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An extracellular ice-binding glycoprotein from an Arctic psychrophilic yeast ☆

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ABSTRACT

A psychrophilic yeast was isolated from an Arctic pond and its culture supernatant showed ice-binding activity. This isolate, identified as *Leucosporidium* sp. based on an analysis of the D1/D2 and ITS regions of its ribosomal DNA, produced a secretory ice-binding protein (IBP). Yeast IBP was purified from the culture medium to near homogeneity by the ice affinity method and appeared to be glycosylated with a molecular mass of ~26 kDa. In addition, the yeast IBP was shown to have thermal hysteresis (TH) and recrystallization inhibition (RI) activities. The full-length cDNA for yeast IBP was determined and was found to encode a 261 amino acid protein with molecular weight of 26.8 kDa that includes an N-terminal signal peptide and one potential N-glycosylation site. The deduced protein showed high sequence identity with other IBPs and hypothetical IBPs from fungi, diatoms, and bacteria, clustering with a class of ice-active proteins.

Introduction

Antifreeze proteins (AFPs) are proteins that depress the freezing point but not the melting point of aqueous solutions by inhibiting the growth of ice crystals [10]. The difference between the freezing and melting points is called thermal hysteresis (TH) [5]. Since DeVries first isolated antifreeze proteins from Antarctic teleost fishes [4], a great number of AFPs and other IBPs with a variety of sequences and structures have been discovered from insects, plants, bacteria, diatoms, and algae [16,18,23] from cold environments. Recently identified IBPs from bacteria, diatoms, and fungi formed a distinct cluster in a phylogenetic analysis [9,17]. Unlike AFPs, these IBPs seemed to have very low TH activity. However like AFPs, they have ice recrystallization inhibition (RI) activity which prevents the growth of larger grains of ice at the expense of smaller grains. Because recrystallization can damage cell membranes, RI activity can increase the freeze–thaw survival of cells. In sea ice or glaciers, RI activity may also conserve the boundaries between ice grains. These boundaries form a network of liquid veins that can harbor microbial communities [14,15]. In the last decade, a number of psychrophilic yeasts were isolated in cold environments such as alpine regions, polar ice and glaciers [3,12,13,19,22]. It is likely that some of these yeasts produce AFPs or IBPs. It has been proposed that fungal IBP genes were acquired by horizontal transfer from prokaryotes [17]. To better understand the evolution of fungal IBPs, it is necessary to find fungi that produce them and to characterize their genes. In this study, we isolated a psychrophilic yeast from an ice core taken from an Arctic pond in the Svalbard archipelago that showed ice-binding activity, purified the IBP and cloned and sequenced its gene.

Materials and methods

Sampling, isolation, and scanning electron microscopy

An ice core sample about 1 m long and 9 cm in diameter was obtained manually using the Mark II Coring system (Kovacs Enterprises, NH, USA) on April, 26, 2006 from the frozen Tvillingvatnet, a freshwater pond (78°49N 11°57E) near the Dasan station, Ny-Ålesund, Svalbard archipelago, Norway. Ice temperature was not measured, but the mean air temperature in late April is about −10 °C. The ice core was cut into three pieces of about 30 cm long and transferred to the laboratory in the Dasan station.

To remove possible contamination the exterior of the ice core was physically scraped off and the scraped ice core was washed with 95% ethanol, followed by a rinse with sterile deionized water. The final ice cores, which were about 7 cm in diameter, were melted and pooled and an aliquot was applied to Nutrient agar (NA; BD Biosciences, San Jose, CA, USA) plates that were incubated at 4 °C. After 2 weeks, about 40 colonies were observed on the plates. Each of these colonies was cultured in Nutrient Broth (NB; BD Biosciences) at 4 °C. DNA was obtained with an *AccuPrep*®Geno-

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mic DNA Extraction Kit (Bioneer, Daejon, Korea) and subjected to PCR using universal primers for 16S ribosomal DNA. If no PCR product was obtained, the PCR was repeated using universal primers for 18S ribosomal DNA. All the 16S and 18S products were sequenced, resulting in 10 non-redundant (identities >97.5%) sequences. Nine of the 10 non-redundant isolates were identified as bacteria: *Cryobacterium* sp. (5 isolates), *Flavobacterium* sp. (2 isolates), *Aquaspirillum* sp. (1 isolate), and 1 unidentified species. The remaining colony was a yeast (described below). Culture supernatants of the isolates were examined for ice-binding activity. Only the yeast strain showed ice-binding activity.

For scanning electron microscopy (SEM), yeast cells were fixed at 4 °C for 2 h in modified Karnovsky's fixative solution (2% paraformaldehyde, 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2), washed 3 times in 0.05 M cacodylate buffer (pH 7.2), and dehydrated in a graded series of ethanol. After treatment with 100% isoamylacetate twice for 15 min, the sample was dried by Critical Point Dryer (CPD). Dried cells were mounted on metal stubs, coated with gold, and then examined in a JSM-5410LV (JEOL, Japan) electron microscope.

Ice activity measurements

Ice-binding activity was measured by the pitting assay previously described by Raymond [16] with a minor modification. Briefly, a single ice crystal was prepared by incubation of 15 ml of deionized water in a petri dish in a −20 °C cold room for 1 h. A single ice slice (2 mm diameter) was separated and placed on a glass slide connected with a long rod-like arm. The glass slide was immersed into a glass viewing tube containing the sample solution. The tube was placed into sample chamber filled with ethanol which was cooled and circulated by a finely controlled water bath at −0.1 °C. The ice basal plane was viewed by Olympus SZ61 stereo microscopes (Olympus, Tokyo, Japan). Photographs were recorded by Olympus C-5060 WZ digital camera (Olympus, Tokyo, Japan) at magnifications between 10× and 180×.

TH activity and ice crystal morphology were examined by a nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand) connected to a cold well stage mounted on a light microscope equipped with a Canon Digital Camera. Briefly, a droplet containing a few nanoliters of sample to be assayed was layered into a well filled with oil. The sample well was placed on the stage and frozen rapidly at around −20 °C. The temperature was raised slowly until a single ice crystal remained. The temperature was lowered again slowly while the ice crystal morphology was maintained. The ice crystal image was pictured at 40× magnification. The freezing point of the sample was taken by the temperature at which the rapid growth of ice crystal was observed.

Ice recrystallization inhibition (RI) activity was measured using sucrose by the method of Smallwood [20] using a Linkam TMHS600 cold stage (Linkam Scientific Instruments, Surrey, UK) mounted with an Olympus BX51 upright microscope. A 2 μl droplet of sample in 30% sucrose was layered between two coverslips and located onto a silver block inside the cold stage. The temperature was quickly lowered to −80 °C at a rate of 90 °C/min, and maintained for 1 min. The sample was then warmed to −6 °C and maintained for 60 min. The image was captured and analyzed.

Purification, peptide sequencing and characterization of AFP

The IBP from the culture supernatant was purified by the cold-finger ice affinity method [11]. Briefly, a single colony from the NA plate was inoculated onto 10 ml of NB medium and grown at 4 °C for 1 week. Ten milliliters of seed culture was transferred into 1 L of NB medium and was cultured at 4 °C for 1 week with shaking. The cells were removed by centrifugation and the supernatant was concentrated using a Tangential Flow Filtration system (Millipore, MA, USA). The cold finger seeded with a thin shell of ice around it was submerged into the pre-chilled (<4 °C) concentrated supernatant. The temperature of the cold finger system was gradually lowered from −0.5 to −2 °C at a rate of −0.1 °C/h. The ice fraction on the cold finger was washed with cold (<4 °C) distilled water and melted for a second round of cold finger purification. The final ice fraction was melted and concentrated by ultrafiltration and stored at −70 °C for later use. The purified protein was visualized by SDS–PAGE and the concentration of protein was estimated by the method of Bradford using bovine serum albumin (BSA) as a protein standard [1].

For *de novo* sequencing of peptide fragments generated from yeast IBP, a single band corresponding to IBP was excised manually from the SDS–PAGE gel after staining with Coomassie blue R-250 and subjected to the standard tandem MS/MS analysis at the Korea Basic Research Institute. To determine if yeast IBP is glycosylated, purified IBP was stained with periodic acid-Schiff (PAS) reagent using a GelCode Glycoprotein Staining Kit (Thermo Fisher Scientific., Waltham, MA, USA) according to the manufacturer's instructions.

Partial genome sequencing and identification of ribosomal DNA and IBP gene

Genomic DNA was isolated from yeast cells using a MasterPure Yeast DNA purification kit (Epicentre, WI, USA) according to the manufacturer's manual. Partial genome sequencing was performed by the pyrosequencing method using the GS-FLX system (Macrogen, Seoul, Korea). About 244,000 reads of approximately 340 bp were obtained (about 83 Mbp). These were assembled into 10,888 contigs with an accuracy of 92% and an average length of 1819 bp. The total size of the contigs was about 16 Mbp.

Because of the high error rate in the reads, which is normal, sequences of interest were confirmed by PCR, and the PCR sequences were taken as the correct sequences. A contig of 7 kbp containing the whole ribosomal DNA was obtained. To confirm the sequence, two sets of primers were used to amplify about 5.7 kbp of whole ribosomal regions: 18SF, 5′-TGG TCC TAA TCG AAC CTT GT and 18SR, 5′-AAG TTC AGC GGG TAA TCC TG; 26SF, 5′-GGA GCA TGT CTG TTT GAG TG and 26SR, 5′-GAG AAT AAG TCC AAC CGA AG. The primers were chosen so that the two sequences overlapped. The amplified sequences were assembled and deposited in NCBI GenBank (Accession No. GQ336996).

To search for AFP- and IBP-like sequences, contigs were searched using local TBLASTN with known AFPs and IBPs. A contig of about 3 kb showed high identity with *Typhula ishikariensis* AFP (BAD02891) and covered the entire *T. ishikariensis* open reading frame (ORF). To confirm the sequence, the gene was amplified using specific forward primer 12gF (5′-TTTCCAGGAGGGTAG CAATG) and reverse primer 12gR (5′-TGCACAAACCAGCAGAAGGA). To clone full-length IBP cDNA, 3 μg of total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and used for the synthesis of first strand cDNA using a CapFishing full-length cDNA Premix Kit (SeeGene, Seoul, Korea) according to the manufacturer's manual. We attempted to obtain the 5′ and 3′ ends by 5′ and 3′ Rapid Amplification of cDNA Ends (RACE), respectively. The 5′-RACE was unsuccessful. Instead, we obtained part of the 5′ end in the 3′-RACE, in which the forward primer (5′-CGGGA CCTTCTCGGAATA) was 120 nt upstream of the start codon. The size and sequence of the PCR product were the same as those predicted from the contig. After A-tailing the PCR product harboring complete partial 5′-UTR, ORF and 3′ end was cloned into TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and sequenced. The full-length cDNA sequence of IBP was deposited in NCBI GenBank (Accession No. GQ336995). Amino acid sequences of the

deduced yeast IBP and other AFPs and IBPs were analyzed with BioEdit. A phylogenetic tree was constructed from these sequences using neighbor-joining method with MEGA4 [21] and bootstrapped 1000 times.

Results

Isolation and identification of a psychrophilic Arctic yeast

Ten isolates were identified from an ice core sample from a freshwater pond on Svalbard and grown on nutrient agar plates at 4 °C for 2 weeks. The culture media of the isolates were tested for ice-binding activity. Only one isolate, a yeast, showed significant ice-binding activity (Fig. 1B). This yeast grew in a temperature range from 0 to 16 °C. The optimum growth temperature was 5 °C, indicating that the yeast is psychrophilic. As shown in Fig. 1A, the isolate is 6–8 μm in length and 2–3 μm in diameter, and divides like a yeast [24].

The yeast was identified by its large subunit D1/D2 and internal transcribed spacer (ITS) rDNA regions, which are commonly used for identifying basidiomycetous yeasts [6]. The whole ribosomal sequence was confirmed by sequencing the PCR product using specific primers designed based on a draft version of genomic DNA sequence of this yeast. The D1/D2 region of this isolate was 100% identical to that of several 'Antarctic yeasts' (AY040645, AY040644) and 98% identical to *Leucosporidium antarcticum* (DQ785787, AF189906). Its ITS sequence was 100% identical to '*Leucosporidium* sp. 1 group' (EU149802, EU149803 and EU149804), newly isolated from the Antarctic soil [2], while it showed relatively low identity (~92%) to *L. antarcticum* (FJ554838, DQ525624). Taken together, these results clearly indicate that the isolate belongs to the genus *Leucosporidium*. However the definition of this species will be described elsewhere. Hence we tentatively identify this isolate as *Leucosporidium* sp. and designate it as strain *Leucosporidium* sp. AY30.

Purification and partial characterization of yeast IBP

The culture medium of *Leucosporidium* sp. AY30 incubated at 0–15 °C showed both ice-pitting activity (Fig. 1B) and RI activity (Fig. 1C), which are indicative of the presence of ice-active substances in the culture medium. Heat (95 °C, 5 min) or proteinase K treatment of the medium completely abolished ice-binding activity, indicating that the ice activity originated from the proteins. Ice-binding proteins in the cell-free culture supernatant were purified by ice affinity using the cold-finger method. SDS–PAGE analysis showed that the ice fraction contained a single protein of ca. 26 kDa (Fig. 2A). The band stained positive for PAS (Fig. 2B) indicating that it was glycosylated.

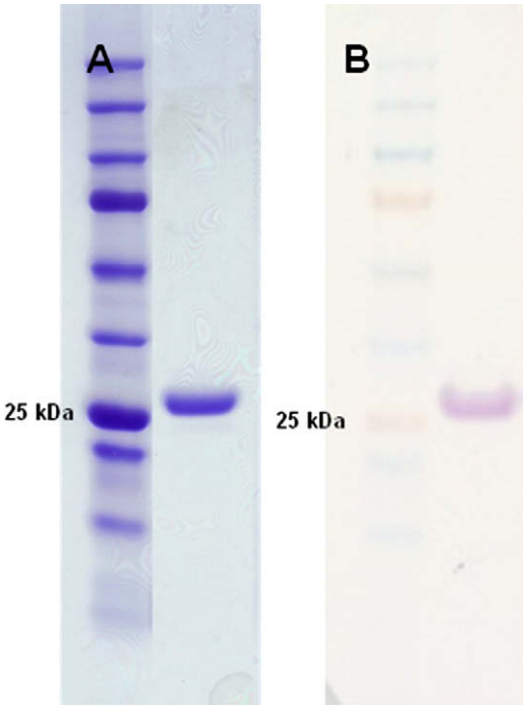

Fig. 2. SDS–PAGE analysis of the purified IBP by ice affinity method stained by Coomassie blue R-250 (A) and by periodic acid-Schiff (PAS) reagent (B).

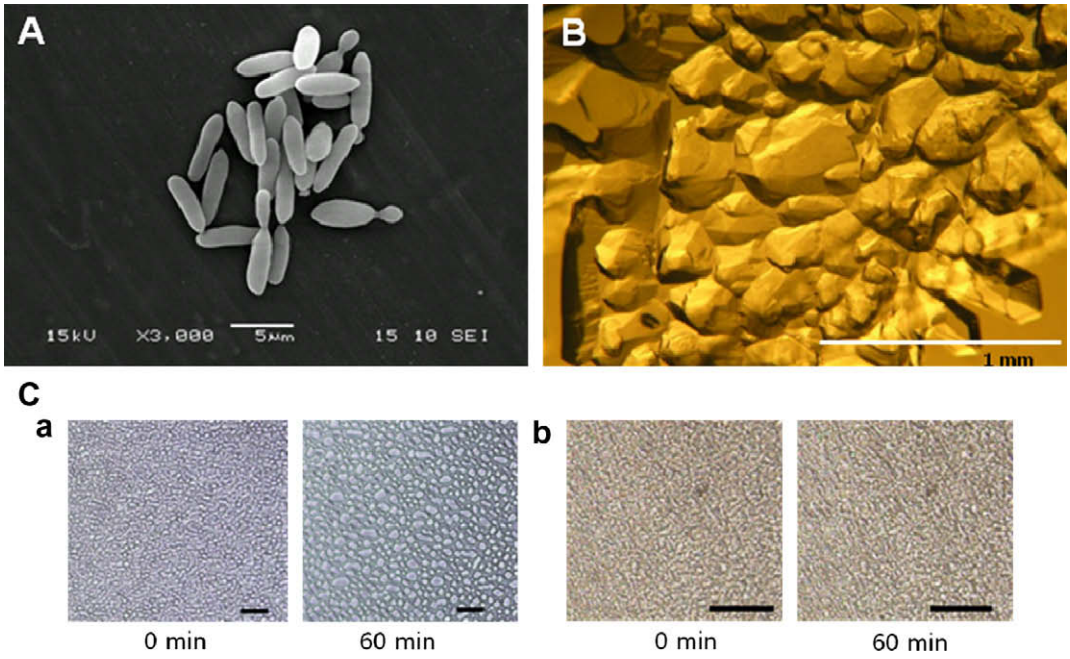

Fig. 1. A psychrophilic yeast, *Leucosporidium* sp. AY30. (A) Scanning electron micrograph. (B) Ice-pitting activity of culture media. (C) Ice recrystallization inhibition (RI) assay of 30% sucrose solution (a) and 30% sucrose solution with the culture medium (b). Scale bars, 500 μm.

The effect of the purified protein on ice crystal morphology was examined with a nanolitre osmometer. *Escherichia coli* extract and phosphate-buffered saline were used as controls. This protein produced a distinct hexagonal ice crystal shape, indicative of protein binding to the ice planes (Fig. 3Ba and b), while the *E. coli* extract and phosphate-buffered saline caused round or irregular shape of ice growth (data not shown). Ice crystal morphology was also affected by protein concentration. At low concentration (~100 μg/ml), the ice crystals were bipyramidal with limited growth on the basal plane (Fig. 3Ba). At high concentration (0.5 mg/ml), ice crystal growth was inhibited but as the temperature decreased, there was a burst of growth along the *c*-axis (Fig. 3A), although the shape was different from the needle-like shape observed with type I fish AFPs. The difference between the freezing and melting points (thermal hysteresis) at a concentration of 0.5 mg/ml was about 0.7 °C.

Tandem mass spectrometry yielded only one peptide sequence, FVT(I/L)(Q/GA)TGSS(I/L)(N/D)GR, which was insufficient for primer design. However, this sequence was highly similar to part of the AFP from the snow mold *T. ishikariensis* [8], which suggested that the protein was similar to other fungal IBPs.

Cloning and sequence analysis of IBP gene

A total of 10,888 contigs of 1819 nt average length were obtained by pyrosequencing. If the genome size is similar to that of *Saccharomyces cerevisiae*, this represents an approximately 1× coverage. Both reads and contigs will be submitted to GenBank. A local TBLASTN search found one contig of about 3 kbp matching the *T. ishikariensis* AFP sequence (BAD02891) [8]. The contig encoded a sequence that was highly similar to the *T. ishikariensis* AFP. No other contigs showed similarity to other AFPs or IBPs. A 2007-bp region of the IBP contig was reamplified by PCR and sequenced. The genomic DNA sequence of yeast IBP (GenBank Accession No. GQ336994) contains 8 introns of 50–68 bp each. The exon–intron boundaries were well conserved according to the GT-AG rule.

The full-length cDNA of yeast IBP contains approximately a 119 nt partial 5′-UTR, a 783 nt ORF, and a 40 nt 3′-UTR (Fig. 4). The 783 nt ORF encodes a protein of 261 amino acid residues. The N-terminal 20 residues were predicted to be a secretory signal peptide by the Signal IP 3.0 program. One potential N-glycosylation site (Asn-X-Thr) was predicted at residue 185N by the NetNGlyc program. The molecular weights of the precursor and the mature IBP were calculated to be 26.8 kDa and 24.9 kDa, respectively.

The deduced IBP protein showed relatively high identities with IBPs of other fungi in the same phylum (Fig. 5): 55% identity with *T. ishikariensis* AFP (BAD02892) [8], 51% with *Lentinula edodes* (ACL27145) [17], and 50% with *Flammulina populicola* (ACL27144) [17]. It also had high identities with IBPs from diatoms and bacteria: 48% with *Navicula glaciei* (AAZ76250) [9,17], 42% with *Colwellia* sp. SLW05 (ABH08428) [15].

Discussion

The optimum growth temperature and phylogenetic analysis of ribosomal DNA sequences revealed that the isolate in this study is

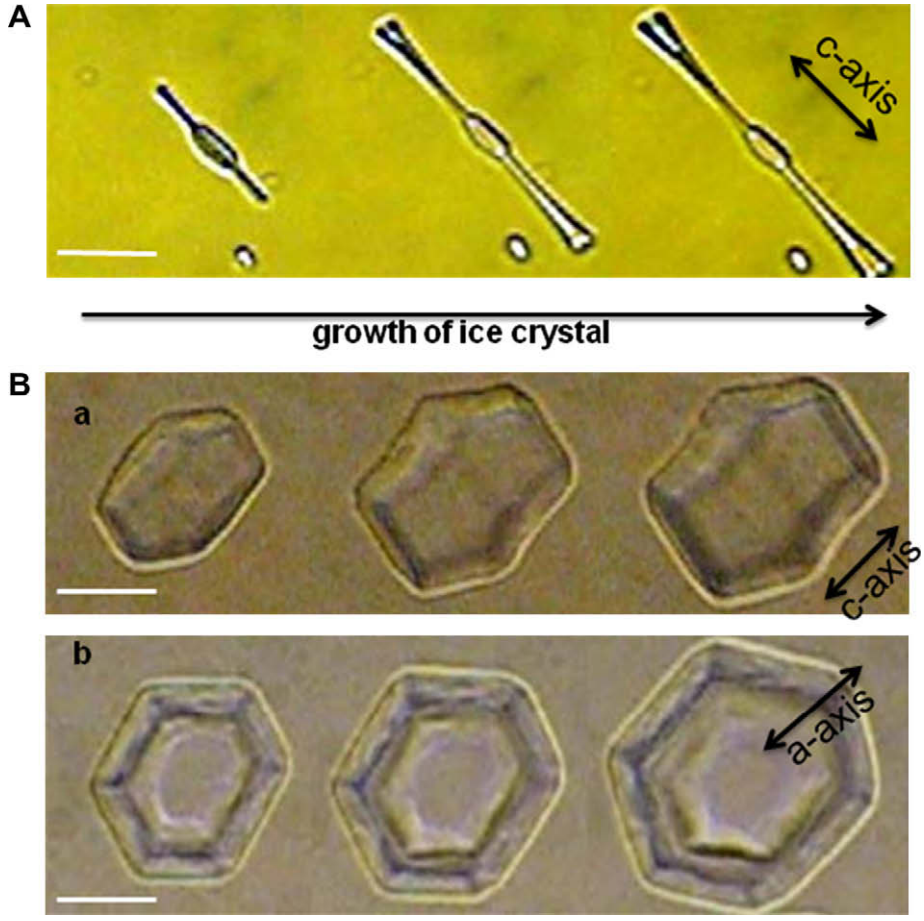

Fig. 3. Ice crystal morphologies and growth of purified IBP from *Leucosporidium*. sp. AY30. (A) Ice growth along the *c*-axis at a concentration of 500 μg/ml. (B) Ice crystal growth along the *a*-axis at a concentration of 100 μg/ml in viewing normal to *c*-axis (a) and *a*-axis (b). Scale bar, 50 μm.

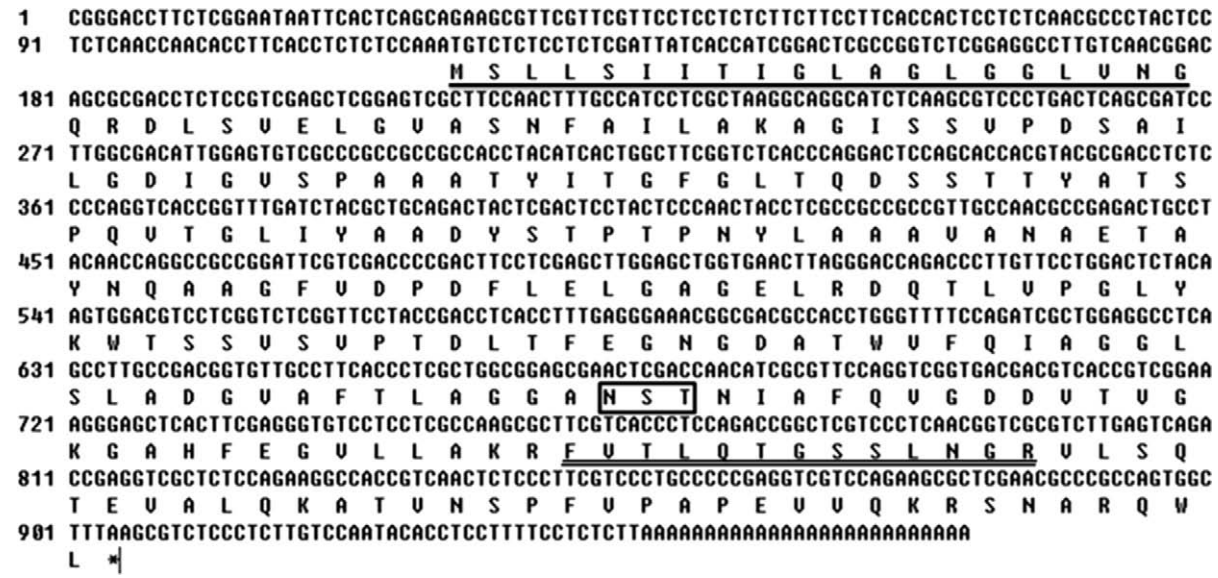

Fig. 4. Nucleotide sequence of full-length cDNA encoding IBP from *Leucosporidium*. sp. AY30. The deduced amino acid sequence is presented under the corresponding nucleotide sequence. The putative signal peptide is underlined. The potential N-glycosylation site is boxed. Peptide sequence identified by MS/MS sequencing is double-underlined.

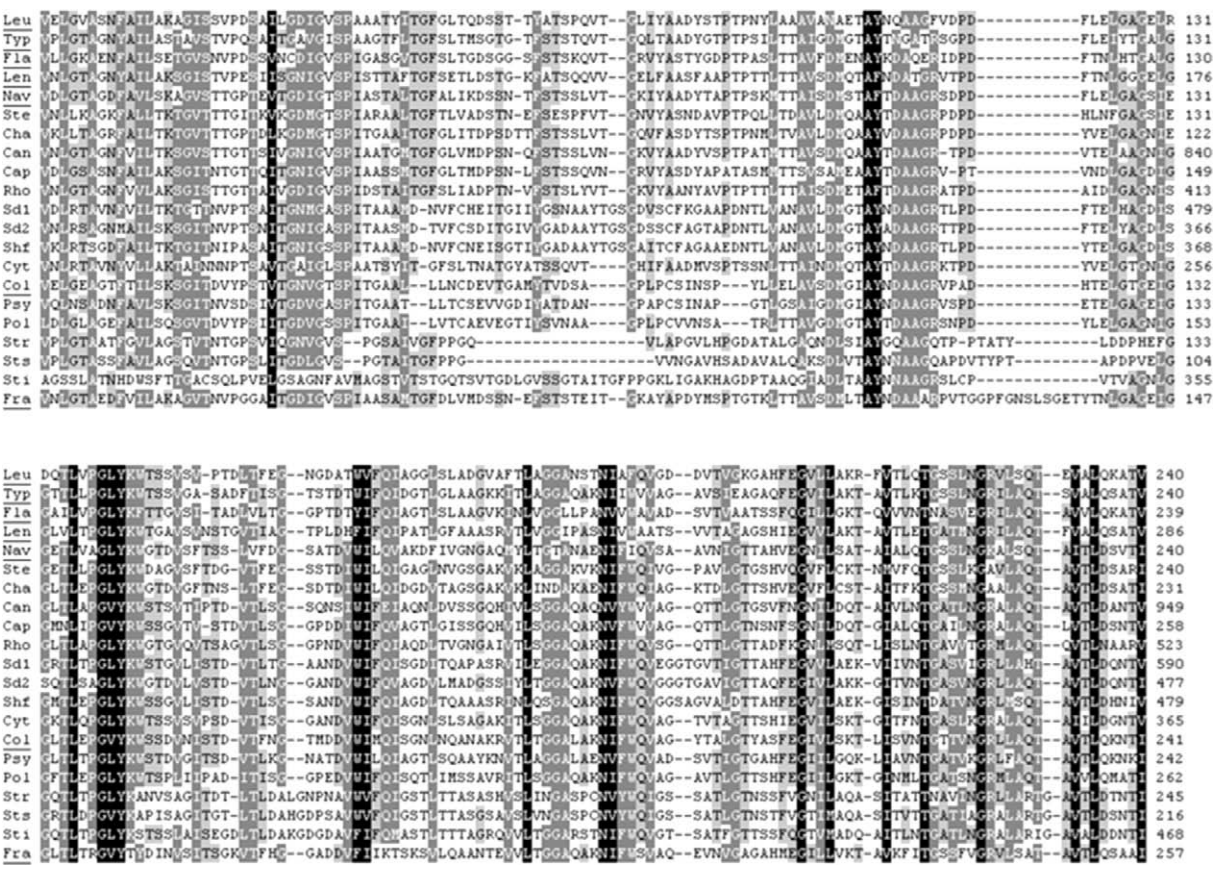

Fig. 5. Multiple alignment of amino acid sequences of known AFPs, IBPs, and hypothetical proteins. The matched portions are only presented. The numbers of amino acid sequences are the original numbers of each sequence. The putative N-glycosylation sites are boxed. The abbreviations of known IBPs or AFPs are underlined. Fla, *Flammulina populicola*; Leu, *Leucosporidium* sp. AY30; Len, *Lentinula edodes*; Typ, *Typhula ishikariensis*; Cyt, *Cytophaga hutchinsonii*; Can, *Candidatus Methanoregular boonei*; Rho, *Rhodoferax ferrireducens*; Nav, *Navicula glaciei*; Ste, *Stephos longipes*; Cha, *Chaetoceros neogracile*; Pol, *Polaribacter irgensii*; Psy, *Psychromonas ingrahamii*; Col, *Colwellia* sp. SLW50; Sd1, *Shewanella denitrificans* 1914; Sd2, *Shewanella denitrificans* 1915; Shf, *Shewanella frigidimarina*; Sti, *Stigmatella aurantiaca*; Str, *Streptomyces avermitilis*; Sts, *Streptomyces sviceus*; Fra, *Fragilariopsis cylindrus*.

a novel psychrophilic yeast belonging to the genus *Leucosporidium*. The D1/D2 domain and ITS rDNA sequences have been used to identify basidiomycetous yeasts at the genus level [6]. The sequence analysis of *Leucosporidium* sp. AY30 revealed that both D1/D2 and ITS sequence are different from that of *L. antarcticum* but the D1/D2 is identical to that of 'Antarctic yeast' and ITS is

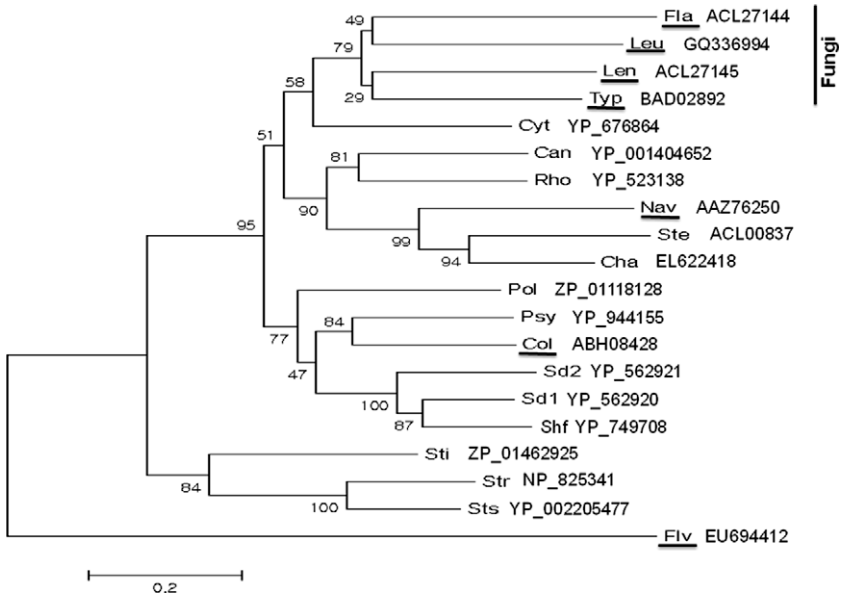

Fig. 6. Phylogenetic analysis among known and hypothetical AFPs or IBPs in GenBank. Bootstrap values at each branch are estimated with 1000 replicates by the neighbor-joining method. The abbreviations of known IBPs or AFPs are underlined. Genbank accession numbers are presented. Flv, *Flavobacteriaceae* bacterium is used as an outgoup.

identical to that of '*Leucosporidium* sp. group 1'. However, due to the limited D1/D2 and ITS sequences and physiological data of species at this genus, it is not possible to further define this isolate. Two yeast species isolated from an Antarctic plateau ice core (*Cryptococcus* sp. and *Rhodotorula* sp.) showed no ice-binding activity [14]. To the best of our knowledge, *Leucosporidium* sp. AY30 is the first yeast to show ice-binding activity.

The purified yeast IBP depressed the freezing point of water to a greater degree than did *T. ishikariensis* AFP [8], but not enough to prevent freezing of cells. Therefore, this secreted protein seems better suited for conferring freeze tolerance than for preventing freezing. RI activity has been described for AFPs and some IBPs. The RI activity of the extracellularly secreted yeast IBP probably serves to control the growth of larger grains of ice rather than to prevent freezing, eventually protecting the cell membranes from damage from extracellular freezing, as is thought to be the case with other IBPs [14,15,18].

The deduced *Leucosporidium* IBP, like *Typhula* AFP, was predicted to have one possible N-glycosylation site and an N-terminal secretory peptide. Yeast IBP was found to be N-glycosylated as judged by the PAS staining.

In a phylogenetic analysis (Fig. 6), yeast IBP distinctively clusters with other fungal proteins, segregating them from diatom and bacterial IBPs. Recently, the possibility of horizontal or lateral gene transfer (HGT) of AFP or IBP genes from prokaryotes to eukaryotes was raised because of the finding of several IBP-like sequences in bacteria and archaea [9,17]. Davies and his colleagues proposed HGT of fish type II AFPs because they exist in three distantly related species (herring, smelt and sea raven) with 85% identity in their amino acid sequences, and also have remarkably higher identity in their genes [7]. The known fungal IBPs do not share such high sequence identities or have similar introns, which suggests that the genes were not horizontally transferred among the fungi. However, it is possible that the genes were acquired independently from prokaryotes. The evolution and source of AFPs and IBPs from organisms in cold climates should become clearer as more genome sequences become available.

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