# P1

## Activity switching of an antifreeze protein from Lolium perenne

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*Lolium perenne* is a perennial grass from the family Poaceae and is cultivated around the world. Its advantage is a fast recovery from stress. Additionally, by expressing a β-helical antifreeze protein (*LpAFP*), its tolerance toward cold stress is increased.

The concept of switching the activity of a protein on and off by using an external stimulus like light has been well established in biochemistry. The basic principle is the covalent linkage of a protein with a photoswitchable unit. A conformational change in the photoswitch thus results in a change in the tertiary structure of the protein. As the activity of antifreeze proteins is dependent on their fold, changing it will have a direct impact on the antifreeze activity. Most of the proteins used in linking and switching studies are α-helical, *LpAFP* as a β-helical protein is part of a new field in science.

*LpAFP* does not contain any Cysteins in its primary sequence. By molecular modeling of the photoswitchable unit, two amino acids were determined for mutation and the mutated protein has been expressed. The photoswitchable molecule on basis of an azobenzene was synthesized, and we will present the results and biophysical and conformational properties of the modified *LpAFP*.

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P2

## Switchable Proteins – The Trp-Cage as novel switching unit

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The ability to switch the function of peptides and proteins by light expands their applicability immensely. By combining an organic photochromic molecule that undergoes a light induced conformational change, the structure dependent activity of peptides and proteins can be switched ″on″ and ″off″. Our goal is to create a universal circuit unit consisting of a photochromic organic molecule and a folded peptide, the Trp-cage. The Trp-cage is a short, 20 amino acid long miniprotein with a very stable 3D-structure (turn, $\alpha$-helical) and cooperative folding behavior. the design and properties of this unit, and first attempts to fuse this unit with an a helical Type 1 AFP to switch antifreeze activity will be presented.

# P3

## Photoswitchable Type I like Antifreeze Proteins

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As the mechanism of the antifreeze activity of AFPs is still not fully understood, the possibility of performing new experiments with spatial and temporal resolution is highly promising. To achieve this goal, our approach is to introduce a photochemical switching unit to cysteine sidechains of different, synthetic type I like sequences. The basis of the photoswitches is an azobenzene moiety which undergoes cis/trans isomerization upon irradiation with light of specific wavelengths. This isomerization leads to a geometrical change of the switch which stabilizes or destabilizes the a-helical structure, depending on chosen linker lengths and cysteine-cysteine distances.
Several sequences derived from HPLC6 with varying lengths and specifically placed cysteine residue have been synthesized and characterized spectroscopically. These were subsequently linked with azobenzene-based linkers. The photochemical and structural properties such as helical content have been determined and will be presented.

P4

# Stabilizing α-Helix Peptide from Antifreeze Protein I by Main Chain Hydrogen Bond Surrogate

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The α-helical structure is a major class of protein secondary structures that plays an important role in protein interactions. For short peptides, comprising an α-helical structure is very important because it stabilizes these peptides and protects from proteolytic cleavage. Houston *et al.* previously showed that the stabilization of the α-helical structure by addition of a lactam bridge in an15-amino acid type 1 antifreeze protein was successful. This modified peptide was able to interact to the ice surface and affect its growth. However, this modification was insufficient to make the peptide fully helical. An alternative promising method that is also able to create mimicking α-helical structure in short peptide is hydrogen bond surrogate (HBS). This method has key feature that the α-helical is created by replacement of N-terminal hydrogen bond with a covalent carbon-carbon bond. This carbon-carbon bond was formed by a ring-closing metathesis (RCM) reaction between olefins on the peptide chain. Hence, all side chains in this peptide are available to contacts to targets. Therefore, we aim to apply HBS in this study to create stable artificial α-helix in shortened type 1 AFP sequences.

P5

# Influence of temperature and pH value on the recrystallization inhibition activity of fish ice structuring protein

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One important criterion for good quality ice cream is the ice crystal size which has a big impact on mouthfeel. The ice crystal size can be influenced by process parameters (e.g. pressure, temperature) or formulation (Bahramparvar and Mazaheri Tehrani, 2011; Miller-Livney and Hartel, 1997). During storage and distribution of ice cream, especially under unfavorable temperature conditions, the size and number of ice crystals change due to recrystallization processes leading to product deterioration. To reduce the recrystallization rate by the use of additives a large array of research attempts has been followed. Traditionally hydrocolloids are added to ice cream, but also ice structuring proteins (ISP) are discussed as very potent agents.

Today there is already industrial manufactured ice cream including ice structuring proteins on the market. However less is published about the influence of process parameters (temperature, pressure) or milieu conditions (pH value) on the recrystallization inhibition (RI) activity of the proteins. Regand and Goff (2005) showed that the activity of ISP from cold-acclimated winter wheat grass extract (AWWE) in sucrose solution was reduced significantly after heating to 85° C for 10 min. In addition pasteurization eliminated ISP activity in a mix of different ice cream components, whereas there was no reduction of ISP activity by pasteurization in ice cream (Regand and Goff, 2006). Studies of the thermal hysteresis (TH) at different pH values showed no influence from pH 2 to pH 11. At pH values 1 and 13 there was a reduction about 25 % of the thermal hysteresis activity. To what extent the pH value has an influence on the RI activity of ISP was not analyzed (Chao et al. 1994; Li et al., 1998).

The objective of this work was to study the influence of temperature and pH value on the recrystallization inhibition activity of the ice structuring protein AFP type III (*macrozoarces americanus*) in sucrose solution. This solution was used as a simplified model system for ice cream. Results showed that heating to 80 ° C for one minute has no influence, whereby increasing the heating time to 30 minutes showed a significant decrease in recrystallization inhibition. Adjusting the pH value with hydrochloric acid or sodium hydroxide increased the RI activity independent of the pH value. Additional experiments with sodium chloride suggest that the concentration of ions is responsible for the increased RI activity and not the pH value as such.

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P6

# Anti-freezing Protein Inhibits Ice Nucleation:Another Mechanism for the Survival of Living Organisms in Subzero Environments

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Antifreeze proteins (AFP) play an irreplaceable role for the survival of many living organisms in subzero environments. The widely accepted mechanism is that AFPs inhibit the growth of microscopic ice crystals at subzero temperatures via absorption-inhibition. We found in our experiments that AFPs can also inhibit the ice nucleation, which complements the absorption-inhibition mechanism. Moreover, we found that the structure of the interfacial water is crucial in tuning the ice nucleation. Our results not only provide a complementally mechanism of the AFPs for the survival of living organisms in subzero environments, but also shed a new light on the fundamentals of ice nucleation. The latter is critical for the promotion or suppression of ice nucleation, which has broad applications in a variety of areas such as cryopreservation of cells and tissues, prevention of the freezing of crops, cloud seeding and snow making etc.

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P7

# Synergistic effects between hydrocolloids and antifreeze proteins from fish on the recrystallization of ice in sucrose solutions

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One important criterion for good quality ice cream is the ice crystal size which has a big impact on mouthfeel. The ice crystal size can be influenced by process parameters (e.g. pressure, temperature) or formulation (Bahramparvar and Mazaheri Tehrani, 2011; Miller-Livney and Hartel, 1997). During storage and distribution of ice cream, especially under unfavorable temperature conditions, the size and number of ice crystals change due to recrystallization processes leading to product deterioration. To reduce the recrystallization rate by the use of additives a large array of research attempts has been followed. Traditionally hydrocolloids are added to ice cream, but also antifreeze proteins (AFP) are discussed as very potent agents.
Antifreeze proteins were first identified in the blood of fish, living in ice-laden seas. Later the proteins were also found in certain insects, plants and microorganisms. AFPs share two interesting properties: Thermal hysteresis in which AFPs depress the temperature of ice growth, called hysteresis freezing point, below the melting point (Kristiansen and Zachariassen, 2005). Furthermore AFPs are potent agents for ice recrystallization inhibition (RI). Regand and Goff (2006) suggested a synergistic effect between AFP and stabilizer in an ice cream matrix. If the addition of hydrocolloids to antifreeze proteins could enhance their antifreeze activity in general this would be a possibility to reduce the necessary amounts of antifreeze proteins for practical application. This is an important factor as one barrier for industrial application of AFPs is their limited availability and as a consequence their exorbitant price.
The objective of this work was to study synergistic effects between several AFP from fish (type III (*macrozoarces americanus*), type I (*pleuronectes americanus*) and AFGP (*gadus ogac*)) and typical ice cream stabilizers (sodium alginate, κ-carrageenan) on recrystallization inhibition in sucrose solutions. This solution was used as a simplified model system for ice cream. Results showed that addition of hydrocolloids to the AF(G)P solutions increased significantly the RI compared to the control without hydrocolloids whereby the influence of κ-carrageenan was higher than that of sodium alginate. Investigation of RI and the ice crystal shape in hydrocolloid solutions indicated a similar RI mechanism of κ-carrageenan and AFP.

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P8

# Chemical synthesis and structural analysis of the antifreeze agent xylomannan fragment from Upis ceramboides

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The novel antifreeze factor xylomannan, first isolated from the freeze-tolerant Alaskan beetle *Upis ceramboides* demonstrates a high degree of thermal hysteresis, comparable to that of the most active insect antifreeze proteins [1]. This substance was subsequently also identified in other diverse taxa [1b]. Although the presence of a lipid component in this factor has not yet been verified, it has been proposed that the glycan backbone consists of a β-D-mannopyranosyl-(1→4)-β-D-xylopyranose disaccharide repeating structure according to MS and NMR analyses. We wish to report the stereoselective synthesis of the tetrasaccharide β-D-mannopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-β-D-mannopyranosyl-(1→4)-D-xylopyranoside, a structural component of xylomannan [2]. Similar synthetic approach to the structural varification of this THF has been also reported by Crich and Rahaman [3] who has employed their β-mannosylation technique under BSP-$Tf_2O$ activation conditions as the key step. Our synthesis features the use of 2-naphthylmethyl (NAP) ether-mediated intramolecular aglycon delivery (IAD) [4] as the key reaction in obtaining β-mannopyranoside stereoselectively. Various glycosyl donors for NAP-IAD were tested to determine the most suitable for the purposes of this synthesis. Fragment coupling between a disaccharyl fluoride and a disaccharide acceptor obtained from a common β-D-mannopyranosyl-(1→4)-β-D-xylopyranoside derivative obtained through NAP-IAD was successfully carried out to afford the desired tetrasaccharide in the presence of $Cp_2HfCl_2$-$AgClO_4$.

Structural analysis of the resulting synthetic tetrasaccharide using NMR techniques and molecular modeling was performed in order to demonstrate the presence of the proposed xylomannan linkages in this molecule. Both NMR and modeling studies also suggested that the 1,4-linkages resulted in the formation of intra- and inter-residual hydrogen bonding, the stabilizing effects of which cause slight conformational differences between these two linkages, causing $(ManXyl)_5$ to form a single helix. In the planar model structure of xylomannan, all the axial HO-C2 groups of mannoside direct to the same phase, resulting in a difference in hydrophobicity between the two phases of the xylomannan chain.

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P9

# Ice-Binding Protein (IBP) Improved the Cryopreservation Efficiency of Diatoms

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Ice binding proteins (IBPs) lower the freezing point non-colligatively and inhibit ice recrystallization in frozen solutions. Microalgae are the emerging targets for biotechnological applications. Strains of microalgae are maintained by subculturing, which is labor intensive and cost ineffective. Cryopreservation could be an alternative method for long-term storage to reduce cost and genetic drift etc. But some of microalgae such as diatoms are susceptible to cryopreservation. Here we present preliminary data of cryopreservation of Phaeodactylum tricornutum using arctic yeast IBP as an additive cryoprotectant. In this study, we used four cryprotectants (DMSO, glycerol, propylene glycol, and ethylene glycol) with and without arctic yeast IBP. Cryopreservation was carried out in the presence of cryoprotectants either by direct freezing in liquid nitrogen (LN2) or controlled freezing using a controlled rate freezer followed by storage in the LN2 tank. Phaeodactylum tricornutum showed more survival rates in all four cryopreservation media when froze in two steps. Addition of IBP further increased the survival rates of *P. triconutum* in cryprotectant-dependent manner. Especially compared to non IBP-added 10% DMSO, 10% DMSO added with IBP solution increased the survival rate of *P. tricornutum* twice. This phenomenon was the same with ethylene glycol. These results indicate that the ability of arctic yeast IBP to inhibit ice recrystallization increases the survival rate of diatoms dramatically.

P10

# Application of antifreeze glycoprotein for macroporous ceramic products prepared by gelation freezing method

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Freezing of ceramic powder dispersed slurry has gathered great attention as a promising technique to fabricate macroporous ceramics, in which the porosity can be formed by growing of ice crystals in the slurry, the sublimation by vacuum drying and subsequent sintering. However, there were serious problems that the size of ice crystals was not homogeneous in a whole frozen body, leading to inhomogeneous cellular morphology in final ceramic products. In this study, the effect of antifreeze glycoprotein on the cellular properties of the macroporous ceramics prepared by gelation freezing route was examined. The relationship between freezing conditions, antifreeze glycoprotein additives and cell properties has been discussed. The engineering properties of macroporous ceramics prepared by the present method has been also reported, in terms of fluid permeability, mechanical properties and thermal insulation properties, suitable for wide range of applications.

# P11

## On the depression of ice/water interface temperature by adding antifreeze protein and ions to water in a narrow space

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The control of ice crystal growth has been an important issue in various fields, such as the quality maintenance of food texture in freezing or thawing food, and the storage of tissues and organs in hospitals. The adding of antifreeze protein to food or organs has been focused on recently because it is appropriate to control ice growth. Also, a 'mixing effect', in which the freezing point of the solution of antifreeze protein and ions is lower than the sum of the freezing point of antifreeze protein solution and the freezing point of ions in a quasi-equilibrium state, is advantageous for the control of ice growth because ions are naturally present in the substances being preserved. This mixing effect was measured using a nanolitre osmometer by Evans et al. (*Comparative Biochemistry and Physiology,* Part A, vol. 148 (2007), pp. 556 – 561) and Kristiansen et al. (*Cryobiology*, vol. 57 (2008), pp. 122 – 129). However, it has not yet been clarified whether or not a similar effect occurs in the case of thermal non-equilibrium states, which can be seen in the freezing or thawing food and the storages of tissues and organs. Furthermore, the mechanism of the mixing effect has not yet been fully understood.

In the present study, we have carried out experiments on the gradual, unidirectional freezing of dilute solutions of winter flounder antifreeze protein, sodium chloride or sodium permanganate in a narrow space 0.02 mm in thickness. In these experiments, we have observed interfacial shapes by cryo-microscopy, and have measured interface temperature by using a micro thermocouple inserted in the narrow space. We have defined the depression of interface temperature as the difference between the interface temperature of the solution and the interface temperature of pure water. Furthermore, we have measured the local, instantaneous concentration of permanganate ion from the intensity of transmitted light and a calibration curve between the ion concentration and the light intensity. In addition, we have measured the local, instantaneous concentration of winter flounder antifreeze protein by fluorescence microscopy. The mechanism of the mixing effect can be discussed from the results of these two measurements. In our experiments, the growth rate of ice and the concentrations of winter flounder antifreeze protein, sodium chloride and sodium permanganate have been varied.

It is found from our temperature measurement that the depression of interface temperature of the mixed solution is more remarkable than the sum of the depression of interface temperature of the antifreeze-protein solution and the depression of interface temperature of the salt solution. Also, it is found that the concentration of antifreeze protein increases with time, while the ion concentration slightly decreases with time. These time changes in the concentrations are different from the time changes in concentrations of solutions of the single solutes. (The protein concentration near the interface does not increase with time, while the ion concentration near the interface increases with time.) The increase in the protein concentration is probably the reason for the low value of the interface temperature of the mixed solution. Furthermore, it is found that the interaction between the antifreeze protein and the bottom edge of the serrated interface becomes noticeable with a decrease in the interface velocity in the case of mixed solutions. This interaction is the reason for the deterioration of the depression of interface velocity due to an increase in the interface velocity.

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P12

# Enhancement of the depression of ice/water interface temperature by heating polypeptide solutions

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Antifreeze protein is a promising additive for the inhibition of ice growth. However, it has a problem of cost and deterioration. Kun and Mastai produced three polypeptides based on parts of winter flounder antifreeze protein (*Peptide Science*, vol. 88 (2007), pp. 807 – 814). Using an osmometer, they clearly measured a thermal hysteresis in the case of the solution for one of these polypeptides. This polypeptide has the following primary structure: aspartate, threonine, alanine, serine, aspartate, alanine, alanine, alanine, alanine, alanine, alanine, leucine. The cost of synthesizing this polypeptide is clearly lower than that of synthesizing the winter flounder antifreeze protein. Thus, the polypeptide is a cheap inhibitor of the ice growth in a quasi-equilibrium state.

On the other hand, Kun and Mastai did not mention whether or not the antifreeze effect of the polypeptide lasts longer than the antifreeze effect of winter flounder antifreeze protein. Furthermore, the cooling rate and ice growth rate in their experiments were lower than those in some applications, such as cryosurgery. Thus, the validity of the polypeptide has not yet been confirmed.

In the present study, we investigate the following two factors for the polypeptide: (1) ice growth in the polypeptide solution in a thermally non-equilibrium state, and (2) the effect of long-time preservation and heating of the peptide solution on the inhibition of ice growth. For these purposes, using cryo-microscopy we have observed interfacial shapes for the unidirectional freezing of the polypeptide solution in a narrow space 0.02 mm in thickness, and have measured interface temperature by using a micro thermocouple inserted in the narrow space.

It is found that the interface temperature decreases with an increase in the concentration of the polypeptide. The interface has a pectinate shape, which is different from the serrated interface whose bottom edges are connecting to narrow liquid layers between sub-crystalline structures in the case of a solution of winter flounder antifreeze protein. Furthermore, the interface temperature in the case of the polypeptide solution is higher than that in the case of the protein solution. Thus, the interaction between the ice surface and the polypeptide is weak compared with the interaction between the ice surface and the protein in the case of unidirectional freezing.

However, it is striking that the effects of the polypeptide are changed by heating the solution for an hour at 80 °C, and doing so just before the unidirectional freezing. The changes are as follows: (1) The interface temperature becomes lower than that in the case of winter flounder antifreeze protein solution or the unheated polypeptide solution at the same concentration: (2) Narrow liquid layers appear between the ice crystals, which is similar to the morphology in the case of winter flounder antifreeze protein solution at the same concentration: (3) The interface temperature in the case of the polypeptide solution being heated for 1 or 5 hours is clearly lower than that in the case of the solution being heated for longer hours. Thus, the enhancement of the depression of the interface temperature depends on the heating period for the polypeptide solution.

To investigate the reason for the changes in the freezing characteristics which result from heating the peptide solution, we have measured the absorption of ultraviolet and visual light passing through the peptide solution by using a spectrophotometer. It is found from the measurement that the ultraviolet light with a wavelength is in the range 250 – 300 nm is more absorbed in the case of the solution being heated, compared with the ultraviolet light in the case of the solution when it is not heated. This shows that the peptide is agglomerated in the solution by the impact of heating. Thus, a short-period heating is effective for enhancing the antifreeze effect of the polypeptide.

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P13

# Plant-derived antifreeze protein and its application in food preservation under frozen conditions

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In the field of foodstuffs, the freezing process is widely used to minimize the quality changes during storage. It is well known that when foodstuffs containing water are frozen, water molecules are combined with one another to form ice crystals, and the aggregate of the grown ice crystals causes the freeze concentration, thereby destroys the internal structure of foodstuffs. For this reason, methods for inhibiting the freeze concentration during food preservation under frozen condition are demanded. Antifreeze proteins (AFPs) control ice crystal growth and prevent recrystallization, therefore having a potential to reduce the damage caused by freeze concentration. We have recently developed natural AFP material from Japanese radish sprout, which shows potent recrystallization inhibiting (RI) activity. In a variety of processed foodstuffs (wheat noodle, white rice and steamed egg custard etc.), we have revealed the beneficial effects of Japanese radish sprout-derived AFP for food preservation under frozen condition. In aqueous solution (colored water containing monascus red pigment) we have recently provided the evidence that addition of AFP yield ice that is homogeneously colored, which support the notion that AFP reduce the deterioration in the quality of frozen food caused by freeze concentration. Futhermore, by using a Cryogenic Micro-slicer Spectral Imaging System (CMSIS), we have directly observed the internal structure of frozen wheat noodle and found that addition of AFP keep the ice crystal small. The quality preservation of frozen food by AFP is a possible measure to reduce food waste, and these results indicate that AFPs have beneficial advantages in frozen food industry.

P14

# Antifreeze protein dendrimers: when two (or more) heads are better than one

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Type III antifreeze protein (AFP) fused to the much larger maltose-binding protein was originally used to show that AFPs acted individually on the ice surface rather than as an aggregate. Mergers of this nature lead to small increases in thermal hysteresis (TH) activity in proportion to the size of the fusion protein. Much larger increases in TH activity have been achieved by lengthening the ice-binding site (IBS) or doubling the number of ice-binding sites by dimerization. In attempting to further increase the activity of AFPs we have linked them to a dendrimer to significantly increase both size and number of ice-binding sites. Using a heterobifunctional cross-linker we have been able to attach multiple type III AFPs (8-13) and *Mp*AFP (3-4) to a second-generation polyamidoamine (G2-PAMAM) dendrimer with 16 reactive termini. The heterogeneous sample of dendrimer-linked type III constructs showed a four-fold increase in TH activity over monomeric type III AFP. Using microfluidics and fluorescent labeling of the cross-linked AFPs we have been able to demonstrate that these AFP constructs retain the same ice plane binding preferences as the individual AFPs. Thus both type III and *Mp*AFP dendrimers generate the same ice crystal morphology as their free AFPs do. The three-dimensional nature of the dendrimers could allow AFP attachment to multiple ice-crystals. This may be particularly helpful in ice-recrystallization inhibition. Initial IRI results indicate dendrimer-linked type III has more IRI activity than monomeric type III. Linking AFPs together via a dendrimer or other polymers will generate novel reagents for controlling ice growth and help explore the relationship between TH activity, ice recrystallization inhibition (IRI), and ice nucleation.

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P15

# Trapping of water molecules by cations and sites of winter flounder antifreeze protein adsorbed on an ice pyramidal facet

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A cooperative effect, in which the freezing point depression for the mixed solution of antifreeze protein and ions is more noticeable than the sum of the freezing point depression for the antifreeze protein solution and the freezing point depression for the ions solution on their own, has been discussed with experimental results (R. P. Evans et al., *Comparative Biochemistry and Physiology,* Part A 148 (2007), 556 - 561; E. Kristiansen et al., *Cryobiology*, 57 (2008), 122 – 129). In these results, various ions such as and proteins such as were used. However, the mechanism of the cooperative effect has not yet been clarified. One of the present authors has previously carried out molecular dynamics simulation for a mixed solution of sodium chloride and winter flounder antifreeze protein with an ice layer covered with pyramidal facets (Hayakari and Hagiwara, *Molecular Simulation*, 38 (2012), p. 26). In this simulation, we predicted that a part of the protein with the hydrophilic amino acid residues approaches the ice surface. This is because the hydration of the sodium ions near the residues weakens the interaction between water molecules in the solution and the residues. As a result, the interaction between the water molecules on the ice surface and the residues is relatively enhanced. However, the mechanism of the cooperative effect has not yet been clarified.

In the present study, we have carried out molecular dynamics simulation for the salt solutions with an identical ice layer to that mentioned above. The winter flounder antifreeze protein is adsorbed on the pyramidal facet of the ice surface. We have observed that some specific water molecules near the ice surface are nearly stationary for some periods. We have measured the distances between the oxygen atoms of these water molecules, ions nearby and the sites of the protein residues nearby. It is found that the water molecules are located in the hydration shell of the calcium ions and the hydration shells of the methyl sites of hydrophobic residues of the protein for these periods. Thus, the water molecule at the overlapped location of the two hydration shells attenuates noticeably the motion of the ions and water molecules in the hydration shells of the ions. Other water molecules located in the first hydration shells of calcium ions are found to stay for periods of approximately 700 ps. On the other hand, water molecules located in the second hydration shells of calcium ions stays for shorter periods. Consequently, the approach of water molecules – which contribute to the ice growth – to the ice surface is inhibited by the stationary ions and stationary water molecules in the hydration shells of the ions.

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# P16

## Characteristics of antifreeze proteins as supercooling-promoting substances

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Supercooling-promoting substances (SCPSs) depress freezing temperatures of solutions by a non-colligative property. Various kinds of SCPSs including a protein and a polysaccharide from bacteria, terpenoids, polyphenols, synthetic polymers, and surfactants have been reported [1, 2, 3, and listed in 4]. Some antifreeze proteins (AFPs) and glycoproteins (AFGPs) have also been identified as a SCPS [5, 6]. It has been suggested that SCPSs inhibit the activity of heterogeneous ice nucleators by which ice nucleation is inhibited [6] and that AFP and AFGP essentially inhibit ice crystal growth by binding to ice crystals after ice nucleation [7]. Although such an ice binding property of AFPs and AFGPs has been widely examined, supercooling promoting activity (SCA) of AFPs and AFGPs has been examined in only a few studies [6, 8, 9]. In the present study, we examined the SCAs of type I and III AFPs in solutions containing different kinds of ice nucleators, including the ice nucleation bacterium *Erwinia ananas*, silver iodide, phloroglucinol and an air-borne ice nucleator, by a droplet freezing assay using 2-μL droplets [10]. The results revealed that these AFPs exhibited SCAs, though the SCAs changed greatly depending on solutions containing different ice nucleators and different concentrations of AFPs showing similar SCAs between type I and III AFPs. SCAs of solutions in the presence of type I or III AFPs were also analyzed in a bulk solution (1 mL in a microtube) under a static condition or with physical vibration, which is known to disturb supercooling of solutions [11]. The results showed that AFPs also have effects to promote supercooling under the condition of physical vibration, though the SCAs were different between type I and III AFPs.

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P17

# Effects of type-III AFP on growth rates of CO2 clathrate-hydrate film

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Gas hydrates, such as the methane hydrate, are paid attentions as the unconventional natural gas resource, as the storage/transportation material, or as one of the important carbon sink and source in the global system. The control technology of gas hydrates were expected to be developed especially for the industrial applications of gas hydrates. In the oil and natural gas production area, the effective and economical inhibitors were important issue to be developed. Since gas hydrate crystals have similar host lattice structures with ice, in which $H_2O$ molecules connected with four neighbouring molecules by hydrogen bonding, the ice binding proteins are one of the promising material for the environmental-friendly gas hydrate inhibition. In order to investigate the inhibition effect of the ice binding proteins for gas hydrate formation, we observed the lateral growth of $CO_2$-hydrate film on the droplet including type-III anti-freeze protein (AFP). Type-III AFP was found to increase the induction period and to reduce the lateral growth rate of $CO_2$-hydrate films. Based on the comparison of these results with those obtained previous investigations, the type-III AFP worked well at low concentrations, indicating that AFP works as a kinetic inhibitor. It was also indicated that AFP would weaken the memory effect of $CO_2$-hydrate formation. As the similar made from natural material, we also investigated the effect of trehalose on the lateral growth rate of $CO_2$ hydrate film. It was found that trehalose had similar inhibition effects on both the induction period and the lateral growth rate, but it had little apparent concentration-dependence on them. Since trehalose also causes the equilibrium conditions of the $CO_2$ hydrate to shift to lower temperatures, it works not only as a thermodynamic inhibitor but also as a kinetic inhibitor, especially as an anti-agglomerant.

P18

# Backbone Dynamics of the Antifreeze-like Domain of Human Sialic Acid Synthase and Fish Type III Antifreeze Protein

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Antifreeze proteins (AFPs) are found in a variety of cold-adapted organisms including fish, insects, plants, bacteria, and fungi, to promote survival at subzero temperatures by binding to ice crystals and decreasing the freezing temperature of body fluids. One of most widely studied classes of AFPs is the type III AFPs from arctic fish. Particularly, the tertiary structure of HPLC12 type III AFP isoform from the ocean pout, *Macrozoarces Americanus*, has been studied with NMR, X-ray crystallography, mutagenesis, and molecular dynamics.

Sialic acids are synthesized by conserved enzymatic pathway including sialic acid synthase (SAS). Mammalian SAS catalyzes the condensation of PEP and ManNAc 6-phosphate (ManNAc-6P) into NeuNAc 9-phosphate (NeuNAc-9P) which is dephosphorylated into sialic acid. This enzyme consist of two distinct domains, N-terminal catalytic domain (NeuB domain, ~250 amino acid residues) and C-terminal antifreeze-like (AFL) domain (~75 amino acid residues). The NMR structural study of AFL domain of human SAS found that the structure comprises one $\alpha$-helix, two single-turn $3_{10}$-helices, and two $\beta$-strands and is similar to those of the type III AFPs.

In this work, we show the structural and dynamic features as well as the thermostability of human AFL domain and HPLC12 isoform. Comparison of these structural and dynamic properties provides the information that the class-specific residues in the human AFL domains are important for their substrate binding, while those of the type III AFPs are gathered on the ice-binding surface.

P19

# Type I AFP of cottid fishes distributing from the Arctic to the North Pacific Ocean

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Sculpins (teleost fishes; Cottidae) inhabit the high-latitude coastal area from Arctic Ocean to the North Pacific. The generation of its origin was assumed in the northwest America for 30 million years ago, and currently this family is grouped into 70 genus and 275 species. Cold-adaptation of sculpins may move them toward colder regions, because proline-containing type I antifreeze protein (AFP) has been identified from some sculpins. Here, we evaluated the activity of AFP for minced muscle solutions of several sculpins: 1 genera 1 species from the Arctic Ocean, July; 6 genus 9 species from Alaska, March; 4 genus 5 species from Hokkaido, April; 5 genus 5 species from Sado Island, February. One species in the Arctic Ocean and three of Hokkaido in winter samples showed the AFP activity. Five-fold condensed sample of the muscle homogenerates of four of five species in spring Hokkaido also showed the activity. Two samples in Sado also exhibited the activity, but the remaining 3 species did not. These results suggest that 1) among the sub-arctic species, distribution- and depth ranges were assumed to be a factor that made AFP activity different from previously reported species such as *Hemilepidotus*, *Radlinopsis*, and 2) AFP is not expressed for some sculpins living in the temperate region where the lowest temperature is generally above 10 °C.

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# P20

## NMR structure of a defective isoform and its activity-improved variant of type III antifreeze protein form Zoarces elongates Kner

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Antifreeze proteins (AFPs) produced in various cold-adapted animals and plants can specifically bind to ice crystals and inhibit their growth (Fletcher et al. 2001; Graether and Sykes, 2004). The ice-binding ability of AFPs depresses the freezing temperature ($T_f$) of water non-colligatively, which leads to the protection of cells and tissues from freezing. The level of $T_f$ depression has been evaluated by measuring the $T_f$ and melting temperature ($T_m$) for an ice crystal created in an aqueous solution of an AFP. The difference between these two temperatures is defined as the thermal hysteresis (TH), which is a measure of the AFP's ice-growth inhibition. AFPs also modify the shape of an ice crystal uniquely, hexagonal bipyramid for example, in the temperature range of TH. These two activities have been assumed to be common for all AFPs; however, it has recently been found that they are not always the rule for every species, such as the AFP type III (denoted AFPIII) found in fish (Takamichi et al. 2008).

Fish AFPIII is a globular protein made up of many short b-strands and one helical turn. AFPIII is generally produced *in vivo* as a mixture of quaternary-amino-ethyl (QAE)-Sephadex- and sulfopropyl (SP)-Sephadex-binding isoforms. These two isoforms possess different pIs and share approximately 50% sequence identity. The Japanese notched-fin eelpout, *Zoarces elongates Kner*, produces 13 different AFPIII isoforms (denoted nfeAFP), which have been divided into six SP (nfeAFP1-6) and seven QAE (nfeAFP7-13) isoforms, and the latter was further divided into QAE1 (nfeAFP7-10) and QAE2 (nfeAFP11-13) isoforms (Nishimiya et al. 2005). Among them, only the QAE1 isoforms are fully active variants exhibiting both TH and ice-shaping activities, while the others only shape the ice morphology. The reason for such defective activity for the SP- and QAE2-isoforms is not well understood.

Recently, we found that alterations of the 9$^{th}$, 19$^{th}$, and 20$^{th}$ residues of a QAE2 isoform make them the same as their counterparts in the QAE1 isoform, leading to the successful conversion of the former into a QAE1-like fully active isoform (Garnham et al, 2012). That is, the QAE-2 isoform nfeAFP11 can only shape ice crystals, but has no TH activity, while its triple mutant nfeAFP11-V9Q/V19L/G20V (denoted nfeAFP11-tri) perfectly exhibits both activities. These residues were thought to construct a "compound" ice-binding site (IBS) that consists of two adjacent flat surfaces inclined at an angle of approximately 150° to each other (Garnham et al. 2010). To determine if the triple mutations in nfeAFP11 led to changes in the structure of the IBS that could affect its ice-binding activity, the multidimensional NMR spectra of both nfeAFP11 and nfeAFP11-tri were examined. Special attention was paid to the surface-bound water molecules, for which the unique role of anchoring the IBS to ice lattice has been postulated (Garnham et al. 2011; Kondo et al. 2012).

The tertiary structures of AFPIII have been determined mostly for the QAE1-isoform HPLC12, which was identified in the ocean pout, *Macrozoarces Americanus*, by NMR and X-ray analysis. The currently available structural database for AFPIII includes NMR structures (Sönnichsen et al. 1996; Miura et al. 2001), X-ray structures (Graether et al. 1999; Antson et al. 2001), and a neutron structure (Howard et al. 2011). On the basis of these data, Howard et al. identified an ice-like geometry for four of the water molecules bound to a pocket of the IBS formed by Gln$^{9}$, Thr$^{18}$, Val$^{20}$, and Met$^{21}$, for which the anchoring role that leads to the AFP-ice interaction was assumed. In the present study, a set of two- and three-dimensional (2D and 3D) NMR spectra for the defective (nfeAFP11) and active (nfeAFP11-tri) isoforms of AFPIII were obtained. Then, the solution NMR structures were determined, and the locations of the surface water molecules in the IBS were examined through nuclear Overhauser effect spectroscopy (NOESY) experiments.

P21

# A 40-residue Type I Antifreeze Protein exhibits an extremely high thermal hysteresis

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*Many species of organisms* accumulates biological substances in their body fluid so as to survive under icy environments. Antifreeze protein (AFP) is one of such substances identified from several fishes, plants, insects and microorganisms, which functions to inhibit growth of ice nuclei generated in their body fluid. Fish AFPs have been categorized into type I–IV on the basis of their structure. Among them, type I AFP is a monomeric $\alpha$-helical peptide, a well-known isoform of which is the 37-residue peptide identified from a flat-fish named Winter flounder. We have recently identified a new 40-residue type I AFP from a righteye flounder living off the northern east coast of Japan, *which* shares 80% sequence identity with that of WfAFP. Significantly, this AFP1 is highly dissolved in water to be a maximal concentration of 700 mg $mL^{-1}$ (approx.), and exhibited 3 °C (approx.) of high thermal hysteresis (TH) activity at that concentration. At the lower limit of TH, the ice crystal bursting uniquely occurs from the prism and/or pyramidal planes, but not from the two tips of the ice bipyramid, in the highly concentrated solution. In the IBP2014 meeting, we will present such a unique properties of this AFP1 exhibiting ultra high solubility, for which FIPA analysis and NMR spectroscopy have also been employed to elucidate the factors responsible for such uniqueness.

P22

# Dendrimers at play as an antifreeze plastic

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Unlike polymers, dendrimers are monodisperse macromolecules with a highly branched, compact three-dimensional structure. The exterior surface of dendrimers can be modified with functional groups leading to diverse multivalent systems. These structural properties have made dendrimers attractive candidates for various applications, such as imaging agent, catalysis, drug delivery and material science. Here, we aim at synthesizing an ice-binding macromolecule with freezing hysteresis (FH) and ice-recrystallization inhibition (IRI) activity by modifying the functional groups on the periphery of poly(propylene imine) dendrimers. Hydroxyl groups are introduced in two simple steps, by first a Michael addition of methyl acrylate followed by a reduction step. A library of dendrimers of different generation and with different end-groups is prepared, after which their physicochemcial properties are characterized and finally tested for ice-binding activity.

P23

# Crystal structures of the wild type and A20V-mutant for the Anti Freeze Protein AFP6 Derived from Japanese fish Zoarces elongatus Kner

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Type III antifreeze protein from the Japanese fish Zoarces elongatus Kner. is naturally expressed as a mixture of AFPs with different net charges. Although nfeAFP6(66aa), one of the sulfopropyl(SP)-resin binding isoforms and nfeAFP8(66aa), one of the quaternary aminoethyl(QAE)-resin binding isoforms share sequence identity approximately 55%, the former cannot arrest the ice crystal growth effectively, the speed of which is 1,000-fold faster than that of the latter.

In order to identify the region responsible for this difference in their biological function, four chimeric AFPs composed of nfeAFP6 and 8 were designed and prepared using E. coli expression system. The result showed that the N-terminal region (1-33) is a key component to the difference. The N-terminal region responsible for AFP activity was further investigated by site-directed mutational analysis on the ice binding sites. As a result of assay for the anti-freezing activity of six kinds of nfeAFP6 mutant, it was found that most of the mutant nfeAFP6s showed similar activity to that of the wild type nfeAFP6 whereas P18L/A19V-mutant nfeAFP6 showed strong anti-freezing activity similar to that of the wild type nfeAFP8, consistent with the result reported previously [1,2]. To further confirm the importance of the two residues, independent mutants, P19L and A20V mutation were incorporated into nfeAFP6 and the opposite mutation L19P/V20A were incorporated into the nfeAFP8. As a result, P19L mutant of nfeAFP6 showed little increase in anti-freezing activity whereas the mutation A20V in nfeAFP6 resulted in similar activity compared to that of nfeAFP8, indicating that Val20 is essential to reveal anti-freezing activity.

Crystal structures of the wild type and the A20V-mutant AFPs were determined to 1.2 and 1.8 Å resolution, respectively, by X-ray crystallography to investigate the difference of hydration structure around the essential area. The space group of both crystals belongs to $C222_1$, and the overall structures of the wild type and A19V-mutant AFPs were almost similar. The side chain of Val20 in A20V-mutant faces to the side chain Gln9, and make van der Waals interactions with Gln9 and Thr18. We found the locations of some bound water molecules at the mutated region in A20V-mutant were different from those in wild type. These observations may illustrate the complexity of what hydration structure constitutes to an ice-binding and anti-freezing activity. Further structure analysis of mutant AFPs using a neutron crystallography is in progress.

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P24

# Structural studies of bacterial ice nucleation proteins and their relevance for atmospheric processes

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Microorganisms have been proposed to play a role in shaping the Earth's climate by acting as ice nuclei (IN), thus influencing the formation of clouds. This can have significant implications for the global distribution of clouds and precipitation, and consequently for Earth's weather patterns and climate. Similar to Earth,, exoplanetary atmospheres may provide information on the possible presence of life on those planets. In this fashion, atmospheric phenomena such as planetary albedo, which may be influenced by ice nucleation active (INA) bacteria, may serve as potential biosignatures.

However, before biologically induced atmospheric processes can be used as reliable biosignatures for life detection in other worlds, a thorough understanding of how and to what extend INA bacteria influence atmospheric phenomena on Earth is needed. Consequently there is a need to determine the ice-nucleating property of living organisms by investigating the structure and properties of INA proteins (INP). Several bacterial species that have been found in the atmosphere produce INP. Situated on the cell-surface of bacteria, INPs have the capability of facilitating the formation of ice crystals in supercooled water by acting as an ice template [1] and inducing ice formation at temperatures much higher than mineral aerosols, which are generally considered the most abundant IN in the atmosphere. We will investigate the role of INA bacteria in cloud formation by answering the fundamental question: by which mechanism(s) do bacterial INPs facilitate ice crystal formation? This will be accomplished by elucidating the atomic structure of an INP.

Hitherto, different groups have presented INP models based on homology modeling efforts revealing an elongated, $\beta$ helical protein and an ice binding mechanism [2][3]. However, little has been done experimentally to validate the models and to determine the tertiary structure of an INP. We aim at experimentally determine the structure of the highly conserved repeat domains of INPs, which may yield insights into their ice nucleation mechanism. Given their important function for bacterial ice nucleation, we will focus on the functional analysis and protein structure determination of individual INP domains. To study INP, we sequenced the INA gene from a bacterial strain designated *Pseudomonas* sp. R10.79 that was isolated from a rain sample,. Additional functional analysis of INP from *Pseudomonas* sp. R10.79 will be continued using molecular biology techniques including molecular cloning, protein purification in combination with X-ray crystallography. In the long-term, we hope that our detailed studies of the molecular structure of INP will hold a key to finding life on other planets.

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# P25

## An Ice Nucleation Protein from an Alpine Tussock

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We describe here the initial characterization of an ice nucleation protein from the New Zealand alpine tussock *Chionochloa macra* (Bannister, 2005; Wharton et al., 2010). Alpine regions in New Zealand have a broad seasonality are cold in winter and warm in summer, but are also subject to freezing in any month of the year (Sinclair, 2001). Plants and animals in these regions show less seasonal variation in their tolerance of freezing temperatures than their northern hemisphere counterparts. Little is known of the mechanisms by which plants tolerate subzero temperatures in the New Zealand alpine region, but we have previously identified an ice nucleating protein in extracts of *C. macra* (Wharton et al., 2010).
The candidate protein was purified from plant extracts using serial ice finger purification (Kuiper et al., 2003). Subsequent purification by preparative gel electrophoresis and size exclusion chromatography revealed a protein of >150 kDa that produced a supercooling temperature of ≈ -5° C in ice nucleation spectrometry (Wharton et al., 2004). Ice nucleation activity was sensitive to NaCl above about 150 mM that precluded the use of ion exchange or hydrophobic interaction chromatography. The activity was stable at 70° C for 2 h but inactive at 90° C after 15 min. Mass spectrometry of tryptic fragments of the protein produced strong, clear spectra but we could find no match in search databases suggesting no homologous protein has been characterized.
Current work involves sequencing RNA from *C. macra* so that a transcriptome can be constructed. These data can be matched to protein data obtained from de novo sequencing by mass spectrometry and the gene so identified can then be isolated and cloned into a suitable vector for expression.

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P26

# Growth mechanism of ice basal planes in antifreeze protein type III solution

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Antifreeze proteins (AFPs) completely inhibit ice growth in water at temperatures even below the equilibrium melting point. The difference between the melting point and the nonequilibrium freezing point at which ice crystals start to grow is called thermal hysteresis. When a small single crystal of ice, up to several tenth micrometers in size, is immersed in a fish AFP solution within the thermal hysteresis gap, the ice crystal grows into a hexagonal bipyramidal shape before it stops growing. This bipyramidal shape makes one to think that the fish AFP molecules interact not with the basal planes of ice, but with pyramidal planes with higher indices. However, when a single crystal of ice that have the basal plane of the order of $mm^2$ or larger is grown in a fish AFP solution within the thermal hysteresis gap, numerous pits, consisting of six (or twelve) pyramidal planes, are left on the basal plane. Assuming that the basal plane is molecularly smooth, this pit formation should be triggered by interaction of the fish AFP molecules with the basal planes.
In this study, to discuss the interaction of fish AFP molecules with the basal planes of ice, we observed pit formation on the basal plane growing in a 5 mg/mL AFP type III solution within the thermal hysteresis gap, and examined the growth mechanism based on the measured growth rate. In the beginning of the observation period, because the basal plane was molecularly rough due to the procedure of ice preparation, extremely high growth rates caused by adhesive growth were observed. Then, the growth rate decreased rapidly with time, the surface changing into molecularly smooth; the growth mechanism was dominated by either spiral growth or 2D nucleation growth depending on the degree of supercooling. The number density of pits per unit volume of ice was evaluated for each growth mechanism. The results showed that AFP type III molecules caused pit formation by interacting not only with rough surfaces, but also with smooth surfaces of the basal planes.

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P27

# Antifreeze activities of an anaerobic bacterium isolated from permafrost

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Many microorganisms have adapted to cold environments by producing cryoprotectants such as sugars, glycerols and so on. Antifreeze proteins (AFPs) also function as a cryoprotectant through binding onto ice crystals to inhibit their growth, resulting in decreasing of the freezing point of water and protecting cells from freezing injury. AFPs have been found in various microorganisms, such as bacteria, fungi, lichen and algae, while anaerobic-microorganism-derived AFP is unknown. In this study, we examined antifreeze activities of an anaerobic bacterium in order to find a new class of AFPs. The bacterium was isolated from a permafrost sample in the Canadian High Arctic and described as *Clostridium tagluense* by Suetin et al. (2009). This bacterium is psychrotolerant and grows at temperatures between 4 and 28 ° C (optimum 15-20 ° C). We cultured it under anaerobic atmosphere at 4 ° C and the antifreeze activity of the culture media was examined by observation of the ice crystal morphology using a microscope equipped with a temperature-controlled stage. It observed a formation of hexagonal ice crystals, suggesting secretion of AFP from *C. tagluense*, which was further verified by SDS-PAGE.

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P28

# Towards large scale expression of hyperactive AFPs for food and cryobiology applications

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Antifreeze proteins (AFPs) are proteins that have evolved in various organisms permitting their survival in subzero environments. AFPs exhibit remarkable structural diversity and molar activities across the various kingdoms. Insects are known to produce highly active proteins (e.g., hyperactive). For example, the longhorn beetle *Rhagium inquisitor* has the ability to supercool to below -25 ° C partially due to the presence of a highly potent AFP (RiAFP) in its hemolymph (Kristiansen, et al. 2005, 2011).

Hyperactive AFPs possess a vast potential in cryopreservation of cells, tissues and organs, however a very limited research was done due to the lack of sufficient availability of these proteins. Our research focuses on large scale production of hyperactive AFPs which are becoming increasingly important not only for scientific research but also for applications. The production and utilization of the AFPs should encompass expression of the proteins in a substantial amount, purification to a desired purity, and investigation of the functionality of these proteins in applications such as cryopreservation.

Hakim and coworkers reported the successful overexpression, purification and crystallization of full-length RiAFP (Hakim, et al. 2012). As a first step we would like to follow on this work by expressing a fusion construct containing an N-terminal enhanced green fluorescent protein (eGFP). Such plasmid was generously donated by Aaron Hakim (Yale University, New Haven). In addition we are developing modified plasmids in order to improve expression.

Our future plans comprise optimization of bacterial expression conditions and media using a fermentor which enables continuous growth and protein production in high-cell-density. Recombinant protein production will be maximized in autoclave bioreactor and measured parameters will be used to determine the specific growth rate, and specific product formation rate.

The next vital step is scaling up purification techniques. AFPs can be purified using ice affinity purification such as the cold finger apparatus developed in the Davies lab (Kuiper, et al. 2003). The next purification step will comprise further development of such techniques.

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# P29

## Ice Recrystallization Inhibition by Ice Binding Proteins

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Ice recrystallization (IR) is one of the major causes of smooth texture loss and deterioration of frozen food such as ice cream during storage. In cryopreservation, recrystallization during thawing damages cells and tissues due to membrane rapture and cell dehydration, reducing the survival rates. Ice Binding Proteins (IBPs) originating from plants, fish, and insects were shown to directly adsorb onto ice crystal surfaces and to inhibit IR, thus may serve as additives for many applications in which ice recrystallization is an obstacle. Nevertheless, the mechanism by which IBPs inhibit IR is unknown and the relation between IR inhibition (IRI) and thermal hysteresis (TH) activity has not been revealed. Particularly, it has been shown that although insect AFPs have considerably higher TH activity than AFPs from fish and plants, their IR inhibition activity is in the same range or lower[1]. In our study, a method which allows quantitative evaluation of IRI activity has been developed based on the work of Budke et al.[2] and our previous work[3]. In this method, protein solutions were diluted in 45% sucrose and frozen at −50 °C. The samples were warmed to annealing temperatures of around −5 °C, at which recrystallization was observed. Determination of recrystallization rate is done by calculating the mean crystal radius at different time points during the annealing period while the total ice volume is constant. Image processing was done by ImageJ software. In order to obtain the recrystallization rate, the theory of Lifshitz Slyozov Wagner (LSW) describing Ostwald ripening processes was employed. Using this system, IRI activity of both hyperactive and moderate IBPs were investigated and compared. Fluorescently-labeled IBPs were also used to investigate protein accumulation on the ice crystals. Initial results show that IBPs accumulate on ice crystals while protein is depleted from the solution. We also found that different IBPs have different levels of IRI and as a result, the lowest concentration in which the protein still has IRI activity varies from protein to protein. We were able to measure significant IRI activity at concentrations of 0.5 μM and lower. We plan to use this method for investigating IRI of more types of IBPs. Additionally we intend to examine the interactions of the protein with ice and suggest a possible mechanism of IRI by IBPs, in particular to explain the inconsistency between IRI activity and the TH activity of hyperactive IBPs. Understanding the mechanism of IRI may improve and expand the use of IBPs in many applications in the medical sector, cryopreservation, and the frozen food industry.

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P30

# On the correlation between adsorption rate of AFPIII to ice and thermal hysteresis; an experimental study

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The nature of AFP's binding to ice is still debated in this field of research; including the issue of whether these proteins bind to ice irreversibly or reversibly. We have previously shown that hyperactive *Tm*AFP remains bound to the ice surface after removal of the AFP in solution surrounding the crystal (Celik, Drori *et al.* 2013). This and other experiments showed compelling evidence for irreversible binding of hyperactive AFPs to ice. Here we have tested the moderately active type III AFP in the same in-house developed microfluidics system by removing the free AFPIII solution from around an AFP-coated bipyramidal crystal. We found that the AFP was irreversibly bound to the ice, because the fluorescence intensity on the ice surface remained constant after AFP solution removal. However, some crystals "burst" during the solution exchange. We found a direct correlation between the size of the basal face at the tips of the crystal and the tendency to burst at higher sub-zero temperatures. AFPIII, like other moderately active AFPs, is unable to bind to the basal plane of ice. Knight and DeVries have suggested that the non-equilibrium freezing temperature is dictated by the AFP adsorption rate (Knight and DeVries 2009). An experimental characterization of the adsorption rate of AFPs is absent from the literature.

We tested the significance of the adsorption rate of AFPIII on freezing point depression by melting the crystal tips with an IR laser. At a high AFP concentration (40 $\mu$M), after melting the crystal tips with laser, ice growth was inhibited. However, at a lower concentration (10 $\mu$M) crystal tip-melting led to an uncontrolled ice growth, showing the importance of adsorption rate on inhibiting ice growth. The adsorption rate ($K_{on}$) of AFPIII was measured using fluorescence microscopy and found to be $0.008 \pm 0.001\ \mu M^{-1}\ s^{-1}$ (Drori, Celik et al. 2014).

In conclusion, we show that moderately active AFPs bind to ice irreversibly. However, unbound moderate AFPs are needed to inhibit ice growth from the bipyramidal crystal tips because, unlike the basal plane-binding hyperactive AFPs, moderate AFPs cannot bind to the basal face; instead, these AFPs minimize its area by inhibiting growth of other crystal planes.

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P31

# Disulfide-braced solenoid fold predicted for new antifreeze protein from a fly

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A novel antifreeze protein (AFP) has been identified in Lake Ontario midges (Chironomidae). The midge AFP is expressed as a family of isoforms at low levels in the adults, which emerge from fresh water in spring before the threat of freezing temperatures has passed. The 9.1-kDa major isoform is glycosylated and has a ten-residue tandem repeating sequence xxCxGxYCxG, with regularly-spaced cysteines, glycines, and tyrosines comprising half the residues. Modeling and molecular dynamics predict a tightly wound left-handed solenoid fold in which the cysteines form a disulfide core to brace the ten-residue coils. The solenoid is reinforced by intrachain hydrogen bonds, side chain salt bridges and a row of stacked tyrosines on the hydrophobic side that forms the putative ice-binding site. A disulfide core is also a feature of the similarly sized beetle AFP that is a $\beta$-helix with twelve-residue coils and a comparable circular dichroism spectrum. The midge and beetle AFPs are not homologous and their ice-binding residues are not alike, with the latter comprised of two parallel arrays of outward-pointing threonines. However, their structural similarities provide information about the physical features needed for a protein to bind ice as well as an amazing example of convergent evolution to cope with change to a colder climate.

# P32

## Novel role for an ice-binding protein – adhering an organism to ice

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Ice-binding proteins (IBPs) have long been known to help organisms resist freezing (antifreeze) or tolerate freezing (ice recrystallization inhibition). A recently discovered third application is host adhesion to ice (1). The IBP found on the cell surface of an Antarctic Gram-negative bacterium, *Marinomonas primoryensis,* is part of an exceptionally large protein of 1.5 MDa. As seen by light microscopy the highly motile *M. primoryensis* swims to ice and attaches to the surface to form bacterial clusters. Here we suggest that *Mp*AFP facilitates the formation of bacterial communities or biofilms underneath lake or sea ice, where oxygen and nutrients are most abundant due to the photosynthetic activity of other microorganisms. *Mp*AFP can be divided into five distinct regions. The N-terminal end (RI) attaches to the bacterial cell envelope, likely by binding to the cell wall peptidoglycan or other polysaccharides on the cell surface. Next are ~ 120 identical tandem repeats of a 104-aa Ig-like domain (RII) that makes up 90% of the protein. Region III, of unknown structure and function, separates the highly repetitive region II (RII) from the moderately repetitive region IV (RIV), The 34-kDa RIV is the ice-binding domain of *Mp*AFP, which folds as a $Ca^{2+}$-dependent $\beta$-solenoid with 19-aa coils that each coordinate an internal calcium ion at RTX repeats along its ice-binding site (2). At the C terminus RV also contains $Ca^{2+}$-binding RTX repeats that may serve as the secretion sequence for the giant protein. All domains of *Mp*AFP bind $Ca^{2+}$ for a total of several hundred. Biophysical analyses show how additional $Ca^{2+}$ bound to the hinge region between Ig-like repeats in RII rigidify the protein into a 0.6-μm rod that projects the ice-binding domain away from the bacterial surface (3, 4). Furthermore, X-ray crystallography showed tandem arrays of the Ig-like repeats bind to each other in an antiparallel fashion. Thus *Mp*AFP not only binds the bacteria to ice but can potentially link the bacteria together in clusters to increase the total number of adhesins binding the group to the underside of ice where the environment is most favourable for growth. This property explains the swarming behaviour of *M. primoryensis* when they encounter ice, and gives insight into the biofilm formation of other bacteria, including those of human pathogens.

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P33

# The ice recrystallization inhibition by AntiFreeze proteins: a game for many players

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Due to their peculiar ability to halt ice crystals growth at very low concentrations, *AntiFreeze* proteins has gained wide attention as an effective tool in the manufacturing of frozen goods. In an attempt to investigate the ice recrystallization inhibition (IRI) properties of AFPs from plants, we produced a recombinant Pathogenesis Related protein (rPRp) which was reported to be expressed in the pool of AFPs isolated from apoplastic fluids of cold acclimated (CA) plants. rPRp failed to inhibit ice crystals growth in *cold stage* microscopy IRI assays in a wide range of concentrations (mg/ml), although experimental evidences suggests that the protein was properly folded by the heterologous host. Given that some AFPs from plants and insects were reported to be more active when they were tested in their physiological *milieu*, we then decided to test whether the rPRp could acquire its functions after being added to apoplastic fluids of CA plants. CA seedlings yielded apoplastic extracts that were effective in reducing ice crystals growth in IRI assay, this effect being protein mediated as confirmed by the lost of activity observed after protease treatment. When the rPRp protein was added to protein extracts from CA plants, the average dimensions of ice crystals were significantly reduced, while no such effect was observed when the protein was tested in extracts from Non Acclimated (NA) plants. IRI enhancement was also noticed when other proteins that were intended to be used as a control were added to IR active extracts, to the same extent of what was observed with the rPRp. The enhancement effect was even greater when the proteins were added to plants extracts that were diluted to a point at which IRI activity was barely detectable. This is not the first report of AFPs being enhanced by other ice binding inactive compounds, as it was shown with AFPs within the body fluids of overwinter insects, where both long range and protein protein interaction were shown to be implicated in the increase of Thermal Hysteresis (TH) values upon addition of small solutes and proteins to the purified AFPs. Based on some experimental evidences and the hyperactivity of larger AFPs, it was speculated that proteins with larger binding sites can stick to the ice more efficiently, as well as preventing further additions of water molecules to it. Preliminary experiments to identify ice binding proteins whose activity may be enhanced by interacting with our rPRp are in progress. Our data taken together suggests that even proteins with no ice structuring ability can act as enhancers of AFPs with IRI properties, in a way that doesn't seem to be dependent on a tight molecular recognition mechanisms.

P34

# Mutagenesis study of an antifreeze protein isoform from a snow-mold fungus, *Typhula ishikariensis*

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Antifreeze proteins (AFPs) facilitate cold-survival of the organisms living under freezing environment by inhibiting ice crystal growth. The binding of AFP to ice results in a depression of the freezing point ($T_f$) of solutions below its melting point ($T_m$), generating a temperature difference between $T_m$ and $T_f$ which is referred to as "thermal hysteresis (TH)". A snow mold fungus, *Typhula ishikariensis,* secretes AFP (TisAFP) as a mixture of seven isoforms exhibiting high sequence identity. We recently solved the crystal structure of TisAFP6, an isoform showing a moderate TH activity. Another isoform, TisAFP8, exhibited a potent TH activity. To clarify the structural basis that determines ice-binding strength of the fungal AFP isoforms, we examined mutational effects on the TH activity of TisAFP8 by referencing to the TisAFP6 structure.

TH activity of a mutant G151D was increased 2.5-fold compared with that of TisAFP8-wt, suggesting the importance of outward-pointing residues. Another mutant L152V lost approximately 50% of TH activity of TisAFP8-wt, which was assumed to be an inward-pointing residue and suggests that L152's inward-pointing side chain is crucial to locate the outward-locating side chains so as to bind TisAFP8 properly with ice. Taking into account that TisAFP8 is capable of binding to the multiple ice planes, the IBS of TisAFP8 may recognize more different sets of ice water atoms compared with TisAFP6.

Besides, significantly, fluorescence-based ice plane affinity (FIPA) analysis showed that the ice binding specificity of TisAFP8 is quite different from the other AFPs.

P35

# A Study on Cold Temperature Acclimation of *Cyprinus Carpio* (Common Carp) in Upland Regions of Himalayas

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The common carp, *Cyprinus carpio* is generally inhabitant of warm water (best growth between 23 °C to 30 °C) and found to be in lakes, ponds, reservoirs and streams in India. Though, it is a warm water species, but it is also found to be adapted to very low temperatures in the Himalayan water system where temperature drops down to 4 – 5 °C during winter. The current study was undertaken to investigate the adaptive mechanisms of this species when it thrives in extreme cold temperature. It has also been reported earlier that in temperate fishes, like Rainbow smelt (*Osmerus mordax)* accumulate high levels of Glycerol in winter that serves as a protecting mechanism to low temperature. Hence, the aim of present study was, to study the enzymatic activities of the enzymes involved in Glycerol production and to isolate the transcript encoding GPDH (Glycerol - 3- phosphate dehydrogenase) gene involved in glycerol synthesis and its gene expression analysis of the common carp in Indian high altitude water bodies.

Live fish samples of *C. carpio* were collected from DCFR, Bhimtal hatchery, when the water temperature was 15 °C. They were transferred to the wet laboratory and acclimatized for 7 days. After acclimatization, fishes (5 in each group) were maintained in three different experimental groups. One tank was maintained at approximately 5 °C by adding ice flakes, one at ambient water temperature (15-18 °C) and other at 30 °C by using the thermostat. The sampling of blood and other tissues were carried out after 96 hours.

After 96 hours, the mean glycerol level in the fishes at 5 °C was found to be 10 mM $L^{-1}$ (20 fold) as compared to 0.542 mM $L^{-1}$ in the fishes at 30 °C.The enzymatic activities of all the three major enzymes i.e, Glycerol - 3- phosphate dehydrogenase (GPDH), Alanine aminotransferase and Aspartate aminotransferase were found to be increased under low temperature. The transcript encoding GPDH gene was PCR amplified, cloned and sequenced. From the deduced sequence, specific RT PCR primer pair were designed to study the tissue specific gene expression and expression analysis under different thermal regimes. Among the different tissues collected, the highest gene expression was found to be in liver. The expression analysis under different thermal regimes shows the concurrent results to the GPDH enzyme activity. All the results are found to be statistically significant ($p < 0.05$) by using one way ANOVA (SPSS V19.0).

Thus these preliminary results revealed that the low temperature challenge is sufficient to active the glycerol production in common carp, *Cyprinus carpio* which makes them to survive at low temperature in the upland regions of the Himalayas during winter.

**Key Words**: Glycerol, cold acclimation, Glycerol - 3- phosphate Dehydrogenase

# P36

## Molecular modelling of Biomimetic Antifreeze Polymers

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Ice crystal growth during storage of tissues presents a serious problem to the cryo-preservation of organs, food and crops in cold climates. In the UK alone the waiting list for organ transplantation currently exceeds 7000[1], and it is important that no organ is wasted. Inspired by antifreeze proteins and glycoproteins (AF(G)Ps), which permit the survival of organisms in sub-freezing climates, researchers are now seeking better and cheaper synthetic alternatives to prevent ice crystal growth or reduce them into manageable sizes. Recently, our collaborators demonstrated the remarkable ability of low concentrations of polyvinyl alcohol (PVA) to preserve mammalian red blood cells after freeze-thawing. However, molecular mechanisms of antifreeze actions of PVA are not known.

Here we employ molecular dynamics computer simulations to address this challenge. This understanding will enable us to correlate the structural properties of this polymer with different antifreeze characteristics e.g. thermal hysteresis, dynamic ice shaping or reduced ice recrystallisation, and facilitate the design of new, improved antifreeze polymers for specific control over crystal size and morphology. Our initial work has focused on PVA, and its antifreeze inactive isomer, polyethylene glycol (PEG). We have simulated various chain lengths of PVA and PEG in solution and at the ice/water interface using the TIP4P/Ice water model and the OPLS-AA forcefield for the polymers.

One hypothesis is that the antifreeze molecules structure liquid water either in an ice-like configuration, which could promote binding to a growing ice crystal, or in a disordered configuration that could inhibit ice crystal growth. Our simulations indicate that PVA does not have significant ordering or disordering effect on surrounding water molecules in solution. On the other hand the O-O separation of PVA in solution is 0.46 nm (compared to 0.31 nm and 0.39 nm in PEG), which corresponds to the O-O separation distance on the prism plane of ice, suggesting that direct binding to ice may play an important role in the mechanism. We are currently investigating the interactions of PVA and PEG with an ice/water interface and how this affects the rate of ice crystal growth from two-phase coexistence studies. Ultimately the development of these polymers may prove as better alternatives to conventional cryprotectants.

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P37

# An ice-nucleation protein fragment with hyperactive antifreeze protein activity

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Ice-nucleation proteins (INPs) initiate water freezing at high subzero temperatures. To examine the relationship between INPs and ice-binding proteins we have produced in *E. coli* a 26.5-kDa truncated *Pseudomonas syringae* INP containing thirteen central 16-residue repeats and the C-terminal region. The $His_6$-tagged truncated INP was purified by Ni-NTA Agarose and anion-exchange chromatographies. It shaped ice during melting into a smoothed hexagonal bipyramid and produced thermal hysteresis values of up to 0.9 ° C. This activity is somewhat greater than that reported by Kobashigawa *et al.* (2005) for a smaller construct of the *P. syringae* INP. When the freezing temperature was exceeded the ice crystal 'burst' away from the *c*-axis in a floral pattern with hexagonal symmetry. This property is consistent with that of a hyperactive antifreeze protein. The circular dichroism spectrum of the truncated INP shows some similarity to those of hyperactive ß-helical antifreeze proteins. We have recently labeled the protein with a fluorescent tag to determine the ice planes to which it can bind. These data support the hypothesis that the functional difference between antifreeze proteins and INPs is due to the respective sizes of their water-organizing/ice-binding surfaces.

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P38

# Is the anchored clathrate water hypothesis a general mechanism for IBP binding to ice?

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Computational simulations first suggested that the ice-binding sites of antifreeze proteins (AFPs) might organize surface waters into an ice-like array that could merge and freeze with the quasi-liquid layer water on the surface of ice. An example of this organized water can be seen all along the ice-binding site of a bacterial *Mp*AFP in its crystal structure. Here the waters, which form cages (clathrates) around methyl groups, are hydrogen-bonded ("anchored") to nearby side chains and backbone peptide amides. In many ice-binding protein (IBP) crystal structures the extensive, flat, relatively hydrophobic ice-binding sites form mutual crystal contact surfaces. Residual waters in the protein sandwich are somewhat ice-like, but are too few to prove the point. Computer simulations using TIP5 water parameters support the inclusion of these waters into ice-like surface arrays from which the extra waters are presumably squeezed out during the formation of protein-protein crystal contacts. We suggest the association of these ice-binding sites in crystallized IBPs might be facilitated by the tendency of the two identical ice-like water networks to fuse together, as predicted for the ice:IBP merger. Support for this idea comes from the crystal structure of Maxi, a large helix-bundle isoform of the alanine-rich type I AFP. In Maxi, the ice-binding i, i+4, i+8 residues (Thr, Ala & Ala), point inwards from all four helices and help coordinate their surface waters into two intersecting networks of ~400 internal ice-like waters that hydrogen bond together to stabilize the protein's fold. Thus protein-protein contacts in IBP crystals and Maxi could be considered as further proof for the anchored clathrate water hypothesis for the ice-binding mechanism. We continue to look for examples of IBSs in IBP crystal structures that are freely exposed to solvent, and will present an example where solvent exposure to an IBS has been achieved by crystalizing an AFP as a fusion to a much larger protein.

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P39

# Stress Responsibility and Structure of Intracellular Antifreeze Protein from Antarctic Marine Diatom

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Antifreeze protein (AFP) has an ability to bind ice crystals and cause the thermal hysteresis (TH) which lowers the freezing temperature. In this study, the structure, protein expression, ice-binding site, and function of antifreeze protein from Antarctic marine diatom, Chaetoceros neogracile, were analyzed. In silico analysis of the Cn-AFP promoter revealed that it contained various stress-responsive motifs such as light- and temperature stress-responsive elements. For identifying the promoter activity of these motifs, Northern and western blot analyses were conducted. The Northern and western analysis showed that there was a dramatic increase of Cn-AFP transcripts and proteins expression levels when the cells were placed under freezing (-20° C), thermal (10° C) and high light (600 $\mu$mol·photons·$m^{-2}s^{-1}$) stress. Also, Cn-AFP clearly showed a thermal hysteresis activity and ice crystal morphology. The maximum TH values of recombinant mature Cn-AFP is 1.28° C at 10 mg/ml protein concentration. The ice crystals formed by Cn-AFP showed burst or snowflake form. In silico 3D-structure analysis exhibits that the Cn-AFP was consisted of seven right-handed $\beta$-helical loops with a triangular cross section. Simulation of the 3D-structure of mutant forms of Cn-AFP in silico predicted an ice-binding site located on the b-face of Cn-AFP. Unexpectedly, two of mutant Cn-AFP increased their antifreeze activity to suggest ice-binding site of this protein. Immunocytochemistry and immunogold labeling results revealed that Cn-AFP is preferentially localized to the intracellular space near the chloroplast membrane.

P40

# Development of Antifreeze Protein Coating Method on Aluminum Surface and Their Anti-Frost Ability

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Antifreeze proteins (AFPs) could be applied to various industrial fields due to their antifreeze functions including thermal hysteresis and inhibition of ice recrystallization. In this study, we developed the coating method of Cn-AFP (Antarctic marine diatom, Chaetoceros neogracile AFP) on industrial metal material (aluminum) to prevent and /or delay frost formation. To coat Cn-AFP to aluminum (Al), the aluminum binding peptide (ABP-Al) was used as a conjugator. Cn-ABP-Al bound well to Al plate and did not significantly influence to the Cn-AFP activity. In addition, we carried out secondary coating on the Cn-AFP-Al plate by trehalose, which could help increasing the protein stability. The trehalose coating on to Cn-AFP-Al plate enhanced the stability of protein on Al plate but this coating did not reduce anti-frost effect on the Cn-AFP activity. This newly developed coating technology of AFP to Al can be further applied to other metals which are important to frozen industry especially, cold storage field where the frequent defrosting process is required.

Submitted abstract for oral presentation (Yoshi Furukawa)

# OSCILLATORY GROWTH OF ICE CRYSTAL IN THE SOLUTION OF ANTIFREEZE GLYCOPROTEIN

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Ice crystal growth in the aqueous solution of antifreeze glycoprotein (AFGP) autonomously oscillates. Kinetic growth behavior at an ice/water interface crystal is strongly modified by adsorption of AFGP molecules. The authors proposed a new model for the adsorption-desorption kinetics of AFGP molecules on the prismatic interfaces of ice crystals, which was called a two-step reversible adsorption [1, 2]. This model said that there were two stages of adsorption for AFGP molecules on the ice/water interfaces. In other words, growth of an ice crystal can be fluctuated in connection with the amount of adsorbed AFGP molecules.

We will present two kinds of experiments which show the fluctuations of growth rates. One-directional growth experiment which was carried out using a thin growth cell with 0.05mm in thickness showed the oscillatory movement of ice (prism face)/water interfaces. On the other hand, a microgravity experiment in the International Space Station (ISS) was carried out to prevent the fluctuation of growth rates originated from the thermal convection around a growing crystal. In this experiment, we measured the growth rates of basal faces using a Michelson type interference microscope as a function of temperature and growth time. As a result, we clearly observed the periodical change in the movement of interference fringes appeared in the basal faces. It means that the growth rates of basal faces also fluctuate on a periodic basis. Since there is not any effect of convection flow, we conclude that the results of both experiments indicate the self-organized oscillatory growth of ice crystals in a supercooled solution of AFGP.

Mechanisms of the oscillatory growth observed will be also discussed in relation to the AFGP adsorption and growth prohibition. Here it should be noted that the effects of AFGP adsorption for ice crystal growth is diametrically opposed for the basal and prism faces. Namely, the growth rates are reduced with increasing amount of adsorbed AFGP for the prism faces, but are enhanced for basal faces. This opposite feature strongly relates to the difference of adsorption-prohibition interaction between the ice interface and AFGP molecules.

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Submitted abstract for oral presentation (Dmitry Vorontsov)

# Growth of ice crystals in the presence of carboxylated ε-poly-L-lysine

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The use of some biological agents gives a potential to control the crystallization processes of ice. Living organisms (polar fish, insects) produce anti-freeze proteins to survive in subfreezing environments. Some agents are used in medicine for preservation and transplantation of cells and tissues [1]. Carboxylated ε-poly-L-lysine (COOH-PLL) is a new cryoprotector which shows lower cytotoxicity [2]. In this study we have investigated the effects of COOH-PLL as an impurity on crystallization of ice in supercooled water. Seed crystals of ice of hexagonal modification (space group $P6_3/mmc$) were created in a glass capillary in supercooled water with COOH-PLL concentrations 0-150 mg/ml $H_2O$. Ice crystal has a disk-like shape at small supercoolings (0.2-0.3° C) where the top and bottom planes are basal faces {0001}. Further increase in supercooling leads to formation of dendrites with branches parallel to the {0001} faces. The change to dendritic growth in the presence of COOH-PLL impurity occurs at lower supercoolings than in pure deionized water. An increase in COOH-PLL concentration inhibits growth rate of the faces of ice crystals at fixed supercooling. Blocking effect of impurity is explained on the basis of Gibbs-Thomson law and under the assumption of Langmuir's dynamics of impurity adsorption. Retardation of ice growth in the presence of COOH-PLL occurs due to blocking of the surface by molecules of impurity and increase in the viscosity of solution with COOH-PLL addition. We used Punin's model for non-equilibrium adsorption [3] as a basis for theoretical description of the shape of growth rate curves. Calculated values for the growth rate correspond to the results of our experiments.

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Submitted abstract for oral presentation (Peter Wilson)

# Determining the effect of IBPs on heterogeneous nucleation: ruling out induction time as a measure

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Often ice binding proteins are added to supercooled solutions to alter the supercooling ability, in both directions. Equally often, induction time is used as a measure of effectiveness. I will outline the problems with this approach and show some novel data on heterogeneous nucleation.

P-withdrew

# Characterization of Afp1, an antifreeze protein from the psychrophilic yeast Glaciozyma antarctica PI12

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The psychrophilic yeast *Glaciozyma Antarctica* demonstrated high antifreeze activity in its culture filtrate. The culture filtrate exhibited both thermal hysteresis (TH) and ice recrystallization inhibition (RI) properties. The TH of 0.1 °C was comparable to that previously reported for bacteria and fungi. A genome sequence survey of the G. antarctica genome identified a novel antifreeze protein gene. The cDNA encoded a 177 amino acid protein with 30 % similarity to a fungal antifreeze protein from *Typhula ishikariensis*. The expression levels of *AFP1* were quantified via real time-quantitative polymerase chain reaction (RT-qPCR), and the highest expression levels were detected within 6 h of growth at -12°□C. The cDNA of the antifreeze protein was cloned into an *Escherichia coli* expression system. Expression of recombinant Afp1 in *E. coli* resulted in the formation of inclusion bodies that were subsequently denatured by treatment with urea and allowed to refold in vitro. Activity assays of the recombinant Afp1 confirmed the antifreeze protein properties with a high TH value of 0.08 °C.