Effect of the Antifreeze Protein from the Arctic Yeast Leucosporidium *sp. AY30 on Cryopreservation of the Marine Diatom* Phaeodactylum tricornutum

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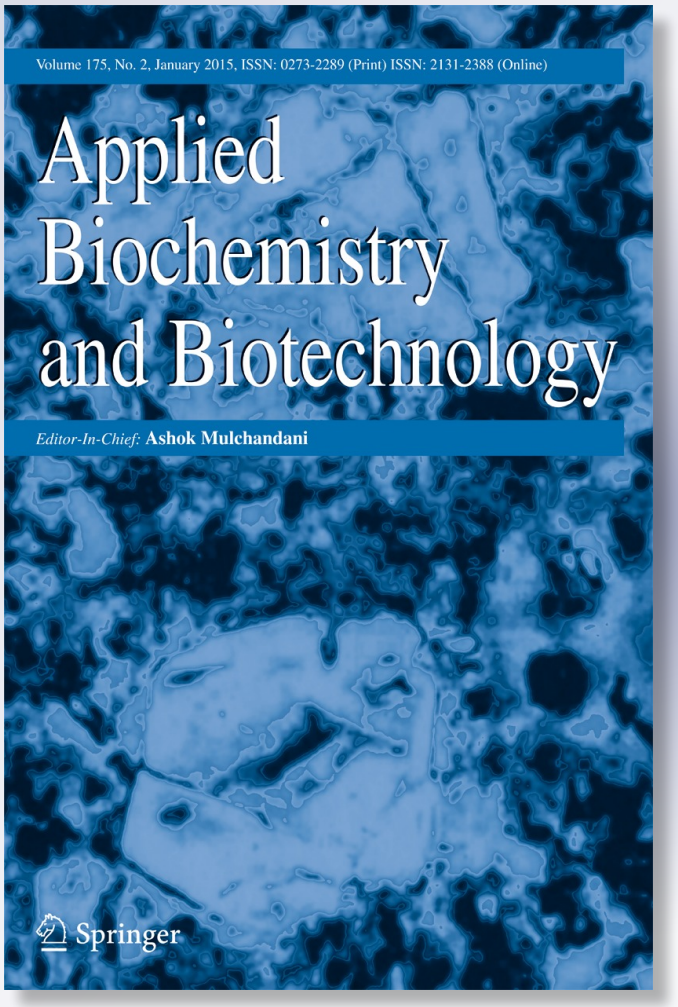

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Effect of the Antifreeze Protein from the Arctic Yeast *Leucosporidium* sp. AY30 on Cryopreservation of the Marine Diatom *Phaeodactylum tricornutum*

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Abstract Antifreeze proteins are a group of proteins that allow organisms to survive in subzero environments. These proteins possess thermal hysteresis and ice recrystallization inhibition activities. In the present study, we demonstrated the efficiency of a recombinant antifreeze protein from the Arctic yeast *Leucosporidium* sp. AY30, LeIBP, in cryopreservation of the marine diatom *Phaeodactylum tricornutum*, which is one of the classical model diatoms and has most widely been studied with regard to its ecology, physiology, biochemistry, and molecular biology. *P. tricornutum* cells were frozen by either a fast or two-step freezing method in freezing medium containing 10 % dimethyl sulfoxide, glycerol, propylene glycol, and ethylene glycol, respectively, with or without LeIBP supplement. When cells were frozen using the two-step freezing method, cell survival was significantly increased and statistically the same as that of unfrozen native cells in the presence of 0.1 mg/ml LeIBP in 10 % propylene glycol or 10 % ethylene glycol at day 11 of post-thaw culture. In the presence of LeIBP, the concentration of chlorophyll a was dramatically increased to 14-, 48-, 1.6-, and 8.8-fold when cells were frozen in freezing medium containing dimethyl sulfoxide (DMSO), glycerol, propylene glycol (PG), and ethylene glycol (EG), respectively. Scanning electron microscopy observations demonstrated that the cells were also successfully preserved and epitheca or hypotheca were not deformed. These results demonstrate that LeIBP was successfully applied to improve cryopreservation of the marine diatom *P. tricornutum*.

Keywords Antifreeze protein · LeIBP · Marine diatom · *Phaeodactylum tricornutum* · Cryopreservation

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Introduction

Diatoms play critical roles in photosynthesis and recycling of carbon and silicon in most marine ecosystems [1, 2]. The pennate Bacillariophyceae *Phaeodactylum tricornutum* seems to be ubiquitous in most coastal areas; in particular, it is found in estuaries and rock pools [3], where temperature and salinity are fluctuating [4]. This diatom has pleiomorphic properties, showing four morphotypes: fusiform, oval, round, or triradiate [5, 3]. It was suggested that different environmental conditions might drive the morphotypic plasticity of *P. tricornutum* [6, 3, 7]. A recent study provided supporting data, showing that the morphological interconversion of *P. tricornutum* can be regulated by changing the culture conditions [8]. Because of this unusual characteristic, *P. tricornutum* has been regarded as a model system to study the response of diatoms to environmental changes [6, 3, 8].

Diatoms have been usually maintained by repeated serial subculture. Even though this method has been utilized in many algal culture collections, it is time-consuming and susceptible to potential contamination and genetic or physiological changes [9–11]. Cryopreservation of cells would preserve the unique phenotype, and other researchers could share the same sources that have identical characteristics, regardless of time or place. It was suggested that cryopreservation would be the best method for long-term storage of microalgae [12]. Although the exact mechanism of cryoinjury of algae has not been fully understood yet [13], some major algal culture collections and the European project COBRA (microalgae and cyanobacteria conservation project) introduced a cryopreservation system [14, 15, 12, 16, 17].

Cryoprotective agents (CPAs) commonly used for cryopreservation of most marine microalgae include methanol, dimethyl sulfoxide (DMSO), and glycerol [18]. They reduce the intracellular water content and lower the cytoplasm crystallization rate; thus, osmotic pressure-induced freezing damage to cells can be avoided [12, 19, 20]. Other CPAs such as polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), hydroxyethyl starch (HES), 2-methyl-2,4-pentanediol, ethylene glycol (EG), and propylene glycol (PG) are rarely used in microalgal cryopreservation protocols, even though these CPAs also dehydrate the cytosol and, thus, minimize the formation of intracellular ice crystals. In spite of the usefulness of these CPAs in cryopreservation, high concentrations of most chemical CPAs cause cytotoxicity [21]. Moreover, some researches have reported that DMSO, for example, may cause unpredicted effects on embryonic stem cells, osteoblastic cells, and embryoid bodies by altering the epigenetic regulation of the cells [22–24]. Therefore, the demand for new candidates of nontoxic or less toxic CPAs has been increasing.

The first antifreeze protein (AFP) was discovered in an Antarctic teleost fish [25]; others were later found in various psychrotolerant organisms such as bacteria, yeast, fungi, diatoms, insects, plants, fish, etc. [26, 25, 27–31]. AFPs depress the freezing point of a solution in a non-colligative manner (thermal hysteresis) and inhibit ice recrystallization [25, 32]. Because of these unique properties, AFPs have been considered strong candidates as biological CPAs. In fact, the potential of these proteins has been demonstrated in the cryopreservation of various cells, including red blood cells, mammalian cells, reproductive cells, etc. [33–39]. The first yeast AFP, LeIBP, was originally found in the Arctic yeast *Leucosporidium* sp. AY30 and has been well characterized in previous studies [26, 35]. Recently, a mass production system for LeIBP was established, and the recombinant protein showed potential in the cryopreservation of red blood cells [35, 40]. In the present study, we demonstrate the cryopreservative effect of LeIBP on the pennate diatom *P. tricornutum*.

Materials and Methods

Strains and Culture Conditions

The marine diatom *P. tricornutum* (CCAP 1055/1) was obtained from the Culture Collection of Algae and Protozoa (CCAP, http://www.ccap.ac.uk). Cells were grown in batch cultures in flasks with a photon fluence rate of 60–80 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes in a 16:8 light/dark (L/D) photoperiod at 12 °C. Cells were routinely maintained in F/2+Si medium [41] and subcultured once a month.

Preparation of Recombinant LeIBP

Recombinant LeIBP was produced in the methylotrophic yeast *Pichia pastoris* and purified as described in a previous study [42]. Briefly, recombinant *P. pastoris* strain X33 cells transformed by pPICZαA harboring the mature LeIBP gene were grown in 3-l flasks containing yeast–peptone–dextrose medium at 30 °C for 2 days. Recombinant LeIBP expression was induced daily by addition of 5 ml methanol to the medium. The culture supernatant was applied to an ion exchange chromatography (QFF) column and eluted with 50 mM Tris–HCl buffer, pH 8.0, containing 400 mM NaCl. The elution fractions were then pooled and further purified on a Superdex 200 size-exclusion column (Thermo Fisher, USA), equilibrated with 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl buffer at a flow rate of 1 ml/min. Purified LeIBP was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses. The protein concentration was determined by measuring the absorbance at 280 nm, using a calculated extinction coefficient of 26,930 $M^{-1} \cdot cm^{-1}$.

Freezing and Thawing Procedures

The cryopreservation procedure was slightly modified from that developed by Mitbavkar and Anil [43]. Solutions of 10 % (*v/v* in sea water) DMSO, glycerol, PG, and EG were prepared. F/2+Si medium instead of CPA was used as negative control. These freezing media were sterilized by 0.2-μm filtration. *P. tricornutum* cells in the exponential growth phase were harvested by centrifugation at 1200×*g* for 5 min at 12 °C. Approximately 1×10^6 cells were then resuspended in 1 ml of each freezing medium supplemented with or without LeIBP. The final concentration of LeIBP was 0.1 mg/ml. Cells were then transferred to 2-ml cryovials.

P. tricornutum cells were subjected to two different freezing procedures: rapid freezing and two-step freezing. For rapid freezing, the cryovials prepared as described above were directly placed in liquid nitrogen (LN_2) and then transferred to LN_2 vapor. For two-step freezing, cryovials containing the cells were transferred to a controlled-rate freezer (Kryo 560, Planner, UK) programmed to cool from 20 to 0 °C at −5 °C/min and then from 0 to −40 °C at a rate of −1 °C/min. They were then rapidly cooled by transferring to LN_2 vapor. Each experiment was performed in triplicate.

After 2 weeks storage, the cryovials were thawed by immersion in a 30 °C water bath for 2 min and centrifuged at 1200×*g* for 5 min at 12 °C, and the supernatant was discarded. Cells were washed three times with F/2+Si medium to remove any remaining CPA. Finally, the washed cells were transferred to fresh F/2+Si medium in a 24-well plate and grown under normal culture conditions as described above.

Cell Viability and Chlorophyll a Measurements

Cultures were grown for 11 days and viability assays were conducted every other day. Viable cells were determined by microscopic observations after staining using neutral red [44]. Briefly, 1 μl neutral red solution (Sigma, Korea) was added to 9 μl cell culture. After incubation in the dark at 12 °C for 10 min, viable red cells were counted using a hemocytometer under a microscope (BX-51, Olympus, Japan). The concentration of chlorophyll a was determined fluorometrically [43]. One milliliter of each sample was taken periodically throughout the cultivation period. The in vivo fluorescence measurements were carried out using a Trilogy laboratory fluorometer (Turner Designs, USA).

Scanning Electron Microscopy

After cryopreservation, whole diatom cells were examined by scanning electron microscopy (SEM). Cells were deposited onto 1-cm round glass coverslips in Petri dishes. Cells were fixed for 30 min by adding several drops of 1 % glutaraldehyde to culture media, rinsed three times with distilled water, and air-dried at room temperature. The preparations were then coated with a complete layer of gold-palladium and observed by SEM (JSM-6610LV, JEOL Ltd., Japan). The cell images presented in the figures are representative for each experimental group.

Statistical Analysis

Excel software (Microsoft, USA) was used for statistical analyses. Student's *t* test was used to determine significant differences, accepting $p < 0.01$ as significant. All experiments were performed independently and repeated at least three times; data are expressed as mean±1 SD.

Results

Cell Viability and Chlorophyll a Measurements

Cells of the marine diatom *P. tricornutum* were subjected to two different freezing protocols using combinations of various chemical CPAs with LeIBP. The cryoprotective effect of LeIBP was determined by measuring cell recovery and chlorophyll a concentration after freezing–thawing. Cells frozen by the programmed two-step freezing method using a single chemical CPA did not reveal obvious cell recovery throughout the period of post-thaw culture (up to 11 days). Cell concentrations were determined as 5, 2, 75, and 34 % in freezing media containing DMSO, glycerol, PG, and EG, respectively, at day 11 of post-thaw culture compared with unfrozen control cells. However, in the presence of 0.1 mg/ml LeIBP, cell concentrations increased to 68, 48, 100, and 100 % in freezing media containing DMSO, glycerol, PG, and EG, respectively, at day 11 of post-thaw culture (Fig. 1). Cells frozen by the rapid freezing method did not show noticeable cell recovery in both CPA-only and CPA-supplemented with LeIBP treatment.

Cryopreserved cells were assessed by measuring the chlorophyll a contents (Table 1). Frozen diatom cells were thawed and cultured for up to 11 days, and the chlorophyll a contents were determined. When the two-step freezing method was used, the chlorophyll a concentration was dramatically increased to 14-, 48-, 1.6-, and 8.8-fold in the DMSO, glycerol, PG, and EG groups, respectively, in the presence of LeIBP compared with that in the absence of LeIBP. The smallest increase was observed in the PG group; however, the absolute chlorophyll a

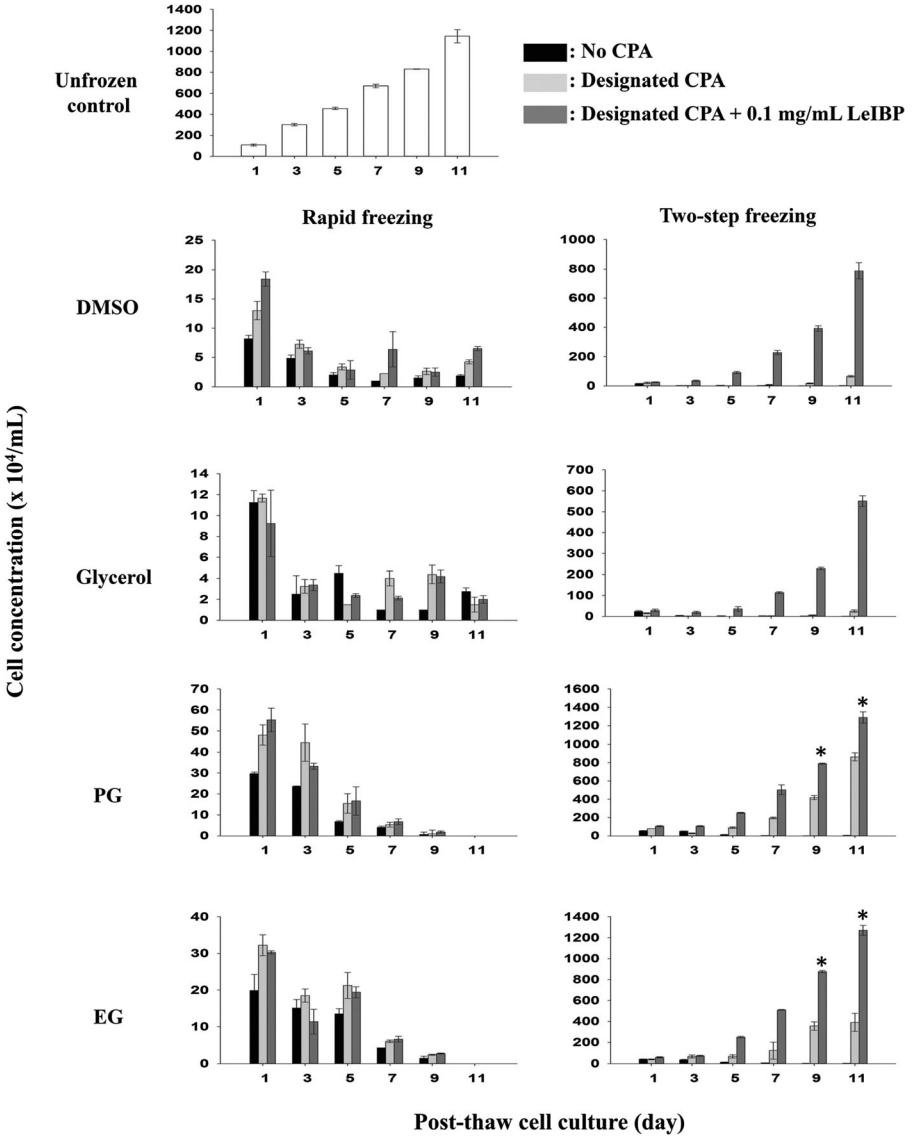

Fig. 1 Cryopreservation effect of cryoprotective agents (CPAs) on cell recovery of *P. tricornutum*. Cells were frozen for 2 weeks in freezing media containing different CPAs with or without 0.1 mg/ml of an antifreeze protein from the Arctic yeast *Leucosporidium* sp. AY30, LeIBP. Fresh cells served as unfrozen control. Post-thaw recovery was determined by neutral red staining using a hemocytometer. *Error bars* represent the standard deviation of three individual experiments. *Asterisks* indicate no significant difference between each CPA and unfrozen control ($p > 0.01$)

concentration was the highest in this group. An almost 9-fold increase was observed in the EG group in the presence of LeIBP; the chlorophyll a concentration in these cells was not significantly different compared with unfrozen control cells (Table 1). A 14- and 48-fold increase was also observed in cells of the DMSO and glycerol groups, respectively; however,

Table 1 Post-thaw chlorophyll a contents of *P. tricornutum*

Freezing method	CPAs (10 %±0.1 mg/ml LeIBP)	Chlorophyll a (μg/l)[a]	
		Day 0 post-thaw	Day 11 post-thaw
Unfrozen control	–	197.9±18.6	2292.7±35.55
Rapid freezing	10 % DMSO	30.5±0.14	9.2±0.12
	10 % DMSO+LeIBP	41.0±0.12	6.5±0.04
	10 % glycerol	34.7±0.17	2.9±0.01
	10 % glycerol+LeIBP	29.7±0.14	2.9±0.01
	10 % PG	25.0±0.13	3.0±0.03
	10 % PG+LeIBP	28.9±0.13	1.9±0.01
	10 % EG	27.4±0.13	1.7±0.02
	10 % EG+LeIBP	21.3±0.1	1.6±0.03
Two-step freezing	10 % DMSO	15.2±11.27	107.9±10.88
	10 % DMSO+LeIBP	25.5±4.89	1558.5±51.84
	10 % glycerol	4.6±3.93	24.1±11.66
	10 % glycerol+LeIBP	31.7±12.96	1155.3±14.81
	10 % PG	137.5±1.85	1727.1±20.74
	10 % PG+LeIBP	194.3±11.11 (#)	2740.9±65.17 (#)
	10 % EG	57.6±8.15	292.2±13.08
	10 % EG+LeIBP	133.6±7.41	2578.6±16.29 (#)

LeIBP antifreeze protein from the Arctic yeast *Leucosporidium* sp. AY30, *CPAs* cryoprotective agents, *DMSO* dimethyl sulfoxide, *PG* propylene glycol, *EG* ethylene glycol

[a] Crosshatches (#) indicate no significant difference between each CPA and unfrozen control ($p > 0.01$)

the absolute chlorophyll a concentration in these cells was lower than that of unfrozen control cells. Rapid freezing did not result in an increase in the chlorophyll a concentration.

Morphology of Cryopreserved Cells

Diatom cells were observed at day 11 of post-thaw culture under an optical microscope and by SEM. Analysis by optical microscopy revealed that the cell walls of several CPA-only treated cells were abnormal, demonstrating separated theca (indicated by solid arrow) and some particles around the cells (open arrows) (Fig. 2). Moreover, several cells did not retain chloroplasts inside the cells (Fig. 2, EG/LeIBP[−] after two-step freezing; representative image). However, with regard to most chloroplasts and the appearance of the cells, there was no significant difference between the CPA-only and CPA supplemented with LeIBP treatments. In order to observe the cell wall damage in detail, SEM was utilized (Fig. 3). In most cells, cracks and deformations of the cell walls were noted (indicated by open arrows and enlarged by boxes). Cell walls were damaged, and big and small holes were visible, which were located in the central or polar region of siliceous frustules. Cells, frozen in the presence of PG using the two-step freezing method, showed a bump that protruded from the surface of the cell wall. In addition, the internal view exhibited doubled cell surface lines, indicating the separation of epitheca and hypotheca of the cell wall due to cryoinjury. However, cells in the PG and EG supplemented with LeIBP groups subjected to two-step freezing exhibited almost the same morphology (undamaged, intact cell walls) as intact unfrozen control cells.

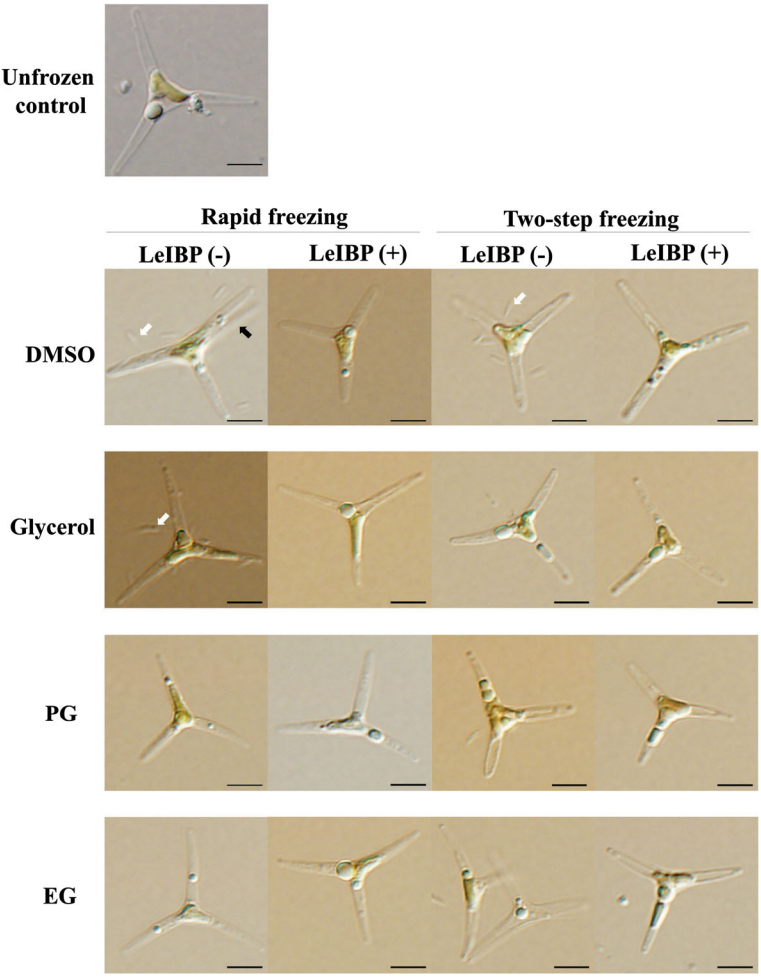

Fig. 2 Microscopic images of *P. tricornutum* at day 11 of post-thaw culture. *Scale bars*, 10 μm

Discussion

Since mammalian spermatozoa were cryopreserved for the first time using glycerol in 1949 [45], subsequent researches have adapted the cryopreservation process for a variety of cell preservations using single or multiple chemical CPAs. Methanol, glycerol, and DMSO were found to prevent cell disruption driven by ice crystallization [46, 12]. These CPAs offered possibilities to protect cells from freeze/thaw-induced damage. However, cryopreservation of microalgae has not been sufficiently studied. Several researches suggested possible protocols to cryopreserve cells by using rapid freezing, two-step freezing, immobilization-dehydration, and lyophilization [47–49, 44]. Extracellular CPAs such as glycerol, HES, PVP, and dextran reduced the extent of freezing-induced cell damage significantly; however, the degree of ice recrystallization can be minimized only by AFPs in the presence of chemical CPAs [50, 33–35]. Unfortunately, some CPAs cause cytotoxicity, eventually resulting in low survival rates; however, a shorter equilibration time solved this problem to some degree [48].

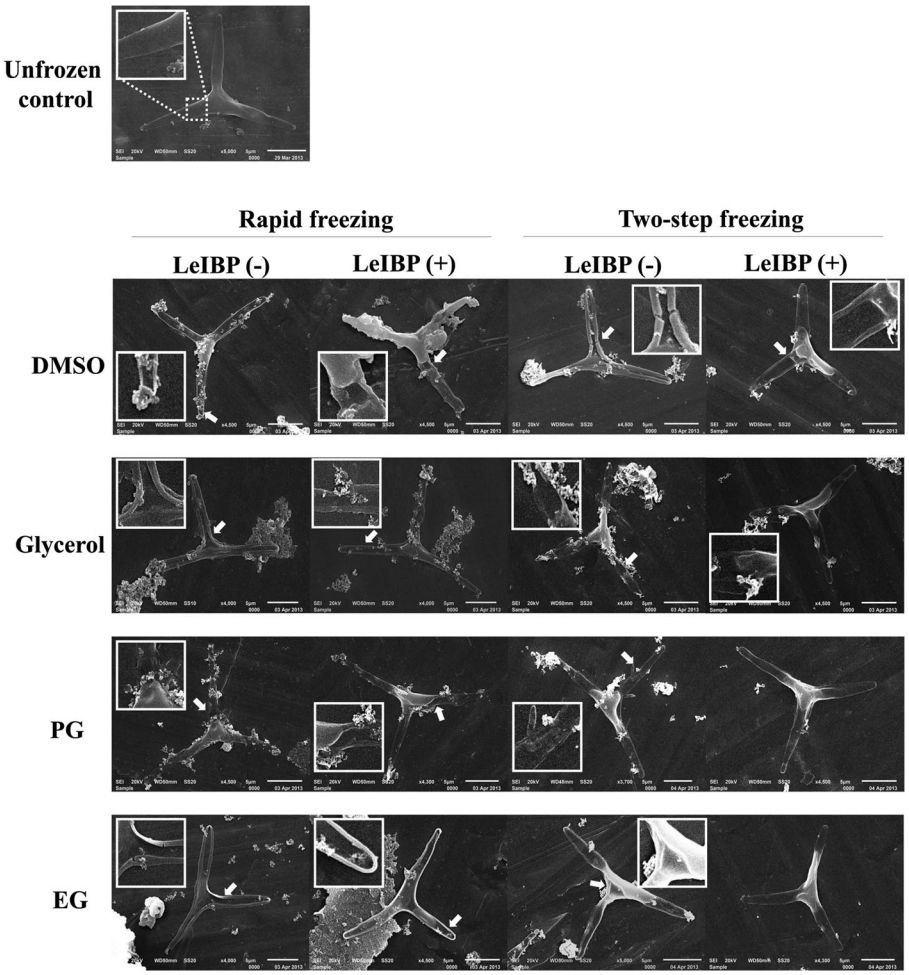

Fig. 3 Scanning electron microscope (SEM) images illustrating the morphology of *P. tricornutum* at day 11 of post-thaw culture. Most groups exhibited deformation compared with unfrozen controls. These groups showed abnormally separated or deformed epitheca or hypotheca (indicated by *white arrows* and *enlarged* in *insets*). Two-step freezing with an antifreeze protein from the Arctic yeast *Leucosporidium* sp. AY30, LeIBP, revealed undamaged appearance as seen in unfrozen controls. *Scale bars*, 5 μm

In the present study, cryopreservation of the marine diatom *P. tricornutum* was evaluated by employing the two-step freezing method using DMSO, glycerol, PG, and EG in the presence or absence of the AFP LeIBP. DMSO has generally been used in most cryopreservation systems for a long time; however, it was not very effective in the present study. Cryopreservation with glycerol and EG also resulted in poor cell viability rates; however, interestingly, the cell concentration after cryopreservation with PG was almost 75 % of that of unfrozen control cells (Fig. 1). Supplementation with LeIBP increased the viability significantly in all experimental groups. In particular, the viability of cells frozen in the presence of PG and EG was statistically the same as that of unfrozen cells at day 11 of post-thaw culture (Fig. 1). This is surprising because addition of a single biological CPA, LeIBP, to a chemical CPA dramatically improved the diatom cryopreservation system. A previous report showed

that LeIBP possessed ice recrystallization inhibition activity, and the authors demonstrated its effect on red blood cell cryopreservation [35]. The result described in the present study might be obtained for most of the other AFPs because of their ice recrystallization inhibition activity. The result of the chlorophyll a measurement is in agreement with that of the viability assay. The chlorophyll a content of the PG and EG groups subjected to two-step freezing in the presence of LeIBP was statistically the same as that of the unfrozen control, whereas the results of the CPA-only groups were unsatisfactory (Table 1). Freezing with PG in the presence of LeIBP resulted only in a 1.6-fold increase in the chlorophyll a content, which seems not very high; however, this could be explained by the already high chlorophyll a content of the PG-only group. The chlorophyll a content of the DMSO and glycerol groups was increased by 14- and 48-fold, respectively, and this increase was even greater than that reported in other previous studies.

Diatom cells are composed of a silica cell wall, which is rigid and, thus, less flexible. Therefore, the diatom cell wall becomes fragile while the cells shrink and expand in freezing medium during freeze–thaw procedures. Microscopic observations proved that chemical CPAs did not protect diatom cells against cryoinjuries (Figs. 2 and 3). Cells cryopreserved in the presence of LeIBP, however, showed no notable cell wall damage; in particular, cells frozen in the presence of EG and PG showed the same intact morphology as unfrozen control cells (Fig. 3). It still remains to be determined if LeIBP can be utilized in general diatom cryopreservation systems and if other AFPs would have the same effect as LeIBP on diatom cryopreservation; moreover, the cellular regulation of cryopreserved diatom cells using LeIBP needs to be further verified by cellular and molecular physiological studies. Nonetheless, it is anticipated that the results obtained in this study help to address these questions.

To the best of our knowledge, the present study is the first report in which the Arctic AFP LeIBP has been successfully applied to improve cryopreservation of the marine diatom *P. tricornutum*.

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