J. Microbiol. Biotechnol. (2015), 25(12), 1989–1996
http://dx.doi.org/10.4014/jmb.1507.07041

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Ice-Binding Protein Derived from *Glaciozyma* Can Improve the Viability of Cryopreserved Mammalian Cells

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Received: July 13, 2015
Revised: August 21, 2015
Accepted: August 22, 2015

First published online
August 31, 2015

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pISSN 1017-7825, eISSN 1738-8872

Ice-binding proteins (IBPs) can inhibit ice recrystallization (IR), a major cause of cell death during cryopreservation. IBPs are hypothesized to improve cell viability after cryopreservation by alleviating the cryoinjury caused by IR. In our previous studies, we showed that supplementation of the freezing medium with the recombinant IBP of the Arctic yeast *Glaciozyma* sp. (designated as LeIBP) could reduce post-thaw hemolysis of human red blood cells and increase the survival of cryopreserved diatoms. Here, we showed that LeIBP could improve the viability of cryopreserved mammalian cells. Human cervical cancer cells (HeLa), mouse fibroblasts (NIH/3T3), human preosteoblasts (MC3T3-E1), Chinese hamster ovary cells (CHO-K1), and human keratinocytes (HaCaT) were evaluated. These mammalian cells were frozen in dimethyl sulfoxide (DMSO)/fetal bovine serum (FBS) solution with or without 0.1 mg/ml LeIBP at a cooling rate of −1°C/min in a −80°C freezer overnight. The minimum effective concentration (0.1 mg/ml) of LeIBP was determined, based on the viability of HeLa cells after treatment with LeIBP during cryopreservation and the IR inhibition assay results. The post-thaw viability of mammalian cells was examined. In all cases, cell viability was significantly enhanced by more than 10% by LeIBP supplementation in 5% DMSO/5% FBS: viability increased by 20% for HeLa cells, 28% for NIH/3T3 cells, 21% for MC3T3-E1, 10% for CHO-K1, and 20% for HaCaT. Furthermore, addition of LeIBP reduced the concentrations of toxic DMSO and FBS down to 5%. Therefore, we demonstrated that LeIBP can increase the viability of cryopreserved mammalian cells by inhibiting IR.

Keywords: Antifreeze protein, ice-binding protein, *Glaciozyma* sp., cryopreservation, ice recrystallization inhibition, DMSO, mammalian cells

Introduction

Cryopreservation allows long-term storage of many biological samples. Generally, a high survival rate after thawing is ensured by the addition of an essential ingredient, cryoprotectants (CPAs) [7]. The main cause of decreased cell viability during cryopreservation is ice recrystallization (IR) [6, 8, 33], during which ice crystals grow larger [17]. However, most commercially available or routinely used CPAs cannot inhibit IR efficiently [19, 20]. Recently Tam *et al*. [41] demonstrated that dimethyl sulfoxide (DMSO), the most commonly used CPA, can inhibit IR at concentrations as low as 1% (v/v). In addition, 3% (v/v) DMSO inhibited IR as effectively as did 0.022 M galactose solution. However, DMSO is considered cytotoxic because of its chemical nature and the high concentration that is required to increase cell viability [7, 9, 40]. More importantly, DMSO can induce reversible branching in mesenchymal stem cells and apoptosis in other cells [4, 7, 9, 12, 14, 30, 34, 40, 42, 45, 47]. Therefore, less toxic or nontoxic CPAs that inhibit IR are needed to improve the efficiency of cryopreservation of valuable biological samples [3, 41, 46].

Antifreeze proteins (AFPs) are a group of proteins that bind ice and inhibit the growth of ice crystals. AFPs have two properties: thermal hysteresis (TH) and IR inhibition. TH activity is quantitatively expressed as the temperature gap between the freezing and melting points created by inhibiting the growth of ice crystals in an aqueous solution *via* the binding of AFP to the ice surface [28]. IR inhibition is also triggered by the ice-binding ability of the proteins. IR inhibition is a freezing-tolerance mechanism of many psychrophiles [10, 35–37]. Recently, we identified, expressed recombinantly in *Pichia*, and characterized ice-binding proteins (IBP) from the Arctic yeast *Glaciozyma* sp. (formerly known as *Leucosporidium* sp.) [25, 26, 31]. IBPs include any protein that binds to ice, such as AFPs, IR inhibition proteins, and ice nucleation proteins [31]. Like other AFPs, the IBP we used, designated as LeIBP, has both TH and IR inhibition activities. Considering the lower TH activity of LeIBP (0.34–0.42°C at 10.8 mg/ml) compared with that of other AFPs and the icy habitat of *Glaciozyma* sp., the IR inhibition activity of LeIBP is probably more important than the TH activity for survival at cold temperatures. The IR inhibition activity of LeIBP has been hypothesized to protect the cells in unfrozen channels between ice crystals by inhibiting the recrystallization of external ice, thereby improving cell viability [13, 26, 36]. This property of LeIBP makes it a strong candidate as an alternative CPA with less cytotoxicity. In previous studies, we tested this hypothesis on red blood cells [27], diatoms [27], and ovarian cells and tissues [24]. In the present study, we aim to assess the effect of LeIBP on cryopreservation of various mammalian cell lines by examining the IR inhibition activity of LeIBP in a cryopreservation solution and the post-thaw cell viability.

Materials and Methods

Materials and Cell Lines

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). We used recombinant LeIBP expressed in methylotrophic *Pichia pastoris* [31]. Freeze-dried LeIBP was dissolved and diluted appropriately in phosphate-buffered saline (PBS). The protein concentration was determined by measuring the absorbance at 280 nm, using a calculated extinction coefficient of 26,930 $M^{-1}\cdot cm^{-1}$. Minimal essential medium (MEM), MEM-α, Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, fetal bovine serum (FBS), antibiotic-antimycotic solution (100 ×), trypsin-EDTA, and TrypLE Express were obtained from Life Technologies (New York, NY, USA). Dulbecco's phosphate-buffered saline (DPBS) was obtained from WelGENE Inc. (Daegu, Korea). Human cervical cancer cells (HeLa) and mouse fibroblasts (NIH/3T3) were procured from the Korean Cell Line Bank (Seoul, Korea), and human preosteoblasts (MC3T3-E1), Chinese hamster ovary cells (CHO-K1), and human keratinocytes (HaCaT) were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Ice Recrystallization Inhibition Assay

A splat cooling assay was conducted to assess IR inhibition, as described previously [19]. Briefly, 10 μl of the aqueous solution containing various amounts of LeIBP in 2.5% and 5% DMSO solutions was released from a height of 2 m onto a polished aluminum plate cooled to −78°C by dry ice. As the droplet splats onto the aluminum plate, it immediately freezes as an ice disc measuring approximately 1 cm in diameter and 20 μm in thickness. The disc was removed from the plate surface, placed between two coverslips, transferred to a Linkam LTS120 cold stage (Linkam Scientific Instruments Ltd., Surrey, UK) held at −6°C, and annealed for 1 h. We used 2.5% and 5% DMSO solutions because solutions with DMSO concentrations greater than 5% are viscous and do not produce reliable data [41]. PBS was used as a control. The cold stage was mounted on a Linkam imaging station. The ice crystals were photographed between crossed polarizing filters.

Cell Culture

HeLa cells were cultured in MEM supplemented with 10% FBS and 1× antibiotic-antimycotic solution. NIH/3T3 and HaCaT cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1× antibiotic-antimycotic solution. MC3T3-E1 cells were cultured in MEM-α supplemented with 10% FBS and 1× antibiotic-antimycotic solution. CHO-K1 cells were cultured in Ham's F-12 supplemented with 10% FBS and 1× antibiotic-antimycotic solution. All cells were incubated in an atmosphere containing 5% CO_2 at 37°C.

Determination of the Minimum Effective Concentration (MEC) of LeIBP for Cryopreservation

We define the MEC as the lowest IBP concentration at which cryopreservation efficacy is maximized. To determine the MEC of LeIBP for cryopreservation, we evaluated the post-thaw viability of HeLa cells at different concentrations of LeIBP: 0.01, 0.025, 0.1, 0.25, or 0.5 mg/ml LeIBP. Freezing and thawing, followed by the cell viability assay, were conducted as described below. All experiments were performed in triplicate.

Cell Freezing and Thawing

Adherent cells (HeLa, MC3T3-E1, CHO-K1, and HaCaT cells) were dissociated from the plate by using trypsin-EDTA, whereas NIH/3T3 cells were disassociated using TrypLE Express. Cells were transferred to a 15 ml conical tube and centrifuged at 1,200 ×*g* for 5 min to remove the medium. Approximately 1 × 10^6 cells were aliquoted, washed twice with DPBS, and suspended in 1 ml of each freezing medium in the absence or presence of 0.1 mg/ml LeIBP. Freezing medium was composed of either 5% DMSO and 5% FBS or 10% DMSO and 10% FBS. Cells were transferred to a 2 ml cryovial. Freezing of cells was conducted overnight at −80°C, at a cooling rate of −1°C/min in a Mr. Frosty freezing container (Nalgene, Rochester, NY, USA). The frozen cells were directly transferred to liquid nitrogen for 1 week. The frozen cells were thawed quickly in a 37°C water bath within 2 min, transferred to 9 ml of medium in a 15 ml conical tube, and collected by centrifugation as mentioned above. The cell pellet was washed twice with DPBS and resuspended in the culture medium. Optimal cell number (1×10^6 cells) for cryopreservation was determined by examining the viability of HeLa cells of 1×10^6, 2×10^6, and 3×10^6 cells/ml using the same freezing media and methods described above.

We examined the cell viability at 48 h after thawing. Approximately $0.5–0.8 \times 10^6$ thawed cells were transferred to a 100 mm dish and incubated for 48 h at 37°C in an atmosphere containing 5% CO_2. After 48 h, we harvested the cells and counted the total number of live cells. All experiments were performed in triplicate.

Cell Viability Assay

After thawing, we evaluated the viability of the cells by immediately counting the live cells by using an ADAM-MC automatic cell counter (NanoEntek, Seoul, Korea), according to the manufacturer's instructions. Briefly, the thawed cells were stained with propidium iodide to distinguish between and count live and dead cells. Two solutions (T, total cells; N, non-viable cells) were diluted with cell suspension (1:1) and 12 µl was transferred to the chip. Unfrozen cells were used as a negative control. Cell images were also acquired using a Motic AE2000 microscope (Motic Inc., Hong Kong) equipped with a digital camera.

Statistical Analysis

Microsoft Excel software (Microsoft, WA, USA) was used for statistical analyses. Student's *t*-test was used to determine differences in relative cell viability after cryopreservation. The difference between groups was defined as statistically significant if $p < 0.05$. The relative viability was expressed as the mean ± 1 SD ($n = 3$).

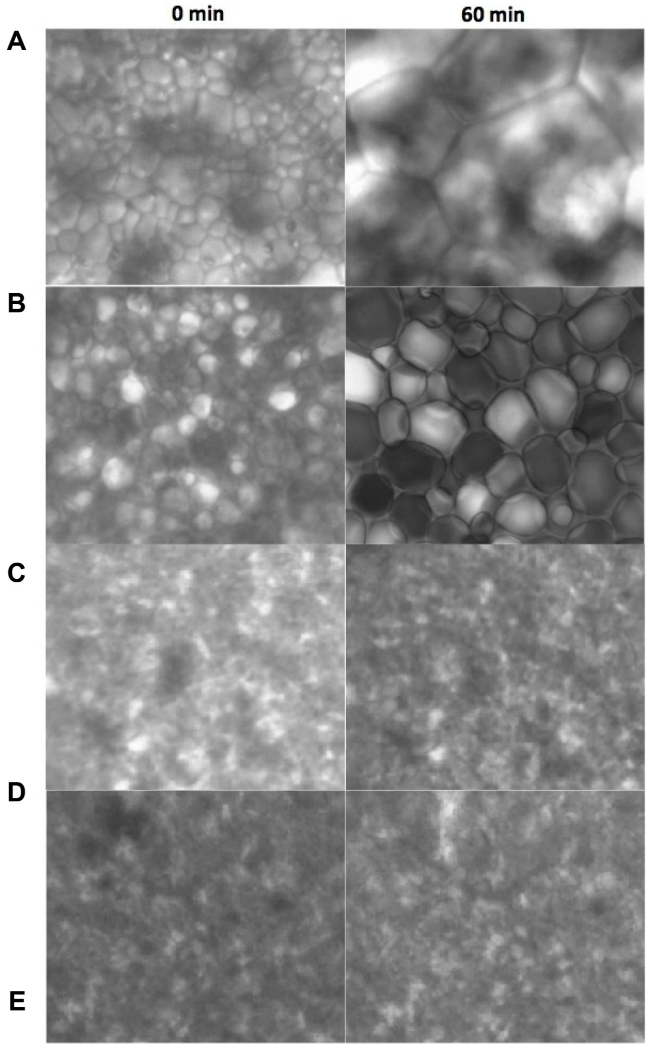

Fig. 1. Ice recrystallization inhibition assay of LeIBP.
Ten microliters of each solution was dropped onto the surface of a precooled aluminum block at −78°C. The ice disc was annealed for 1 h at −6°C. During the annealing, the ice recrystallized. The images captured at 0 (left column) and 60 min (right column) are shown. (**A**) PBS alone, (**B**) 0 mg/ml LeIBP, (**C**) 0.001 mg/ml LeIBP, (**D**) 0.1 mg/ml LeIBP with 5% DMSO.

Results and Discussion

Ice Recrystallization Inhibition of LeIBP in DMSO Solution

In the IR inhibition assay, the ice grain size in 5% DMSO alone (Fig. 1B) was smaller than that in PBS control (Fig. 1A). Similarly, smaller ice grains were observed in 2.5% DMSO than in PBS control (data not shown). These results are consistent with the results obtained by Tam *et al.* [41], who reported that DMSO inhibits IR. We investigated the IR inhibition activity of LeIBP in the presence of 2.5% and 5% DMSO. In PBS and in 2.5% and 5% DMSO solutions containing LeIBP, IR was inhibited at concentrations as low as 0.001 mg/ml (Figs. 1C and 1D) [31]. Therefore, LeIBP remains active in the presence of DMSO. Chaytor *et al.* [3] reported that the IR inhibition activity of DMSO and carbohydrates can be augmented when they are mixed together for use as cryoprotectants; however, a synergistic effect is likely negligible in this case since LeIBP is a much stronger IR inhibitor than DMSO.

Minimum Effective Concentration of LeIBP for Cryopreservation

The MEC of LeIBP was tested in HeLa and NIH/3T3

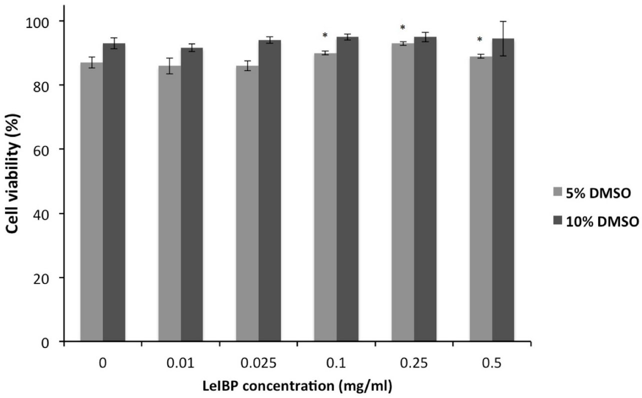

Fig. 2. Determination of minimum effective concentration of LeIBP for mammalian cell cryopreservation.

HeLa cells were treated with LeIBP concentrations ranging from 0 to 0.5 mg/ml in 5% or 10% DMSO. After thawing, the viability of HeLa cells was evaluated using propidium iodide staining. The beneficial effect of LeIBP was distinct from 0.1 mg/ml. Asterisks indicate significant difference ($p < 0.05$) relative to 10% DMSO. These data represent the mean ± SD of three samples.

cells. The MEC for HeLa cells was 0.1 mg/ml LeIBP in 10% DMSO and 0.25 mg/ml LeIBP in 5% DMSO (Fig. 2). The difference in viability at concentrations ≥0.1 mg/ml was subtle. Interestingly, at 1 mg/ml LeIBP, the viability decreased slightly, which is similar to that observed with the MECs of red blood cells (RBCs), ram spermatozoa, rat smooth muscle cells, and immature rat oocytes when exposed to AFPs/IBPs [11, 15, 27, 32]. In those experiments, the use of high amounts of AFPs/IBPs was related to a decrease in the viability of cryopreserved biological samples. For cryopreservation of RBCs, 0.4–0.8 mg/ml LeIBP was more effective than was 1.0 mg/ml LeIBP; at higher concentrations of LeIBP, hemolysis increased. For NIH/3T3 cells, the MEC was 0.1 mg/ml in both 5% and 10% DMSO (data not shown). These results are in accord with recent findings by Lee *et al*. [23], who reported that the optimal concentration for the vitrification of mature mouse oocytes was 0.1 mg/ml LeIBP in 15% ethylene glycol, 15% 1,2-propandiaol, and 0.5 M sucrose. Additionally, 0.1 mg/ml LeIBP increased the survival of the marine diatom *Phaeodactylum tricornutum* during cryopreservation [21]. Taken together, the MEC for the cryopreservation of mammalian cells is 0.1 mg/ml LeIBP. However, owing to the variation between cell types and the effect of the composition of the cryopreservation solution on IBPs, the MEC should be verified empirically before adopting this strategy for cryopreservation [1, 2, 11, 15, 21, 22, 24, 27, 39, 44]. For example, for the vitrification of mouse ovarian tissues, the MEC of LeIBP was 10 mg/ml in DPBS containing 20% FBS, 7.5% ethylene glycol, 7.5% DMSO, and 0.5 M sucrose [24], whereas for the cryopreservation of RBC, 0.8 mg/ml LeIBP was optimal in 40% glycerol [27].

Viability of Cryopreserved Mammalian Cells in LeIBP

To assess the effect of LeIBP on the cryopreservation of mammalian cells, we used five different mammalian cells (Table 1). A cell density of 1×10^6 cells/ml was optimal for cryopreservation compared with $2{\sim}3 \times 10^6$ cells/ml (data not shown). Freezing was conducted in either 5% DMSO/5% FBS or 10% DMSO/10% FBS with or without 0.1 mg/ml LeIBP. As shown in Fig. 3, the cell viability was mostly increased in the presence of LeIBP, particularly at 48 h post-thawing. We observed no significant difference in cell viability immediately after thawing (0 h); however, there was a drastic improvement in cell viability at 48 h after thawing for all cell types cryopreserved with LeIBP in 5% DMSO/5% FBS (HeLa: 20% increase; NIH/3T3: 28% increase; MC3T3-E1: 21% increase; CHO-K1: 10% increase; HaCaT: 20% increase). Our results demonstrate that 0.1 mg/ml of LeIBP in 5% DMSO/5% FBS has better cryopreservation efficiency over 10% DMSO/10% FBS, suggesting that the

Table 1. Cell lines used in this study.

Cell line	Species of origin	Tissue of origin	Culture medium	Source
HeLa	Human	Cervix adenocarcinoma	MEM 10% FBS	KCLB
HaCaT	Human	Normal keratinocytes	DMEM 10% FBS	ATCC
NIH3T3	Mouse	Embryo fibroblasts	DMEM 10% FBS	KCLB
CHO-K1	Hamster	Ovarian cells	Ham's F-12 10% FBS	ATCC
MC3T3-E1	Mouse	Preosteoblasts	MEM-α 10% FBS	ATCC

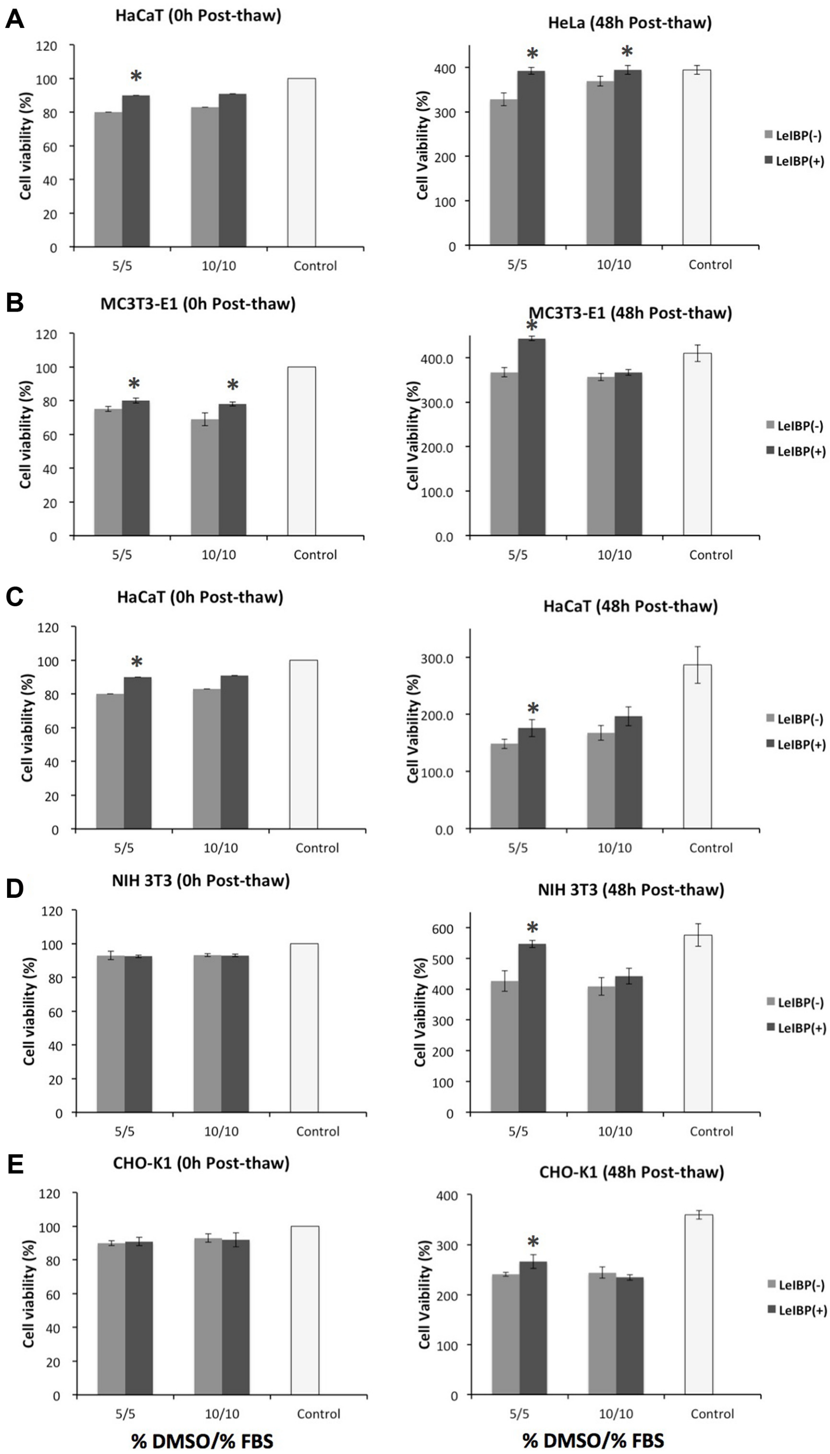

Fig. 3. Cryopreservation efficacy of LeIBP based on the viability of various mammalian cells.

(**A**) HeLa, (**B**) MC3T3-E1, (**C**) HaCaT, (**D**) NIH/3T3, and (**E**) CHO-K1 cells. Cells were frozen in the absence (grey bars) or presence (black bars) of 0.1 mg/ml LeIBP combined with either 5% DMSO/5% FBS or 10% DMSO/10% FBS. The frozen cells were thawed quickly in a 37°C water bath within 2 min. Post-thaw viability was determined using propidium iodide staining. Unfrozen samples of each cell were used as the control. Data are presented as the mean ± SD for at least three replicates and are compared with the 10% DMSO/10% FBS control. Values on the x-axis indicate % (v/v) concentrations of DMSO and FBS. Significant differences between means were determined by Student's *t*-test; asterisks indicate the significant difference ($p < 0.05$) relative to 10% DMSO/10% FBS.

addition of LeIBP improves the viability of the mammalian cells tested and reduces the cytotoxic DMSO concentration. Complete replacement of DMSO with LeIBP was unsuccessful (data not shown).

The increase in cell viability is attributed to the IR inhibition ability of IBPs in the cryopreservation solution, corroborating the hypothesis described above [1, 2, 11, 15, 21, 22, 24, 27, 39, 44]. The DMSO concentration can be lowered by the addition of other molecules that inhibit IR, such as sugars [3, 30, 46]. Contrary to IBPs, sugars inhibit IR in a colligative manner similar to DMSO [3, 41, 46], such that IR inhibition is proportional to the concentration. To achieve substantial IR inhibition, millimolar quantities of sugars are needed [3]. Alternatively, nanomolar or micromolar concentrations of AFPs/IBPs can inhibit IR since they behave in a noncolligative manner and are more effective at inhibiting IR [18, 20, 43, 48]. The endpoint (below which IR inhibition is no longer detected) of LeIBP, *Flavobacterium frigoris* IBP (FfIBP), and type III AFP are 1 μg/ml, 69 μg/ml, and 5 μg/ml, respectively [5, 31, 43]. Thus, 0.001 mg/ml LeIBP was the lowest amount to inhibit IR [31]. Interestingly, the MEC of LeIBP in this study is 100-fold higher than the endpoint reported in a previous study [31], possibly owing to the composition of the solutions and its effect on the function or solubility of LeIBP. In the study by Park *et al*. [31], assays evaluated IR inhibition of LeIBP solutions prepared in water or PBS, whereas we used 10% DMSO/10% FBS. Overall, these data are in agreement with the previous results obtained using disaccharides [3]. Since DMSO inhibits IR, addition of potent IR inhibitors may lead to a synergistic effect; however, this is not the case for IBPs. For the vitrification of mouse ovarian tissue, 10 mg/ml LeIBP, FfIBP, and type III AFP was used [24, 29, 38]. Alternatively, 1 μg/ml *Dendroides canadensis* AFP (DcAFP) in the cryopreservation solution containing ~7% DMSO was effective for the cryopreservation of mouse A10 smooth muscle cells [11]. More surprisingly, only 0.5 μg/ml type III AFP was used for the vitrification of mouse immature oocytes [16]. These results are interesting because they used 10–20-fold lower amounts of AFP than the values reported for the minimum IR inhibition activity [18, 43], possibly because hyperactive AFPs/IBPs can be used in lesser quantities than moderately active ones. FfIBP and DcAFP are classified as hyperactive, based on their TH activity, whereas LeIBP and type III AFP are moderately active; however, their IR activities are not proportional to their TH activities. Compared with hyperactive AFPs, moderately active LeIBP and type III AFP have relatively higher IR inhibition activity. Yu *et al*. [49] showed that there is no direct correlation between TH and IR inhibition properties. Hence, further research to explore this discrepancy will provide insights into how AFPs/IBPs affect cell viability. Morphologically, there were no discernible changes between unfrozen and frozen cells (data not shown).

In summary, we showed that the addition of LeIBP significantly increased the post-thaw viability of various mammalian cells during cryopreservation. This result is in agreement with the growing body of evidence that IR inhibition by other AFPs, similar to that observed with LeIBP, can improve cell viability. Furthermore, LeIBP can substitute toxic DMSO; in this study, we were able to reduce the DMSO concentration to 5%. These data suggest that LeIBP has potential for use as a cryoprotectant.

Acknowledgments

The authors appreciate Hye Yeon Koh and Dr. Sung Gu Lee for conducting preliminary experiments. This work was supported by a grant from the National Fisheries Research and Development Institute (R2015004).

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