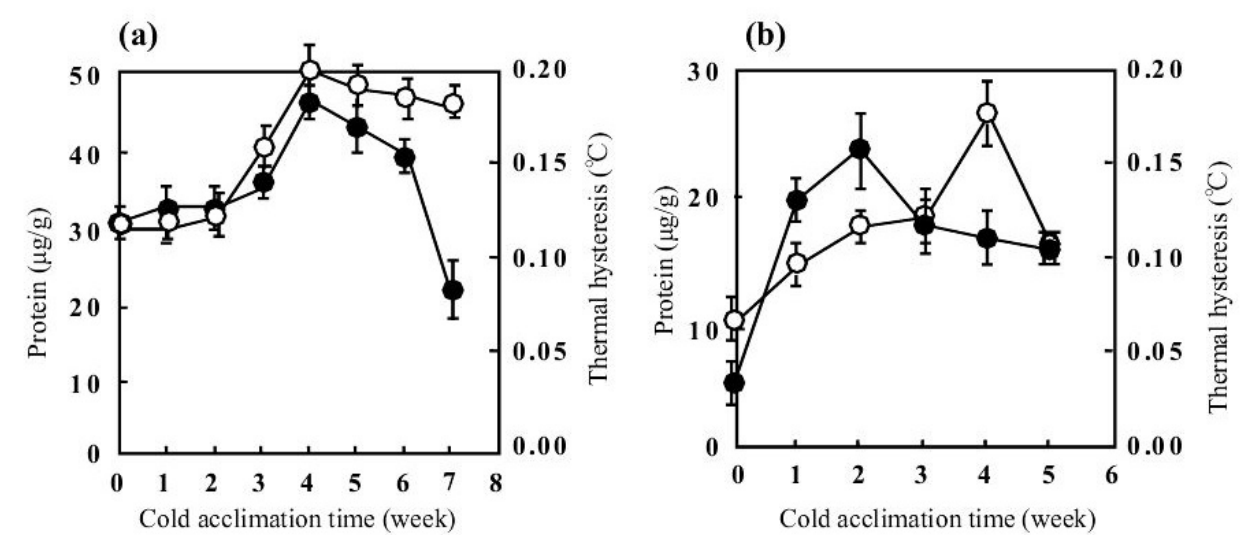
Table 2. Structure and properties of AFP and AFGP from various fishes.

Based on the presence of TH activity in the extract of various plants, some grains like winter and spring rye, some vegetables including cabbage and carrot, *Ammopiptanthus mongolicus*, *Solonum dulcamara*, *Lolium perenne* and tobacco have been chosen to investigate AFPs [84, 85] as shown in Figure 8. In Japan, many vegetables are harvested during the winter. The Japanese radish particularly is one of the typical winter vegetables and the most productive vegetable with the largest amount in Japan. However, AFPs in the Japanese radish leaf and tuber were found to accumulate in the apoplastic spaces of vegetables harvested until April. We examined the effect of cold acclimation time on the AFP production by measurement of protein amount and TH activity [86]. The protein amount and TH were almost constant during 2 weeks of acclimation time. Each maximum value (46.5 $\mu\text{g/ml}$ and 0.20°C, respectively) was attained after 4 weeks of cold acclimation time. The TH was almost constant (0.18 - 0.20°C) until 7 weeks of cold acclimation time had elapsed. When the Japanese radish tuber was stored at 4°C for 7 weeks, the protein amount in its apoplastic space diminished remarkably (22 $\mu\text{g/ml}$) (Figure 9). Some proteins in this apoplastic fraction reacted with the anti-glucanase-like protein (GLP) antiserum and anti-chitinase-like protein (CLP) antiserum produced against isolated winter rye AFPs. Also, these prepared proteins exhibited chitinase and β -1,3-glucanase activities. The structure of the chitinase-type AFP and glucanase-type AFP from winter rye leaf were elucidated by sequencing the gene of each AFP [87], but the binding sites in these AFPs were unclear. Also, the structures of these proteins from Japanese radish remain unknown.

Brassicaceae Garlic mustard Barbarea vulgaris Brussels sprout Tenderstem broccoli Cabbage	Poaceae Oats Kentucky bluegrass Barley Winter wheat Winter rye Perennial ryegrass Triticale Annual meadow grass	Apiaceae Salicaceae Carrot Fremont cottonwood Papaveraceae Euphorbiaceae Rudolph Ghostweed Asteraceae Liliaceae Aartarian aster Daylily Dandelion Leek Crown daisy
Winter cultivation in Japan (vegetable) Cabbage Japanese Mustard Spinach Turnip Greens Japanese Greens Chinese Cabbage Japanese Radish Broccoli Turnip Green pak choi	Plantaginaceae Fagaceae Apocynaceae Ribwort Plantain White oak Periwinkle Greater Plantain Caryophyllaceae Solanaceae Chickweed Woody nightshade Violaceae Oleaceae Potato sweet violet Golden bells	

This list is modified in table from reference No. 83

Figure 8. List of some plants having antefreeze activity (TH activity).



Each value is the mean \pm SD (n=4). Values obtained from different cold acclimation times are significantly different at $p < 0.05$ ●, protein; ○, Thermal hysteresis

Figure 9. Effects of cold acclimation times on the apoplastic protein and thermal hysteresis. (a) tuber (b) leaf

Other than AFP having high TH activity (0.1 – 0.2°C), some proteins having high RI activity were isolated from various plants. Two related genes encoding ice recrystallization-inhibit-

ing protein from wheat were identified and characterized by assay of IR activity. Two proteins share homology with two subsets of proteins: their N-terminal parts are similar to the Leucine rich repeat-containing regions present in the receptor domain of receptor-like kinases, while their C-terminuses are homologous to the RI domain of AFPs [88]. This C-terminal part is homologous to LpAFP, a partial gene coding for an AFP from the rye grass *Lolium perenne*. The encoded, incomplete 118 residue LpAFP has an RI activity higher than that of other AFPs [24]. This primary structure shows a series of highly conserved repeated motifs with regularly spaced serine and threonine residues that may form hydrogen bonds with the ice surface [89]. One model predicted that two ice binding sides were each on side of the AFP molecule, with xxNxVxG and xxNxVx consensus motifs [90]. This consensus motif is different from the ice-binding motif (xTxTx) for TH activity in some beetle AFPs [91]. It was predicted that this unusual duplication of putative-binding sites on opposite sides of the protein could be responsible for the high RI activity. Homology search within this RI domain (113 amino acids of C-terminal region) has shown that this region exists only in four species that are known to be cold-tolerant cereals from the *Pooideae* subfamily of the *Poaceae* (*Gramineae*) family: wheat, *L. perenne*, rye, and barley. The consensus sequence of some AFPs may be found from some organisms in the same subfamily.

Then, how is this antifreeze activity measured? Both activities, that is, TH activity and RI activity, were measured by different methods. TH activity was measured using a nanoliter osmometer (for example, Otago osmometers). The osmometer was calibrated using deionized water (Milli-Q) and osmolarity standards. Droplets of the test sample were transferred to the sample wells of the osmometer, which were filled with oil. The droplet was frozen by rapidly cooling it to about -30°C and then was observed under a dissecting microscope. The temperature was then raised rapidly until close to the expected melting temperature, at which the last ice crystal melted, and the melting temperature was determined and the osmolality calculated. Then the temperature was decreased to refreeze the sample and increased to melt the sample back to a single small ice crystal. In the use of nanoliter osmometer, the temperature was lowered by $0.02^{\circ}\text{C}/\text{min}$ until discernable growth of the ice crystal occurred. This was taken as the hysteresis freezing point and from this the mount of thermal hysteresis (TH) was calculated. The shape of the ice crystal upon growth at the hysteresis freezing point was noted using the microscope [69]. Other the method using a micro-osmometer, a microscope with temperature-controlled freezing stage (Model THM 600, Linkham Scientific Instruments, Surrey, UK) was used to measure TH activity [83] (Figure 10). One microliter of protein sample was applied to the center of a temperature-controlled freezing stage on a circular glass cover. The freezing stage was fitted onto the stage of a conventional microscope and was connected to a pressurized air supply cooled by liquid N_2 . The stage temperature was controlled by a programming unit (Model TMS 90, Linkham Scientific Instruments, Surrey, UK). After sample application, the stage was heated to 20°C , then cooled to -40°C at the rate of $100^{\circ}\text{C}/\text{min}$ to freeze the sample, after which it was heated at the same rate to -5°C . The warming was slowed to $5^{\circ}\text{C}/\text{min}$ to thaw the sample until only a single ice crystal was present. Subsequently, the temperature was slowly ($1^{\circ}\text{C}/\text{min}$) lowered to observe ice crystal growth. The time (s) at which the ice crystal growth started, were measured and the TH value ($^{\circ}\text{C}$) was calculated this time 60^{-1} . Under these conditions, high

levels of antifreeze activity were indicated by the multi-faceted or bipyramidal shape of the ice crystal, whereas low levels of antifreeze activity were indicated by the flat, hexagonal shape of the growing ice crystal. In the absence of AFPs, the ice crystals were round and flat. Measurement of RI activity was performed with various methods. The assay for the inhibition of ice recrystallization was performed using the method described by Smallwood et al. [92]. This method was called the 'sucrose sandwich method'. Each sample contained a known dilution of the AFP preparation and 30% sucrose in water. This mixture (1.5 μ l) was 'sandwiched' between two labeled, 13 mm diameter circular glass cover slips. The sandwich was cooled to -80°C using the programming unit and then maintained at -6°C. The sandwich was observed using a phase-contrast microscope with a 10X objective and with a temperature-controlled freezing stage (Figure 11). Visual assessment of any recrystallization was made by comparison of the test sample with a control sample (30% sucrose solution without AFP) after 30 min. The presence of RI activity in the sample was compared with crystal sizes in photographs of both the sample and positive control, that is, a fish AFP sample after 30 min of annealing. Before the development of the sucrose-sandwich method, RI activity was measured by a technique known as the 'splat cooling assay' [93]. In this method, a small volume of sample liquid (10 μ l) is dropped from a height of about 2 m onto a polished metal block precooled in solid carbon dioxide. The polished block is usually at a temperature of about -78°C. The resulting ice splat is about 1 cm in diameter and is a thin disc of polycrystalline ice. This is then transferred to a cold stage on a microscope where it is maintained at -6 to -9°C and is observed between crossed photographs to determine changes in the average size of the ice crystals over time. Also, to compare each ice crystal size in serial diluted samples, a capillary method using 10 μ l glass capillaries was developed [94]. Serial dilutions of sample are prepared to determine the concentration below which RI activity was no longer detected, termed the RI endpoint. All of the diluted samples can be assayed and evaluated simultaneously. Also, Warlton et al. have developed a new technique to measure and qualify levels of crystallization in a sample solution using an optical recrystallometer [95]. However, these assay methods for RI activity were not defined in terms of the unit of RI activity, such as the enzyme unit for various enzyme activities. As shown in Figure 11, photographs of both the test sample and control sample after 30 min at -6°C were analyzed by average area (in pixels) of one crystal using Image Factory (Ruka International Co., Japan). The value of RI was calculated as the relative rate of the average area of one crystal from both the control and test samples. On high RI activity this high activity equal to the decrease of this RI value (Figure 12). Also, one unit of RI activity was defined as activity with the relative ratio of RI=0.5. To confirm the presence of AFP in crude extract from various organisms, the best method is the separation of those proteins having the affinity ability against ice crystal surface. The cold finger method [96] can purify ice-binding protein like AFP from a crude mixture and confirm the presence of ice-binding protein.

Many companies around the world have been expecting to apply AFPs to frozen food. The representative application of AFP is quality preservation in various processed frozen foods. Unilever Group developed AFP type III HPLC 12 preparations produced by recombinant baker's yeast, which is used commercially for the quality preservation of commercial ice cream. They established the safety of this recombinant AFP based on a set of *in vitro* and *in*

in vivo genotoxicity assays (bacterial mutation, chromosome aberration, mammalian cell gene mutation and rat bone marrow micronucleus) as well as a 3-month repeat-dose gavage study on rats [97]. This recombinant protein, that is, ice structuring protein (ISP), is used as a food additive and ice cream containing ISP is sold in North America. Also, concentrated carrot protein (CCP) containing 15.4% (w/w) carrot (*Daucus carota*) AFP was found to potentially improve the fermentation capacity of frozen dough [98]. The effects of pre-slaughter administration of AFGP to lambs were assessed on lamb meat quality after thawing [99]. Meats were vacuum packed and stored frozen at -20°C for 2-16 weeks. Upon thawing, meats were assessed for drip loss and sensory properties (foreign flavour, storage flavour, texture, tenderness, juiciness and overall acceptability). Injection of AFGP at either 1 or 24 h before slaughter reduced drip loss and ice crystal size. Ice crystals were smallest in the lambs injected with a final concentration of $0.01\text{ }\mu\text{g/kg}$. AFGP, particularly when injected 24 h before slaughter. These results suggest that the addition of AFGP could reduce damage owing to frozen storage of meat. Perhaps the qualities of various frozen meats and fishes could be improved by the injection of AFP and AFGP. However, the applications of non-recombinant AFPs for these applications are marred by high cost due to low yield. Applications for frozen food may require future technology in which highly active extracts can be manufactured inexpensively.

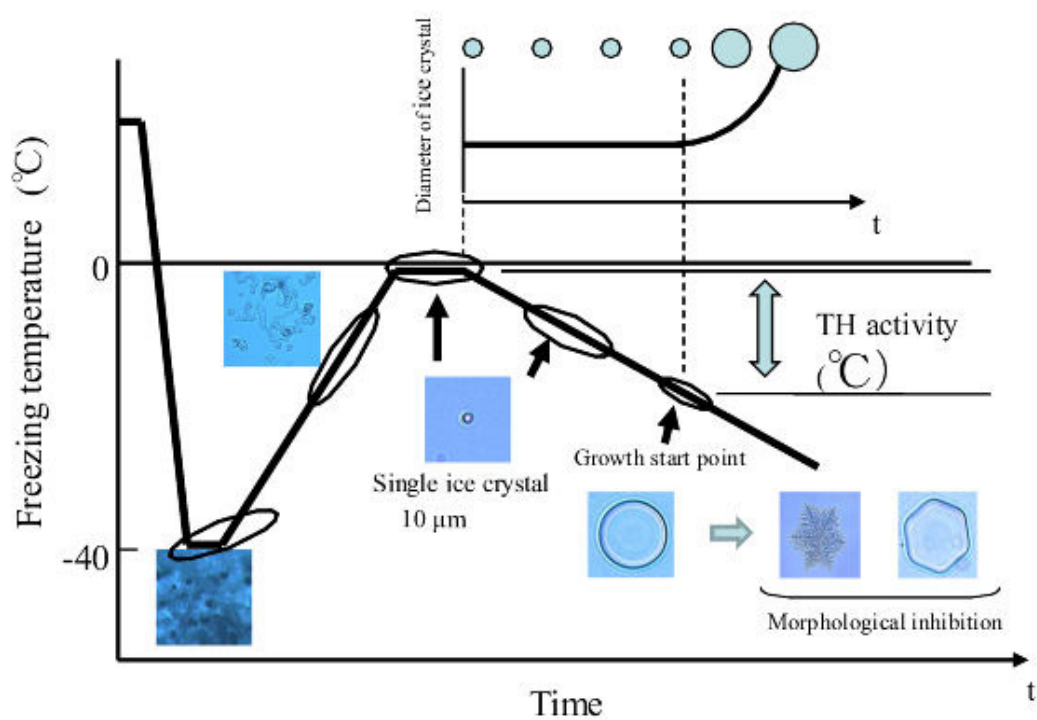


Figure 10. Scheme of freezing curve and ice morphology of the TH activity using microscope with temperature-controlled sample stage.

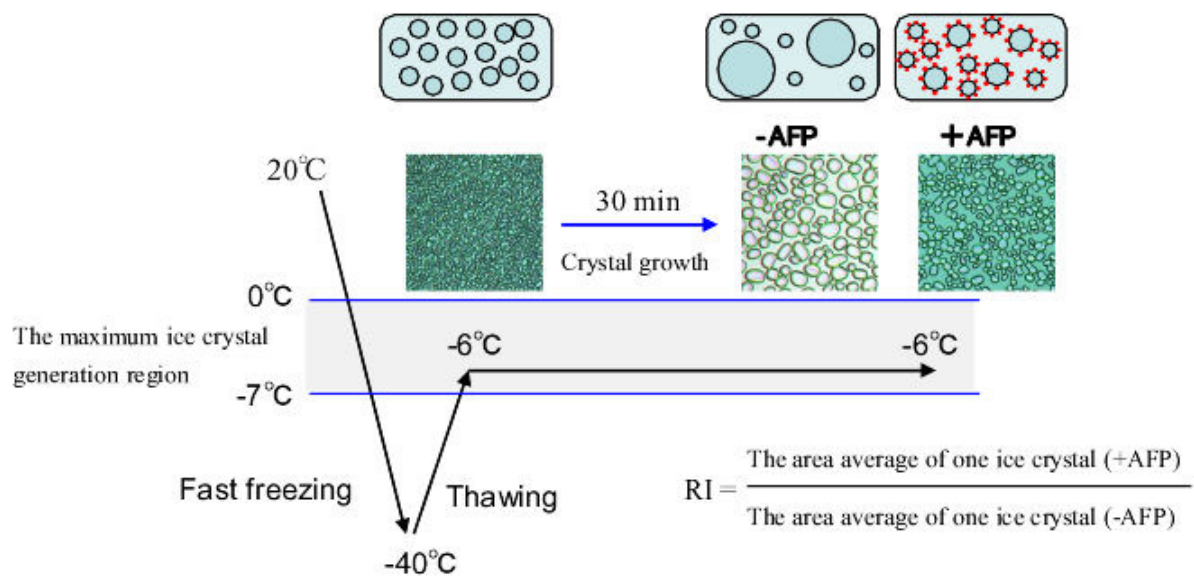
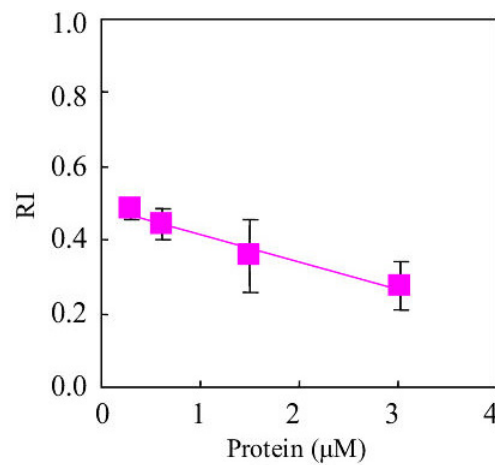


Figure 11. Scheme of freezing curve and ice morphology of sucrose sandwich assay using microscope with temperature-controlled sample stage.



Each value is the mean \pm SD (n=3)

Figure 12. Effect of proteins concentration of RI value in the purified fish type II AFP.

6. Conclusion

All steps related to the processes from nucleus generation as shown in Figure 1 without sublimation have correlated with the survivals of various organisms under subzero temperature. However, the sublimation of ice take place under frozen conditions (-10 °C) during a long term over 1 month. The sublimation-controlling ability must be the materials with the ice-binding ability. These organisms have developed the ability to tolerant freezing condi-

tions by producing ice crystal-controlling materials in their extracellular or intracellular spaces. Although these materials have different functions, all of them seem to have ice-binding sites and therefore a high affinity for ice lattice surface. Among all materials, some AFP groups had consensus sequence or consensus tertiary structure. Also, both INPs and AFPs exhibit their each opposite functions in the case of special regions containing ice binding sites on all amino acid sequence [100] or aggregations of each molecule [101]. This phenomenon is an important factor in consideration of AFP's potentially harmful effect on the quality of frozen food processing and the viability of cultured cells after cryopreserving owing to excess concentration. However, these materials can be advantageous in various industries concerned with freezing and preservation through use of more diluted concentrations. Among these materials, sublimation-inhibiting or -facilitating materials remain unexplored. As AFPs and INPs had ice binding site on each molecule, both proteins may have sublimation-inhibiting ability. From now we will try to confirm an assay system for sublimating-inhibiting activity. In the future, this inhibitor will likely be discovered and applied to various processed frozen food.

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