

Isolation and Characterization of Antifreeze Proteins from the Antarctic Marine Microalga *Pyramimonas gelidicola*

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Abstract Antifreeze proteins (AFPs) play an important role in the psychrophilic adaptation of polar organisms. AFPs encoded by an Antarctic chlorophyte, identified as *Pyramimonas gelidicola*, were isolated and characterized. Two AFP isoforms were found from cDNAs and their deduced molecular weights were estimated to be 26.4 kDa (Pg-1-AFP) and 27.1 kDa (Pg-2-AFP). Both AFP cDNAs were cloned and expressed in *Escherichia coli*. The purified recombinant Pg-1-rAFP and Pg-2-rAFP both showed antifreeze activity based on the measurement of thermal hysteresis (TH) and morphological changes to single ice crystals. Pg-1-rAFP shaped ice crystals into a snowflake pattern with a TH value of 0.6 ± 0.02 °C at ~15 mg/ml. Single ice crystals in Pg-2-rAFP showed a dendritic morphology with a TH value of 0.25 ± 0.02 °C at the same protein concentration. Based on in silico protein structure predictions, the three-dimensional structures of *P. gelidicola* AFPs match those of their homologs found in fungi and bacteria. They fold as a right-handed β -helix flanked by an α -helix. Unlike the hyperactive insect AFPs, the proposed ice-binding site on one of the flat β -

helical surfaces is neither regular nor well-conserved. This might be a characteristic of AFPs used for freeze tolerance as opposed to freeze avoidance. A role for *P. gelidicola* AFPs in freeze tolerance is also consistent with their relatively low TH values.

Keywords Antifreeze proteins · Antarctic marine prasinophyte · Ice binding · Thermal hysteresis · In silico protein modeling

Introduction

Antarctica has one of the harshest environments on earth, with strong winds, frequent blizzards, and very low temperatures. This cold climate results from the continent's location at the South Pole and its isolation by a circumpolar current in the Southern Ocean. Marine and freshwater Antarctic planktonic habitats exhibit consistently low temperatures, ranging from -1.9 °C to 5 °C (Priddle et al. 1986), and support many psychrophilic organisms.

Pyramimonas gelidicola (*Pyramimonadaceae*, *Pyramimonadales*, Prasinophyceae, Chlorophyta) is a mixotrophic primary producer in Antarctic saline lakes and oceans (Laybourn-Parry 2002). Even though this microalga is a dominant species and occupies a crucial position within Antarctic aquatic ecosystems, little is known about *P. gelidicola* at the molecular level.

Cold-adapted or cold-acclimated microorganisms are physiologically and ecologically successful in cold environments due to the unique characteristics of their proteins, membranes, and genetic responses to thermal changes (Deming 2002; Teoh et al. 2004). Where ice is an accompanying environmental challenge one effective strategy for either freezing avoidance or freeze tolerance is production and secretion of ice-binding proteins (IBPs), traditionally called antifreeze proteins

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(AFPs) (Hoshino et al. 1998; Raymond et al. 2009). IBPs are widely distributed in organisms exposed to cold environments such as fish, insects, plants, fungi, and bacteria. In plant tissues, IBPs prevent physical damage from the growth of ice crystals in extracellular spaces by inhibiting ice recrystallization, which would otherwise produce large ice crystal grains under freeze–thaw conditions (Smallwood and Bowles 2002; Griffith and Yaish 2004). The name ‘antifreeze’ does not accurately describe this ice recrystallization inhibition role. However, plant IBPs are still generally referred to as AFPs. IBPs secreted from Antarctic microorganisms are capable of modifying the structure of surrounding lake and sea ice to preserve local ice-free pockets (Raymond and Fritsen 2001; Raymond and Knight 2003; Raymond 2011). This activity is distinct from that of animal and plant AFPs in that the effect of the IBPs occurs outside the organism, and is more akin to that of animal AFPs in preventing rather than ameliorating freezing.

As a consequence of binding to ice, AFPs lower the freezing temperature below the melting point (Raymond and DeVries 1977). This thermal hysteresis (TH) and is used to detect and quantify AFPs (Scotter et al. 2006). Proteins that show TH are also active in ice recrystallization inhibition, which is presumably also a manifestation of the adsorption–inhibition mechanism (Knight et al. 1984).

AFPs have been used in many biotechnological applications. Due to their ability to inhibit ice recrystallization, AFPs have been used to reduce damage to red blood cells undergoing a freeze–thaw cycle (Kang and Raymond 2004). However, they are also used to destroy harmful cells during cryosurgery because they change the morphology of ice (Koushafar et al. 1997). In addition, they can be used to help improve foods such as ice cream and meats by keeping ice crystals small (Regand and Goff 2006; Boonsupthip and Lee 2003).

Given its habitat, *P. gelidicola* was a candidate to produce IBPs to survive the stresses of an icy environment. Recently, a cDNA library was constructed and ESTs of *P. gelidicola* expressed at cold temperatures were analyzed and deposited in GenBank (Jung et al. 2012). Two potential IBP sequences of *P. gelidicola* were identified in this EST collection (JQ743499 and JQ743500 as NCBI accession numbers) and these two sequences were described by Raymond and Kim (2012). The aims of this study were to produce and purify a recombinant *P. gelidicola* IBP in *Escherichia coli*, characterize its activity, and compare its properties to the natural antifreeze activities secreted by the microalga under conditions that mimic its polar environment. In addition, we used in silico protein structure analysis to produce a reliable three-dimensional protein model of *P. gelidicola* AFP, which demonstrated that its putative ice-binding site, like that of its orthologs, is not well conserved.

Materials and Methods

Culture Conditions of *P. gelidicola*

P. gelidicola was collected at Maxwell Bay near the King Sejong Station, King George Island, Antarctica (62°13' S, 58°47' W). The strain was isolated and purified by successive dilution and by picking colonies using a Pasteur pipette. *P. gelidicola* was grown and maintained in f/2 media (Guillard and Ryther 1962) at 3 °C and under 25 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (continuous light) with horizontal shaking. To determine optimal growth conditions, the microalgal strain was incubated at several temperatures (–2 °C, 0 °C, 2 °C, 4 °C, 8 °C, 12 °C, 16 °C and 20 °C) with continuous light and shaking. The growth rate of *P. gelidicola* was determined by measuring chlorophyll *a* concentrations extracted by 90 % acetone from samples collected every 3 days.

Molecular Identification of *P. gelidicola*

To identify the microalgal strain, we used molecular biology methods. An aliquot (10 ml) of culture was harvested by centrifugation. Genomic DNA was extracted from the fresh f/2-washed microalgal pellets using a gDNA extraction kit according to the manufacturer's protocols (i-genomic plant DNA extraction kit; Intron, South Korea). Sequences of 18S rDNA (SSU) were determined by polymerase chain reaction (PCR) of nuclear DNA using two sets of primer pairs (G01/G14 and G04/G07) (Saunders and Kraft 1994), and a complete SSU sequence was assembled by overlapping two fragments of partial SSU sequence. The nuclear SSU sequence was compared to sequences in the NCBI database by BLASTn.

Isolation of Open Reading Frames of Antifreeze Proteins from *P. gelidicola*

Jung et al. (2012) previously constructed a cDNA library from *P. gelidicola* to analyze the expressed genetic information. From this database, we identified the sequences predicted to have antifreeze functions based on analysis of orthologous groups of eukaryotes (Genbank acc. nos. FS592795, FS594306; Genbank acc. nos. FS594586, FS594590, FS594546 and FS594790) and assembled them into two contiguous sequences. To isolate the open reading frames (ORFs) coding for the AFPs of *P. gelidicola*, a DNA walking experiment was performed using DNA Walking SpeedUp kit (Seegene, Seoul, South Korea). Three target-specific primers (TSPs; TSP-1, 5'-GCA CCT CCG TTG AGA TTC AA-3'; TSP-2, 5'-GTA GAT ATT CGC GCC GAT GGT-3'; TSP-3, 5'-CCT TCA TGT GTG CTT TCT CGC T-3') were designed based on the partially analyzed ORF sequences. PCR bands amplified by the DNA walking method were sequenced and

compared to the sequences that overlapped with the amplified regions using DNA walking annealing control primer (DW-ACP) and TSP-1 primers. The isolated ORFs for two *P. gelidicola* AFP isoforms (Pg-1-AFP and Pg-2-AFP) were compared to sequences in the NCBI database using tBlastx. In addition, the deduced amino acid sequences of Pg-1-AFP and Pg-2-AFP were analyzed using the SignalP v4.0 (Peterson et al. 2011) and TargetP v1.1 (Emanuelsson et al. 2000) programs in order to identify the signal peptides and predict the cellular localization of the two AFP isoforms.

Phylogenetic Relationship of IBPs and AFPs from Various Microorganisms with *P. gelidicola* AFPs

The deduced amino acid sequences of the two *P. gelidicola* AFP isoforms were compared to the sequences of other IBPs and AFPs originating from a variety of microorganisms including bacteria, fungi, and diatoms, as well as plants, in order to identify the phylogenetic positions of the Pg-AFPs. The peptide sequences analyzed to have high identity with AFPs of *P. gelidicola* by Blastp were collected from the NCBI database (*Fragilariopsis cylindrus* IBP, ACX36851; *Fragilariopsis curta* IBP, ACT99642; *Typhula ishikariensis* AFP, BAD02895; *Lentinula edodes* IBP, ACL27146; *Leucosporidium* sp. AFP, ACU30807; *Navicula glaciei* IBP, AAZ76254; *Chaetoceros neogracile* AFP, ACU09498; *Glaciozyma antarctica* AFP, ACX31168). The IBP amino acid sequences were aligned using the ClustalW algorithm in the BioEdit program package (Hall 1999). Phylogeny was tested by the distance method with 5,000 bootstrap repetitions. The UPGMA method was used for construction of a phylogenetic tree using the MEGA5 program (Tamura et al. 2011).

Construction of Expression Vectors for Heterologous Overexpression of *P. gelidicola* AFPs in *E. coli*

The coding regions for mature Pg-AFPs, without the signal peptides, were amplified by forward primers containing a *Nde*I restriction enzyme site (Pg-1-AFP, 5'-CAT ATG CGC ACT CTG CTT CAA-3'; Pg-2-AFP, 5'-CAT ATG CGC GCT CTG ACA ACC-3') and reverse primers with an *Xba*I site (Pg-1-AFP, 5'-TCT AGA CTA AAC AAC ATA ATC GGG TTT-3'; Pg-2-AFP, 5'-TCT AGA TTA GAG GGC TTC ATC GGA GAT-3'). The sequences of each ORF were directly verified by nucleotide sequencing. pColdI expression vector (Takara, Japan) was used to express Pg-AFPs with a His-tag at the N-terminal region of the proteins at temperatures as low as 15 °C. The purified PCR products were digested with *Nde*I and *Xba*I and ligated into these sites of the pColdI expression vector. The ligation products were transformed into DH5 α competent cells. The plasmids containing the *P. gelidicola* AFPs (Pg-1-rAFP and Pg-2-rAFP) were transformed into BL21 (DE3) competent cells. A single colony of each type

of Pg-AFP was inoculated into LB media and incubated at 37 °C. When the OD₆₀₀ reached approximately 0.5, the culture was cooled at 15 °C for 30 min and 1 mM of isopropyl β -D-thiogalactopyranoside (IPTG) was then added to induce AFP gene expression. After the addition of IPTG, the cultures were further incubated with shaking for 24 h at 15 °C to allow for the production of the AFPs. The cultures were then centrifuged in a fixed-angle rotor at 18,000 \times g for 30 min at 4 °C. The cell pellets were resuspended in lysis and binding buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 50 mM PMSF) and lysed by sonication on ice for 5 min (5-s pulse and 10-s pause). The lysates were centrifuged at 18,000 \times g for 20 min at 4 °C and the resulting supernatants, which were the soluble fractions, were collected in new tubes on ice. The Pg-AFPs in the soluble fraction were purified by affinity chromatography using Ni-NTA resin used according to the manufacturer's protocols (Elpisbio, Korea). Purified fractions of the recombinant proteins were visualized on 12 % SDS-PAGE and concentrated using Centricon centrifugal filters with a 10 K molecular weight cutoff (Millipore, USA). The concentrations of the purified recombinant Pg-AFPs were calculated using extinction coefficient values of 14,503 and 13,138 cm⁻¹ M⁻¹ for Pg-1-AFP and Pg-2-AFP, respectively.

Activity Characterization of Native AFP and the Two Isoforms of Recombinant *P. gelidicola* AFP

To examine the ice-binding activity of native Pg-AFP, the culture medium was separated from the algae by centrifugation, followed by filtration through a 0.2- μ m pore microfilter to completely eliminate cells and other cellular components. Ice-binding activity of the spent medium was measured by observation of pitting on the basal plane of the ice (Raymond and Fritsen 2001). The TH activities and single ice crystal morphology corresponding to native Pg-AFP and various concentrations of purified Pg-rAFP were analyzed by use of nanolitre-osmometer (Otago Osmometers, New Zealand) as previously described (Gwak et al. 2010). The stability of Pg-rAFP after heat and proteinase treatment was checked following incubation at 95 °C or in 1 mg/ml of proteinase K (purified from *Tritiachium album*; Sigma), respectively. After incubation for 30 min, TH activity and ice crystal morphology were evaluated by use of a nanolitre-osmometer.

Homology Modeling and Prediction of the Protein Structures of the Two Isoforms of Pg-AFP

The peptide sequences of IBPs and AFPs showing high identity to Pg-AFPs were aligned with the two isoforms of Pg-AFPs using the ClustalW program. To predict the structures of the Pg-AFPs, in silico analyses were performed by secondary structure prediction and homology modeling. The PSIPRED program was used to predict secondary structure. The

template proteins for protein modeling were selected based on the results of analyses using the CPHmodels V3.2 (Lund et al. 2002; Nielsen et al. 2010) and Phyre2 (Kelley and Sternberg 2009) comparative modeling programs. Homology modeling was performed by manual alignment of amino acids with template proteins by Modeller v9.9 (Sali et al. 1995). Fifty models derived from Modeller were aligned and visualized using PyMol (Delano 2002). The best models were selected by analysis of the molpdf and DOPE scores.

Results and Discussion

Identification of the Strain and Determination of Its Optimal Growth Temperature

P. gelidicola was observed by optical microscopy to be 10 μm long and pyramidal in morphology (Fig. 1). The distal parts of four thick flagella were bent over along the surface of the cells, which is characteristic of the morphology of the genus *Pyramimonas* (Marchant 2005). To identify the species, the 18S rDNA sequence (SSU) of the strain was analyzed. Two fragments of SSU (SSU-1 (G01/G14) and SSU-2 (G04/07)) were found to overlap for 50 nt at the 3' region of SSU-1 and the 5' region of SSU-2. By the analysis of BLASTn results and pairwise alignment (data not shown), the combined partial SSU sequence (GenBank acc. no. EU141942) had 99.5 % sequence identity with those of *P. gelidicola* (HQ111509, HQ111510 and HQ111511) collected from other locations.

The optimum temperature for *P. gelidicola* growth was close to 4 $^{\circ}\text{C}$ (Fig. 2). The cells could still grow at 0 $^{\circ}\text{C}$ and cell growth was even observed at -2°C in the presence of ice slurry. However, this strain was not able to grow at temperatures over 8 $^{\circ}\text{C}$. These results indicate that *P. gelidicola* is a

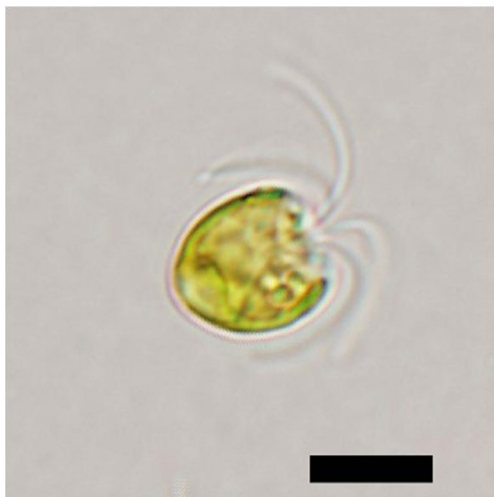


Fig. 1 *Pyramimonas gelidicola* cultured in-house at KOPRI. These microalgal cells have a rounded pyramidal shape with four thick flagella. Scale bar, 10 μm

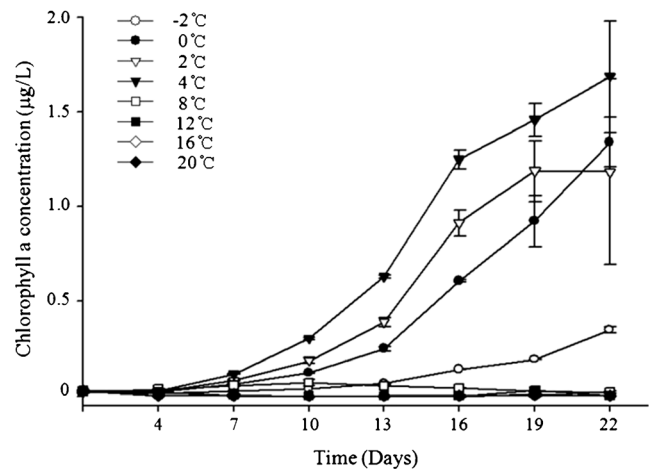


Fig. 2 Growth curves of *Pyramimonas gelidicola* at different temperatures. *P. gelidicola* growth was measured as a function of chlorophyll *a* concentration over a period of 22 days. Readings at -2°C (open circles), 0°C (closed circles), 2°C (open triangles), 4°C (closed triangles), 8°C (open squares), 12°C (closed squares), 16°C (open diamonds), 20°C (closed diamonds), were taken in duplicate and averaged

psychrophilic microorganism, capable of growing in temperatures as low as -2°C , and that *P. gelidicola* must have a mechanism for survival and even growth in freezing environments. The freezing temperature of Antarctic seawater is approximately -1.9°C .

Cloning of Two Isoforms of AFP from the cDNA of *P. gelidicola* and Their Phylogenetic Analysis

From a transcriptome analysis of *P. gelidicola*, two partial AFP sequences have been reported (Jung et al. 2012). To obtain the full sequence of the ORF DNA-walking experiments based on partial contiguous sequences were performed. In total, two complete ORFs of *P. gelidicola* AFP were found (Pg-1-AFP, GenBank acc. no. JQ743499; Pg-2-AFP, GenBank acc. no. JQ743500), which consisted of 828 and 864 bp, respectively (Table 1). The Pg-1-AFP sequence

Table 1 Summary of information on the two AFP isoforms from *P. gelidicola*

	Antifreeze proteins from <i>Pyramimonas gelidicola</i>			
	Pg-1-AFP		Pg-2-AFP	
	Preprotein ^a	Mature ^b	Preprotein	Mature
Nucleotides (bp)	828	762	864	795
Amino Acid	275	254	287	265
molecular weights (kDa)	28.4	26.4	29.3	27.1
Isoelectric points (pI)	4.90	4.78	4.62	4.54

^a Preprotein: the protein with signal peptide

^b Mature: the protein without signal peptide

encoded a protein of 275 amino acids with a molecular weight of 28.4 kDa. The Pg-2-AFP sequence encoded a 287-residue protein with a molecular weight of 29.3 kDa. The protein sequences of Pg-1-AFP and Pg-2-AFP were deposited as Genbank acc. nos. AFK64811 and AFK64812 in the NCBI database. Using the SignalP program (Peterson et al. 2011), we found that the ORFs commenced with signal peptides of 21 and 22 amino acids for Pg-1-AFP and Pg-2-AFP, respectively (Fig. S1). Therefore, mature Pg-1-AFP and Pg-2-AFP, which lack the signal peptides, would be 254 and 265 amino acids in length, and the expressed proteins would be 26.4 and 27.1 kDa, respectively. Amino acid sequence alignment of Pg-1-AFP and Pg-2-AFP by the ClustalW program showed the two isoforms share 50.0 % sequence identity and 60.8 % similarity. Results from the TargetP program (Nielsen et al. 1997) predicted there was over 90 % probability that the Pg-AFP precursors would be secreted out of the cell. This is consistent with the findings of a study which reported that native AFP recovered from *P. gelidicola* medium, when cultured at low temperatures, showed strong antifreeze activity (Raymond and Kim 2012). Note that a high degree of ice-pitting was observed by optical microscopy (Fig. 3a) when the ice-pitting assay (Raymond and Fritsen 2001) was performed using samples of the algal culture media. In addition, single ice crystals in the extracellular supernatants took on hexagonal shapes, with dendritic ice crystal growth patterns forming

along the a-axes when the temperature fell below the TH value (Fig. 3b). All these observations are consistent with secretion of the AFP into the medium.

From the results of alignment and phylogenetic analyses based on amino acid sequences, Pg-AFPs were found to have homology with IBPs produced from various psychrophilic microorganisms (Figs. 4 and 5). Pg-1-AFP and Pg-2-AFP showed high identity with AFPs from Antarctic marine diatoms, *Fragilariopsis cylindrus*, *Fragilariopsis curta*, *Navicula glaciei* and *Chaetoceros neogracile*, the snow mold *Typhula ishikariensis*, and other fungi, *Lentinula edodes* and *Leucosporidium* sp. The phylogenetic relationship of the proteins closely related to Pg-AFPs was strongly supported by 100 % bootstrap repetitions, and the clade composed of these proteins was separated from the other clades by a difference in sequence identity of below 40 % bootstrap repetitions. Based on these analyses, the microalgal Pg-AFPs are closely related to IBPs originating from the fungi, *Leucosporidium* sp., *T. ishikariensis* and *L. edodes* and Antarctic marine diatoms, *F. cylindrus*, *F. curta*, *N. glaciei*, and *C. neogracile*. AFPs and IBPs of plants, insects and bacteria were analyzed but had sequence identity with Pg-AFPs below 10 %. It was noted that the genes for microalgal IBPs and AFPs have homologs in various microorganisms living in polar regions via horizontal gene transfer (HGT) (Bayer-Giraldi et al. 2010; Janech et al. 2006; Kiko 2009). Furthermore, sea ice could serve to

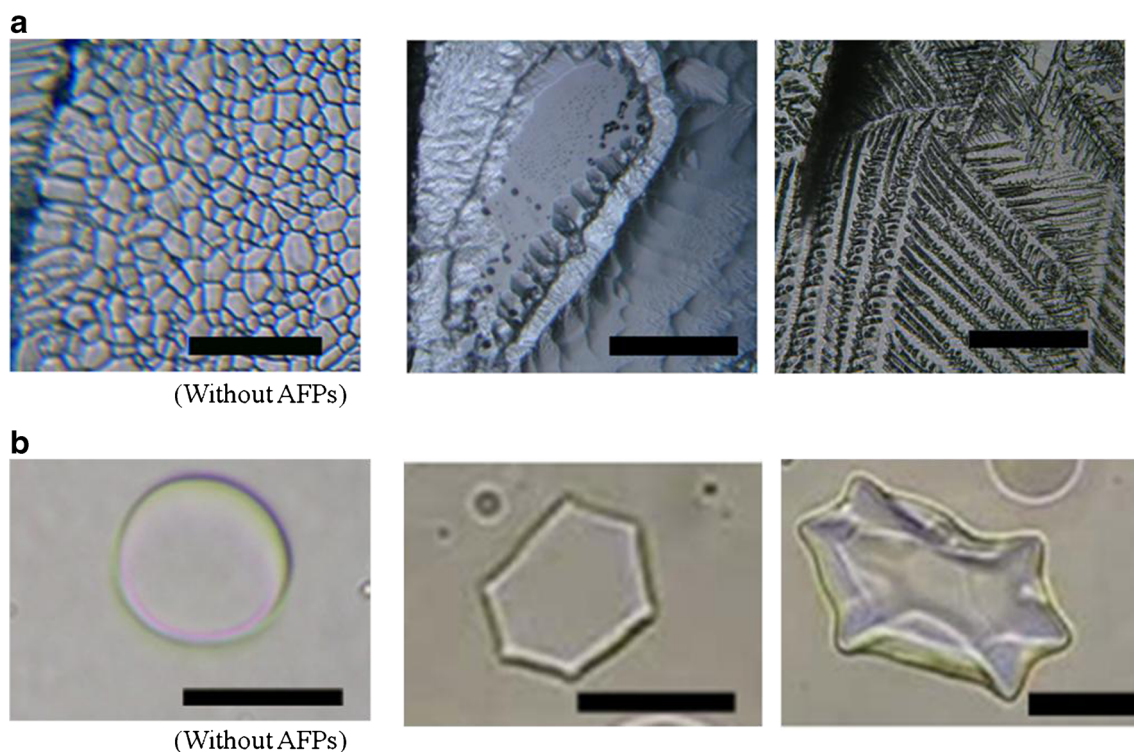


Fig. 3 Ice crystal morphology in the presence of native Pg-AFP. **a** Deformation of ice surface produced by extracellular culture media from which microalgal cells and any particles were eliminated by 0.2- μ m

filters. Scale bar, 1 mm. **b** Morphology of single ice crystals observed in the nanolitre-osmometer. Scale bar, 50 μ m. In **a** and **b**, the leftmost panel is the control developed in the absence of any AFP

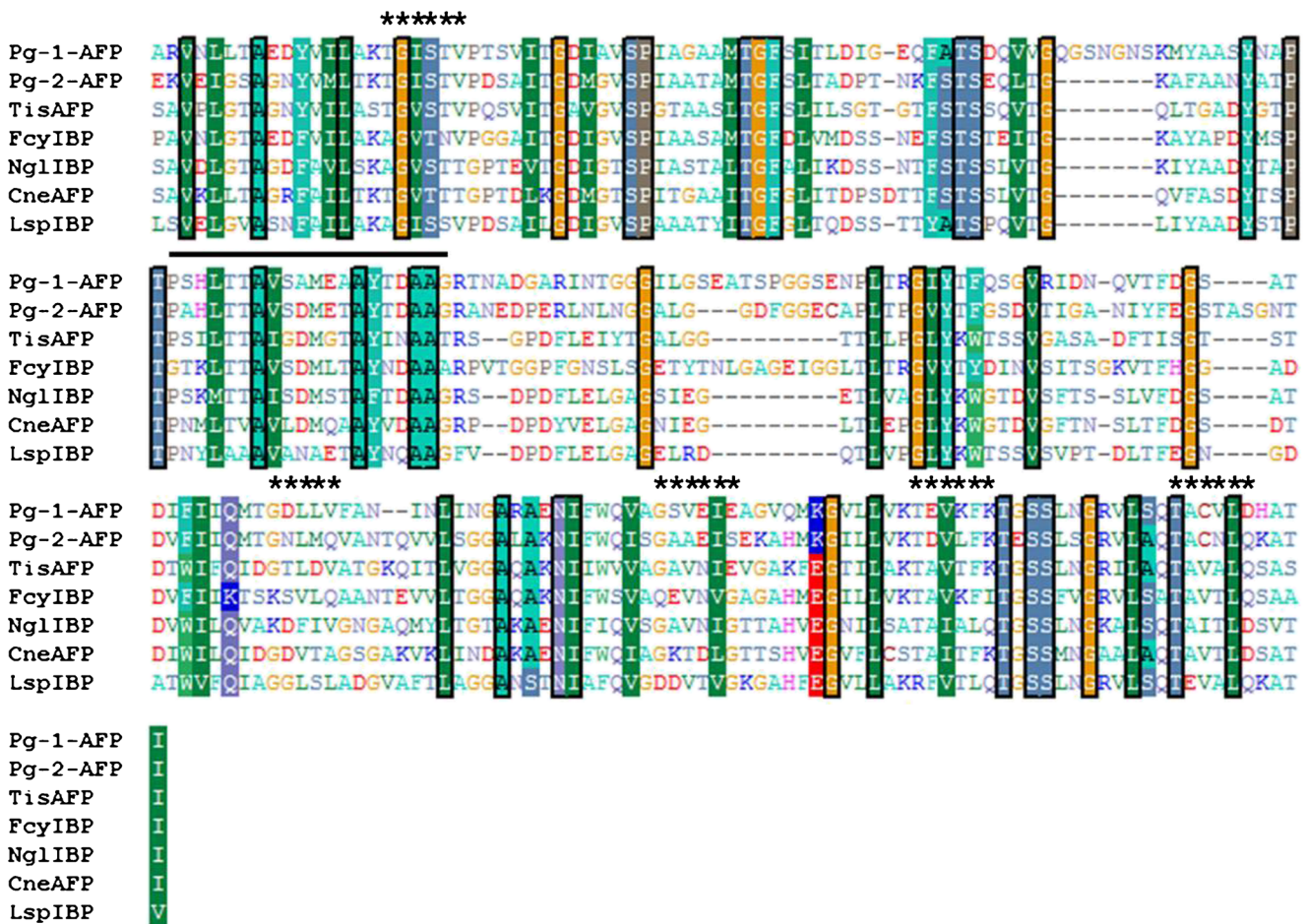


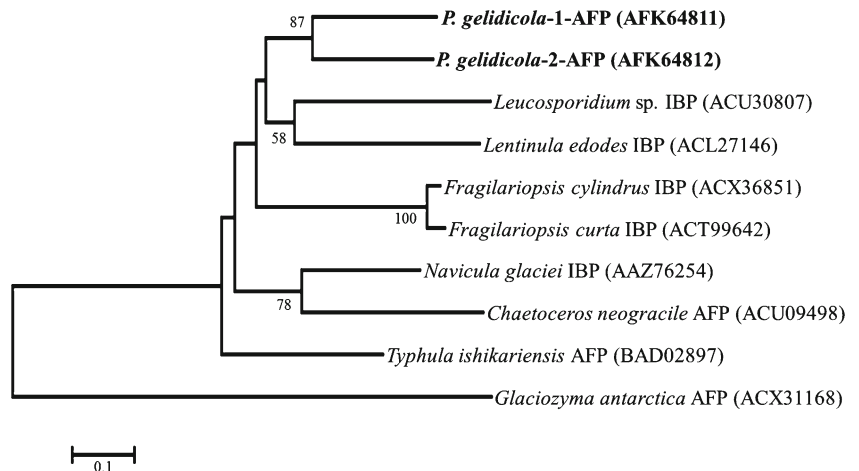
Fig. 4 Amino acid sequence alignment of homologous AFPs from microorganisms. Proteins listed were Pg-1-AFP and Pg-2-AFP of *Pyramimonas gelidicola*; FcyIBP of *Fragilariopsis cylindrus* IBP; TisAFP, *Typhula ishikariensis* AFP; NglIBP, *Navicula glaciei* IBP; CneAFP, *Chaetoceros neogracile* AFP; LspIBP, *Leucosporidium* sp.

IBP. Asterisks indicate the residues predicted to be bound to ice surfaces based on the protein structure of TisAFP. Black line indicates six turns of α -helix region. The letters on black boxes indicate identical amino acids among the proteins. Similar amino acids are indicated by highlighted characters. Gaps in the alignment are indicated by dashes

concentrate extracellular DNAs and facilitate HGT between sea ice algae that would help these organisms survive in a cold environment. (Collins and Deming 2011; Raymond and Kim

2012). *P. gelidicola* is a mixotrophic microorganism that can use autotrophy and heterotrophy (Bell and Laybourn-Parry 2003). Thus it is quite likely that the *P. gelidicola* AFP genes

Fig. 5 Relatedness of amino acid sequences of AFPs and IBPs from various types of organisms as determined by the MEGA5 program (Tamura et al. 2011). The tree was generated using the UPGMA algorithm and values at each branch represent 5,000 bootstrap replicates. Bootstrap values below 40 % support were omitted from the phylogenetic tree

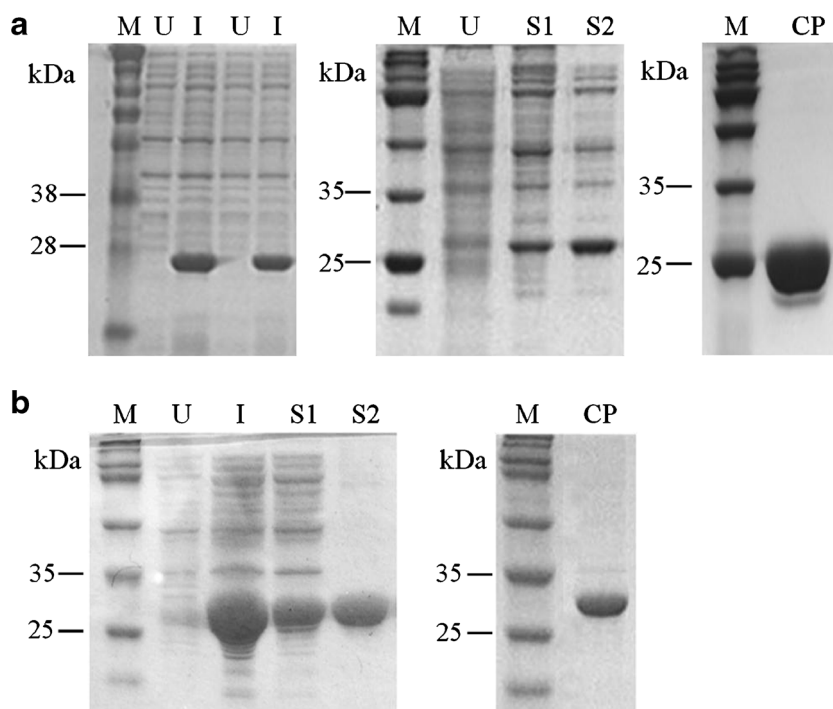


were obtained from a nearby microorganism by an HGT mechanism including the heterotrophic uptake of AFP-producing bacteria.

Heterologous Expression of Recombinant *P. gelidicola* AFPs and Characterization of Antifreeze Activity

Secreted bacterial, fungal and microalgal IBPs have been shown to have ice-binding activity based on the observation of pitting morphology on ice surfaces (Raymond and Janech 2009; Raymond et al. 2009). However, it is difficult to characterize the optimal properties of these proteins due to the limited amounts produced by their slow-growing psychrophilic hosts. To facilitate their analysis and use, the Pg-AFPs were produced by recombinant protein expression in *E. coli*. To express the two AFP isoforms from *P. gelidicola* as soluble proteins, we used the pColdI-expression system (Takara, Japan) with long protein expression times at low temperature. The yields of recombinant Pg-AFPs purified from the soluble fraction of the lysate by Ni-NTA affinity chromatography were 30 mg/l of culture (Fig. 6). Mature recombinant Pg-1-rAFP and Pg-2-rAFP were assayed by observation of single ice crystal shapes and measurement of TH (Fig. 7). Pg-1-rAFP (16 mg/ml) modified the single ice crystal shape to a snowflake-like morphology and also depressed the freezing point to 0.6 ± 0.02 °C below the melting point. The shape of the single ice crystal became more hexagonal with decreasing Pg-1-rAFP concentration. Pg-2-rAFP (14 mg/ml) produced dendritic-shaped single ice crystals, which also became less angular with decreasing Pg-2-rAFP concentrations.

Fig. 6 Heterologous expression of two recombinant Pg-AFP isoforms (Pg-rAFPs) analyzed by SDS-PAGE. The induced cells of Pg-rAFPs were collected at 24 h after adding 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) to the cell culture. Uninduced cells were harvested without adding 1 mM IPTG at the same time of collection as the induced cells. **a** Protein expression and purification of Pg-1-rAFP. **b** Protein expression and purification of Pg-2-rAFP (*M* protein marker, *U* uninduced cell extracts, *I* cell extracts induced by 1 mM IPTG; *S1*–*S2* soluble fractions from first and second round of sonication, *CP* concentration of purified recombinant AFPs by affinity chromatography)



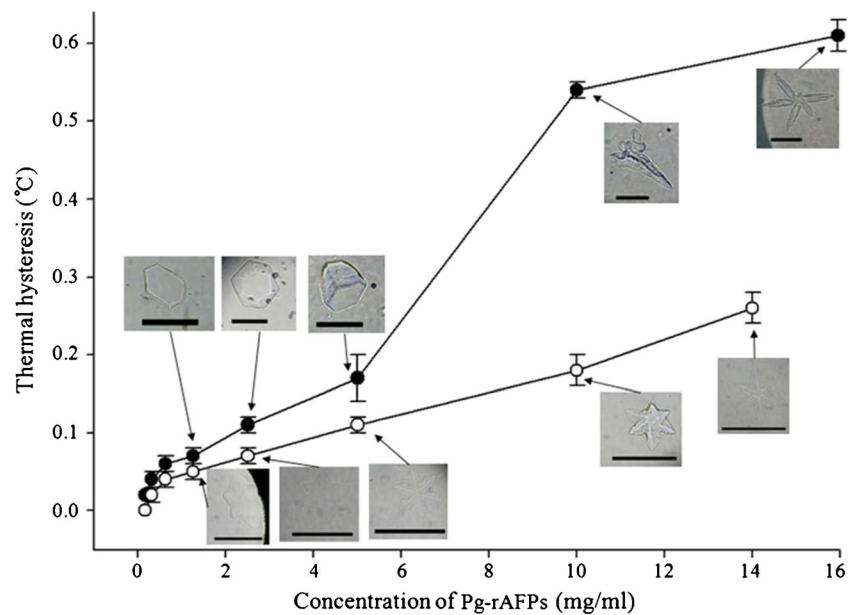
Antifreeze activity of *P. gelidicola* was compared with the activity of AFP from the Antarctic marine diatom, *Fragilariopsis cylindrus*. Recombinant AFPs of *F. cylindrus* (Uhlig et al. 2011) had TH values of approximately 0.1 °C to 0.2 °C at a concentration of 200 μ g/ml. The morphology of single ice crystals formed by both Pg-rAFPs and *F. cylindrus* recombinant AFP was hexagonal in shape at these concentrations. These results indicate that recombinant AFPs from an Antarctic marine diatom and a prasinophyte have similar TH activity and single ice crystal morphology at low protein concentrations.

To examine the stability and confirm the proteinaceous nature of the antifreezes, Pg-rAFPs were incubated at 95 °C or treated by proteinase K. The antifreeze activity of both Pg-rAFPs was abolished after thermal denaturation at 95 °C or proteinase K treatment for 30 min (Fig. 8). These results clearly showed that the antifreeze activity of recombinant Pg-AFPs is protein-mediated, and that their activity is very sensitive to thermal and proteolytic deactivation.

Homology Modeling and Prediction of the Protein Structure of the Two Pg-AFP Isoforms

To date, there have been many studies of the protein structures of AFPs and IBPs in order to elucidate the relationships between ice-binding activity and protein structure (Baardsnes et al. 1999; Garnham et al. 2008; Kondo et al. 2012; Lee et al. 2012). To study the structural characteristics that predict ice binding and antifreeze activity of Pg-AFPs, we performed computational structure modeling. The amino acid

Fig. 7 Measurement of thermal hysteresis (TH) for two isoforms of Pg-rAFPs. Pictures of single ice crystals were taken at specific TH values and related to the concentration of AFPs. Pg-1-rAFP and Pg-2-r-AFP are represented by *solid* and *open circles*, respectively. TH values at each point were measured and represented by the average of triplicated results. Scale bars, 100 μm



sequences of IBPs that showed high sequence similarity with mature Pg-AFPs were aligned to discover the conserved regions in Pg-AFPs (Fig. 4). As shown in Fig. 5, phylogenetically different microorganisms, such as Antarctic marine diatoms, bacteria and fungi produced homologous IBPs and AFPs, which is speculated to be the result of HGT (Raymond and Kim 2012). The high sequence identity indicates that Pg-AFPs likely have a structure similar to that of other proteins in that group (Fig. 4). Therefore, in silico protein structure analyses were deemed sufficient to predict the protein structure of Pg-AFPs and to locate the ice-binding surfaces. From the results of homology modeling, *Typhula ishikariensis* AFP (TisAFP, PDB ID: 3VN3) was selected as the template protein for modeling Pg-AFPs. Pg-1-AFP and Pg-2-AFP have 495 and 47 % identity with 3VN3 (Kondo

et al. 2012), respectively. 3VN3 showed 100 % confidence in structural homology to both Pg-AFPs. The identity of the amino acid sequences to two orthologs (above 45 %) is high to do modeling the structures with confidence because molecular modeling can be almost accurate. Fifty models based on the alignment of the amino acid sequences generated from Modeller showed stable and regular patterns among the models. This result indicated that TisAFP was a structurally reliable template for predicting the protein structure of Pg-AFPs. After the selection of the best model for Pg-AFPs from the Modeller program, an in silico 3-D protein structure of Pg-AFPs was assessed by comparing it with the structures of TisAFP and *Leucosporidium* sp. IBP.

From the protein modeling analysis, we found that Pg-AFPs shared the following structural characteristics with

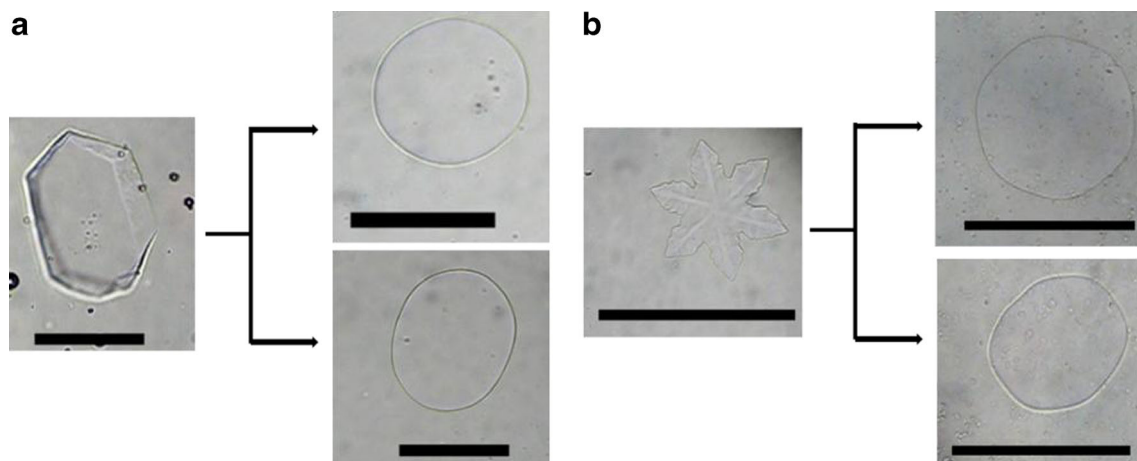
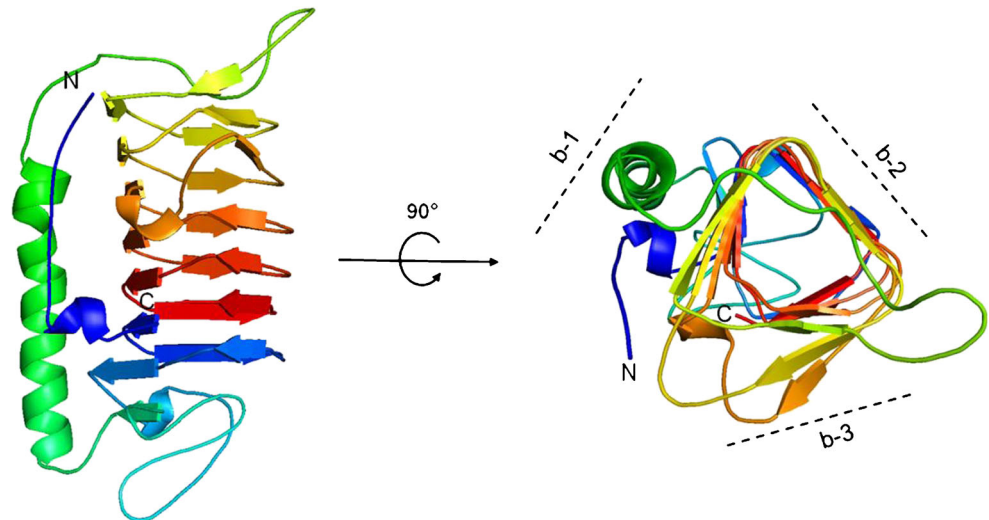


Fig. 8 Characterization of recombinant AFPs of *P. gelidicola*. **a** Thermal denaturation and proteinase K treatment of 1 mg/ml Pg-1-rAFP. **b** Thermal denaturation and proteinase K treatment of 5 mg/ml Pg-2-rAFP. The shapes of the single ice crystals obtained with the PgAFPs prior to

treatment are shown on the *left*. The shapes on the *right* were obtained after thermal denaturation (*top*) or proteolysis (*bottom*) of the AFPs. Scale bars, 100 μm

Fig. 9 Prediction of the 3D structure of *P. gelidicola* AFP by in silico protein modeling. The modeled structure of PgAFP is shown in ribbon format with rainbow colours indicating proximity to the N-terminal (blue) and C-terminal (red) ends. Arrows indicate beta-strands and the coiled region (green) represents alpha-helix. In the top-down view on the right, dotted lines indicate the three faces (b1–b3) of the structure

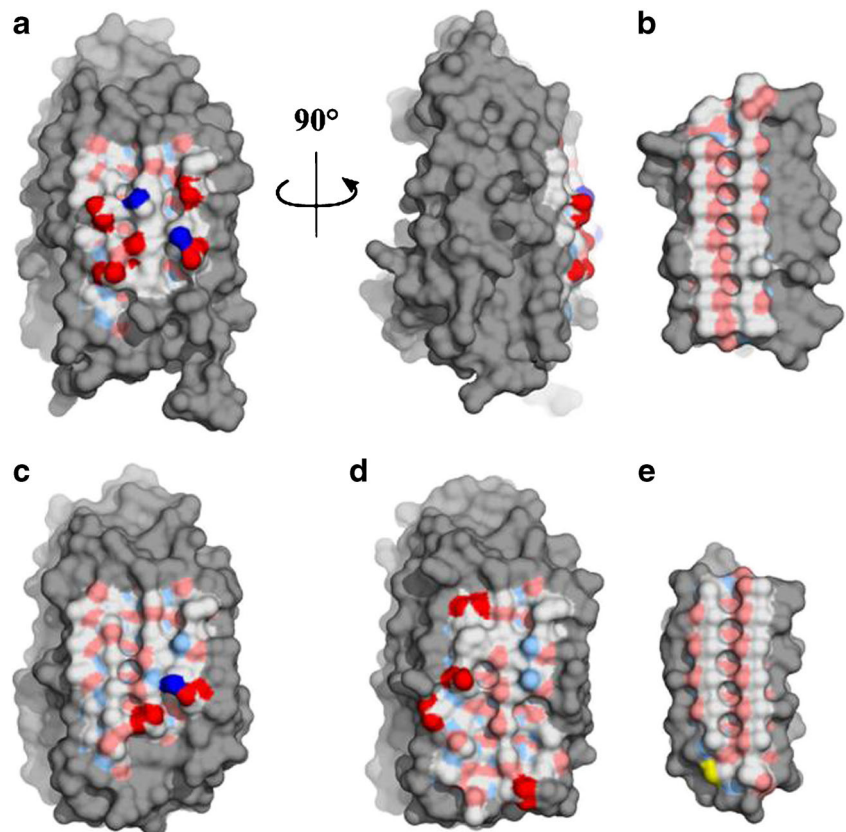


TisAFP: (1) a right-handed β -helix comprising six β -helical loops with a triangular cross-section, and (2) one α -helix with six turns that lies along the length of one of the three flat β -sheets (b-1) comprising the β -helix (Fig. 9). The β -loops of the Pg-AFPs consisted of 20 residues on average. The inward-pointing residues of the loops formed a hydrophobic core using residues such as Phe, Trp, Val, Leu, and Ile.

By analogy with LeIBP and TisAFP, the b-2 face of Pg-AFP was predicted to be the ice-binding surface. The ice-

binding surface of TisAFP was determined by single amino acid substitutions to be the b-face the β -helix (Kondo et al. 2012). It was also the flattest surface of the AFP and flatness is known to be a critical factor for antifreeze activity (Yang et al. 1998). The b-2 face of Pg-AFP is the structural equivalent of the b-face in TisAFP and it also shows a remarkably flat conformation with a regular distance between beta-strands on the b-2 sheet (Figs. 9 and 10). In addition, the alignment of the amino acid sequences from the six β -strands forming

Fig. 10 Comparison of AFP structures and ice-binding faces. The structures of **a** *Pyramimonas gelidicola* AFP (Pg-AFP), **b** spruce budworm AFP (sbwAFP), **c** *Typhula ishikariensis* AFP (TisAFP), **d** *Leucosporidium* sp. IBP (LeIBP) and **e** *Tenebrio molitor* AFP (TmAFP) are represented in space-filling mode and coloured grey. The putative or experimentally determined ice-binding sites are coloured light grey with the exception of O atoms (pink, or red if charged), and N atoms (light blue, or dark blue if charged). **a** Flatness of the b-2 face of Pg-AFP is illustrated by rotation of the structure through 90°



the b face between TisAFP and Pg-AFPs resulted in a sequence identity of 57.2 %, which is significantly higher than the overall sequence identity. Based on these results, it is proposed that the b-2 face of the Pg-AFPs is an ice-binding region that adsorbs to ice to stop its growth.

One of the interesting emerging observations about IBPs is that those proteins optimized for freeze tolerance seem to have rather irregular and poorly conserved ice-binding sites (Kondo et al. 2012; Middleton et al. 2012). This is illustrated by the space-filling diagrams for the microalgal (a) and fungal (c and d) AFPs in Fig. 10. In contrast, AFPs that prevent an organism from freezing, like the hyperactive AFPs in spruce budworm and *Tenebrio molitor*, tend to have highly regular well-conserved ice-binding sites (Fig. 10b and e). Pg-AFPs, just like TisAFP, do not possess the classical ice-binding motifs such as TXT seen in tandem arrays in some insect AFPs (Marshall et al. 2002) or TXN found in a bacterial AFP (Garnham et al. 2011).

In summary, this is the first report to present a genetic and molecular analysis of the AFPs from an Antarctic prasinophyte. Recombinant AFPs were expressed as soluble proteins using a heterologous expression system, and were found to show moderate TH activity. The protein structures of AFPs from *P. gelidicola* were predicted by in silico homology modeling with a high level of confidence to be right-handed β -helices with three β -faces, one of which functions as the ice-binding site.

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