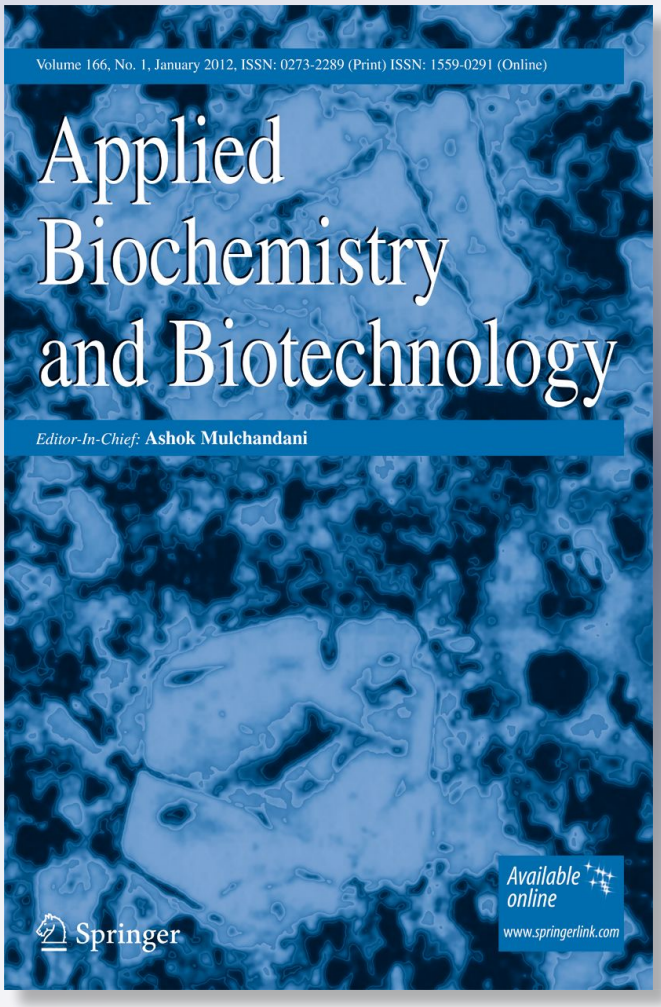
Cryopreservative Effects of the Recombinant Ice-Binding Protein from the Arctic Yeast Leucosporidium *sp. on Red Blood Cells*

Sung Gu Lee, Hye Yeon Koh, Jun Hyuck Lee, Sung-Ho Kang & Hak Jun Kim

Applied Biochemistry and Biotechnology
Part A: Enzyme Engineering and Biotechnology

ISSN 0273-2289
Volume 167
Number 4

Appl Biochem Biotechnol (2012) 167:824-834
DOI 10.1007/s12010-012-9739-z



Springer

Appl Biochem Biotechnol (2012) 167:824–834
DOI 10.1007/s12010-012-9739-z

Cryopreservative Effects of the Recombinant Ice-Binding Protein from the Arctic Yeast *Leucosporidium* sp. on Red Blood Cells

Sung Gu Lee · Hye Yeon Koh · Jun Hyuck Lee · Sung-Ho Kang · Hak Jun Kim

Received: 16 January 2012 / Accepted: 14 May 2012 /
Published online: 24 May 2012

Abstract Antifreeze proteins (AFPs) have important functions in many freeze-tolerant organisms. The proteins non-colligatively lower the freezing point and functionally inhibit ice recrystallization in frozen solutions. In our previous studies, we found that the Arctic yeast *Leucosporidium* sp. produces an AFP (LeIBP), and that the protein could be successfully produced in *Pichia* expression system. The present study showed that recombinant LeIBP possesses the ability to reduce the damage induced to red blood cells (RBCs) by freeze thawing. In addition to 40 % glycerol, both 0.4 and 0.8 mg/ml LeIBPs significantly reduced freeze–thaw-induced hemolysis at either rapid- (45 °C) or slow-warming (22 °C) temperatures. Post-thaw cell counts of the cryopreserved RBCs were dramatically enhanced, in particular, in 0.8 mg/ml LeIBP. Interestingly, the cryopreserved cells in the presence of LeIBP showed preserved cell size distribution. These results indicate that the ability of LeIBP to inhibit ice recrystallization helps the RBCs avoid critically damaging electrolyte concentrations, which are known as solution effects. Considering all these data, LeIBP can be thought of as a key component in improving RBC cryopreservation efficiency.

Keywords Antifreeze protein · Recombinant · LeIBP · Red blood cells · Cryopreservation · Cell size distribution

Sung Gu Lee and Hye Yeon Koh contributed equally to this manuscript.

S. G. Lee · H. Y. Koh · J. H. Lee · H. J. Kim
Division of Polar Life Sciences, Korea Polar Research Institute, Incheon 406-840, Republic of Korea

S.-H. Kang
Division of Polar Climate Research, Korea Polar Research Institute, Incheon 406-840, Republic of Korea

S. G. Lee · J. H. Lee · S.-H. Kang · H. J. Kim (✉)
Department of Polar Sciences, University of Science and Technology, Incheon 406-840,
Republic of Korea
e-mail: hjkim@kopri.re.kr

Introduction

Antifreeze proteins (AFPs) lower the freezing point in a non-colligative manner, which leads to a difference between the melting point and freezing point, known as thermal hysteresis [1, 2], and inhibit ice recrystallization in frozen solutions [3, 4]. Consequently, many AFP-expressing freeze-tolerant organisms are able to survive at temperatures below subfreezing temperatures [5–11]. Since the protein was first isolated from fish [12], many AFPs have been found in other diverse organisms, including fish [7, 13–18], plants [19], bacteria [20–22], fungi [23], insects [24, 25], and yeast [26]. The ability of AFPs to inhibit ice recrystallization may create tremendous potential in many applications, in particular, in the field of cryopreservation. These include cryopreservation of stem cells, umbilical cord blood and blood, human organs, food, etc.

Red blood cells (RBCs) have significance in both military and civilian communities from the transfusion aspect. Since Rous and Turner first successfully stored RBCs using citrate and glucose [27], efforts have been made to enhance the efficiency of the storage process [28–31]. However, the maximal period of storage is still quite limited. Under the best conditions, approximately 82 % of the cells can be recovered after 6 weeks of storage [32]. The Food and Drug Administration (FDA) has approved the storage of frozen RBC for up to 10 years. As indicated by the FDA's decision, cryopreservation can be an ideal alternative to extend the storage period; nonetheless, the problems associated with cryodamage persist. These include the solution effects, extra/intracellular ice formation, and dehydration, which is still a leading problem in cryopreservation research. During freezing, the solutes become more concentrated because they are excluded from the growing extracellular ice crystals. This could drive cell damage or death, which is known as the solution effects [33, 34]. Glycerol was first adapted as an additive to reduce the cryodamage of RBCs [35], and was further utilized to develop the high glycerol/slow freeze and the low glycerol/rapid freeze techniques [36, 37]. Extracellular cryoprotectants such as hydroxyethyl starch (HES), dextran, polyvinyl pyrrolidone, and serum albumin have been studied as well. These macromolecules minimize osmotic stress due to the addition and removal of glycerol, and facilitate the transfusion procedure without deglycerolization. These additives greatly solve the challenges involved in freezing, but the risk in thawing continues to be another issue because of ice recrystallization.

In previous studies, the AFPs from microalgae and fishes were shown to have cryopreserving effects on RBCs, by inhibiting ice recrystallization in the presence of glycerol or HES [38–40]. Recently, a novel ice-binding protein (IBP) was identified and purified by our lab from the psychrophilic Arctic yeast *Leucosporidium* sp. [26]. This yeast IBP showed high similarity to the algal protein used in previous RBC cryopreservation study [40]. We previously obtained the coding sequence for this protein from cDNA, and the recombinant protein was highly produced in the methylotrophic yeast *Pichia pastoris* [41]. In the present study, the effect of recombinant LeIBP on the cryopreservation of human red blood cells was presented, in particular, by comparing the hemolysis, RBC cell recovery, and the cell size distribution of cryopreserved RBCs.

Materials and Methods

Recombinant LeIBP Preparation

Recombinant LeIBP was produced using a *Pichia* expression system, and purified as described in a previous study [41]. Briefly, codon-optimized mature LeIBP gene was cloned

into pPICZαA vector, and the plasmid was transformed into *P. pastoris* strain X33 (Invitrogen). The recombinant cells were grown in 3-L YPD medium at 30 °C for 2 days, and the recombinant LeIBP expression was induced daily using a 5-ml methanol supplement. 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 pooled and further purified in a Superdex 200 size-exclusion column (Thermo Fisher Co., USA), equilibrated with 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl buffer at a flow rate of 1 ml/min. The purified LeIBP was identified by SDS–PAGE 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} cm^{-1}.

RBC Preparation and Glycerolization

Human blood was collected from healthy volunteers, placed into heparinized tubes, and centrifuged at 2,000×*g* for 10 min. Plasma supernatant and the buffy coat were removed, and the RBC pellet was washed three times with equal volumes of phosphate-buffered saline (PBS). In the third wash, the hematocrit was adjusted to 70 %. Equal volumes of freezing solution containing 80 % (*w*/*v*) glycerol and 0.9 % NaCl were added drop-by-drop to the RBCs. The RBCs were then centrifuged at 2,000×*g* for 10 min, and the supernatant was removed to yield a final pellet containing 70 % hematocrit and 40 % (*w*/*v*) glycerol.

Freezing and Thawing

Prior to freezing, 75 μl of glycerolized RBCs was mixed with 25 μl of LeIBP solution (LeIBP and 40 % glycerol in PBS) in a 2-ml cryovial. The concentration of LeIBP ranged from 0 to 1.5 mg/ml. The mixtures containing 52.5 % hematocrit, 40 % glycerol, and LeIBP were immersed in liquid nitrogen for 10 min and then kept in liquid nitrogen vapor. The frozen RBCs were thawed either by placing in a 45 °C water bath for 1 min (rapid warming) or by leaving in air at 22 °C (slow warming).

Hemolysis Measurement

Post-thaw percent hemolysis was determined for all freezing experiments by comparing supernatant hemoglobin to total hemoglobin with the cyanmethemoglobin method [42, 43]. Briefly, the thawed RBC mixtures were centrifuged at 13,000 rpm for 1 min. An aliquot (5 μl) of the supernatant was incubated with 195 μl of Drabkin's reagent (Sigma-Aldrich, USA) in a 96-well plate for 15 min. The absorbance was measured at 540 nm by Multiskan GO (Thermo Scientific, USA).

RBC Cell Count Recovery and Cell Size Measurement

RBC recovery and cell sizes were microscopically determined. Before measuring the cell size of the cryopreserved RBCs, frozen cells containing 0, 0.4, or 0.8 mg/ml LeIBP in 40 % glycerol were thawed at either 45 °C (rapid warming) or 22 °C (slow warming). Non-frozen intact RBCs served as a control. The cells (5 μl) were fixed on a glass slide with methanol, stained with Giemsa solution (Sigma-Aldrich, USA), washed with distilled water, air-dried, and then observed under a microscope at ×500 magnification (Olympus BX51; Olympus, Japan). The cell count and size were determined using TOMORO ScopeEye™ software

(Saramsoft, South Korea). Recovery values were expressed as percentages of the positive control (non-frozen RBCs).

Recrystallization Inhibition Assay

Ice recrystallization inhibition was measured by the method of Smallwood [44], with slight modification. Briefly, LeIBP was solubilized in PBS containing 30 % sucrose to a final concentration of 0, 0.4, or 0.8 mg/ml. A 2-μl aliquot of each sample was sandwiched between two round cover slides (diameter, 16 mm). The sandwich was pre-chilled at −1 °C for 3 min to prevent surface frost. Next, the sandwich was transferred to a cryostage (THMS600 cold stage; Linkam Scientific Instruments, Surrey, UK). The sandwich was then cooled quickly at a rate of 90 °C/min to −80 °C and maintained for 1 min. The temperature was raised to −6 °C at the same rate and maintained for 60 min to allow ice recrystallization. Ice crystals were observed under a microscope at ×200 magnification (Olympus BX51).

Statistical Analysis

Excel software (Microsoft Co., Redmond, USA) was used for statistical analyses. A Student's *t* test was used to determine significant differences, accepting P values of <0.001 or <0.005 as significant. The data were expressed as mean±1 SD and $n=3$ for each experiment.

Results

Hemolysis of Cryopreserved RBCs

LeIBP, recombinant Arctic yeast IBP expressed in a *Pichia* system, was evaluated as a potential cryoprotectant in the RBC cryopreservation process. To demonstrate the effect of reducing RBC hemolysis, human RBCs containing LeIBP (concentrations ranging from 0 to 1.5 mg/ml) in the presence of 40 % glycerol were frozen in LN_2 and then thawed at either 45 °C (rapid warming) or 22 °C (slow warming). In the absence of or at low concentration (below 0.2 mg/ml) of LeIBP, 31–41 % hemolysis was observed at rapid warming temperature, whereas 0.4, 0.8, and 1 mg/ml LeIBP significantly reduced the hemolysis to <16 %; in particular, 0.4 mg/ml LeIBP reduced the hemolysis down to 9.5 % (Fig. 1). The slow warming method induced considerably high hemolysis, almost up to 90 %, when LeIBP was added below 0.06 mg/ml; however, 0.2, 0.4, and 0.8 mg/ml LeIBP dramatically suppressed hemolysis to approximately 31, 15, and 13, respectively (Fig. 1). Concentrations greater than 0.8 and 1 mg/ml did not significantly lower the freeze–thaw-induced hemolysis at slow and rapid warming temperatures, respectively.

RBC Cell Count Recovery

Cryopreservation of RBCs using LeIBP in the presence of 40 % glycerol revealed different RBC recoveries with rapid and slow warming methods. The cell count recovery was significantly enhanced by slow warming, at both 0.4 and 0.8 mg/ml LeIBPs (Fig. 2). More interestingly, the recovery of RBCs frozen in the presence of 0.8 mg/ml LeIBP and thawed by slow warming was almost the same as that of non-frozen intact RBCs, whereas the recovery of RBCs frozen in the absence of LeIBP was <30 %. The recovery at the rapid

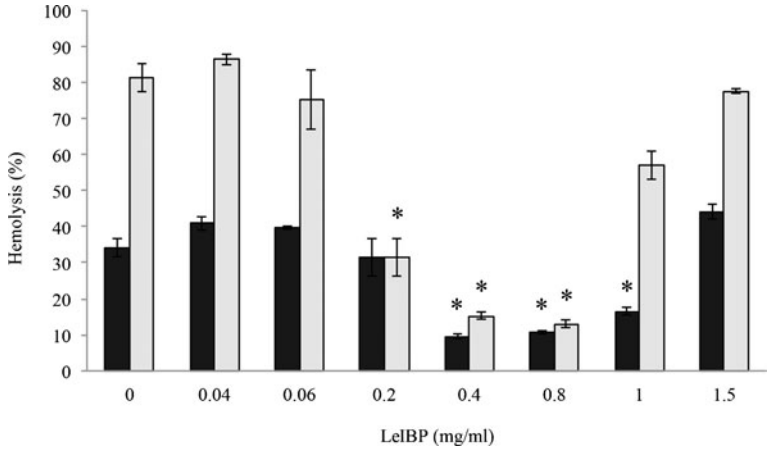


Fig. 1 Influence of LeIBP on RBC hemolysis. RBCs containing LeIBP ranging from 0 to 1.5 mg/ml in 40 % glycerol were frozen in LN_2. The frozen RBCs were thawed at either 45 °C (rapid warming, *black bars*) or 22 °C (slow warming, *gray bars*). The cell pellet was spun down, and the amount of released hemoglobin in the supernatant was measured at 540 nm by using Drabkin's reagent method. LeIBP (0.4 and 0.8 mg/ml) significantly reduced freeze–thaw-induced hemolysis at both rapid and slow warming conditions. *Asterisks* denote $P<0.001$. These data represent the mean±1 SD of three samples

warming temperature appeared to be enhanced by 0.4 or 0.8 mg/ml LeIBP supplementation, but the increase was not statistically significant (Fig. 2).

Morphology and Cell Size Distribution of Cryopreserved RBCs

Morphological changes were assessed after thawing at either rapid or slow warming methods (Fig. 3). The RBCs supplemented with LeIBP exhibited almost the same round morphology

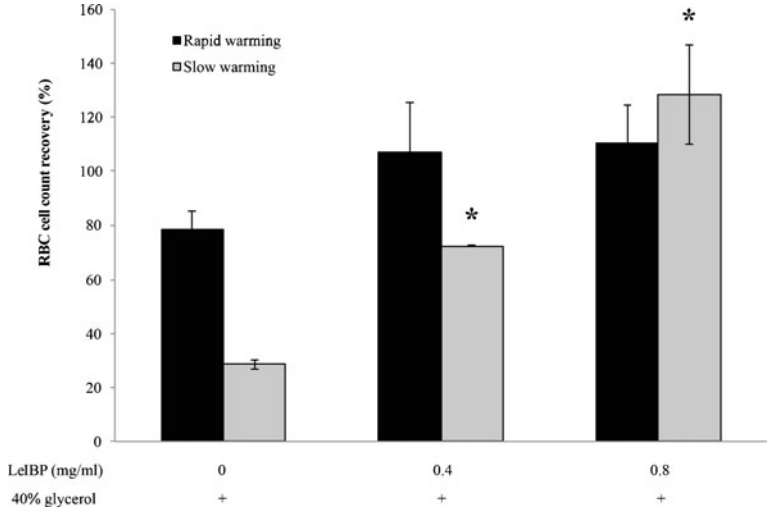


Fig. 2 Effect of LeIBP on RBC cell count recovery after cryopreservation. RBCs containing 0, 0.4, or 0.8 mg/ml LeIBP in 40 % glycerol were frozen in LN_2. The RBCs were thawed at either 45 °C (rapid warming, *black bars*) or 22 °C (slow warming, *gray bars*). Intact RBCs (non-frozen) were used as a 100 % control. The RBC recovery values were converted to percentages of the positive control. *Asterisks* denote $P<0.005$. The data represent the mean±1 SD of three samples

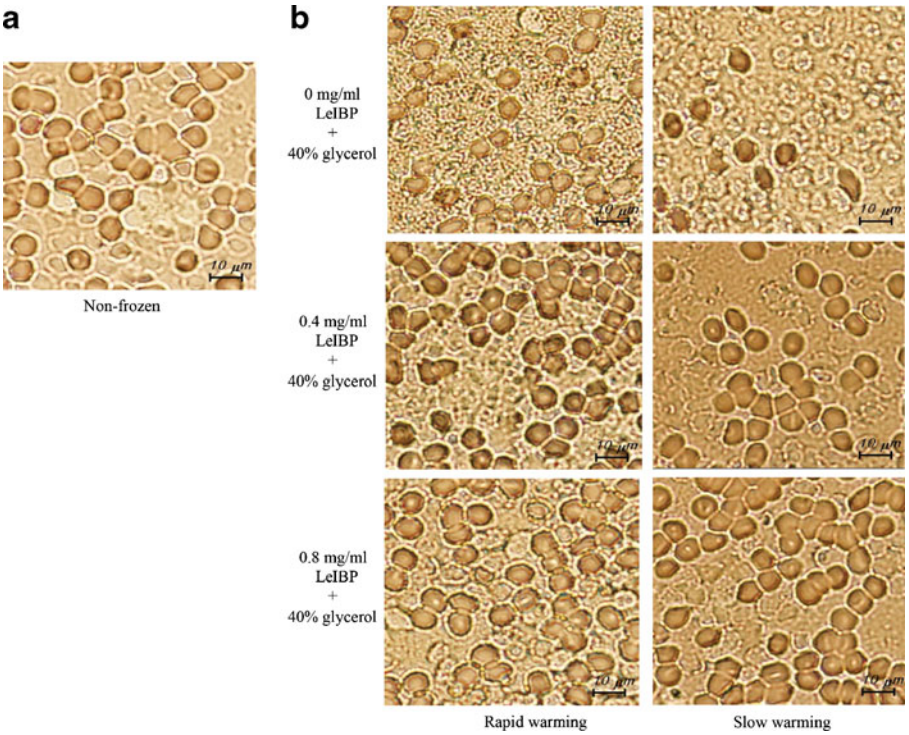


Fig. 3 Post-thaw morphology assessment of RBCs. **a** Non-frozen RBCs with no additives. **b** RBCs containing 0, 0.4, or 0.8 mg/ml LeIBP in 40 % glycerol were frozen in LN_2. The cryopreserved RBCs were thawed at either 45 °C (rapid warming) or 22 °C (slow warming). The RBCs were stained with Giemsa solution and were observed under a microscope at ×500 magnification. *Scale bar*=10 μm

as the non-frozen RBCs (Fig. 3a), whereas RBCs with no LeIBP were shrunken (Fig. 3b, rapid warming). Ghost cells were present in all the treatments. Many irregular-sized RBCs were distributed in the non-LeIBP-containing samples thawed at either rapid or slow warming temperatures (Fig. 4). More than 80 % of non-frozen RBCs were of typical size, which ranges from 7 to 9 μm in diameter. However, in the absence of LeIBP, only 13 and 32 % were of the typical size at rapid and slow warming temperatures, respectively. In contrast, LeIBP treatments resulted in almost typical cell size distribution even after thawing by either rapid or slow warming method. Interestingly, 0.8 mg/ml LeIBP preserved cell size distribution better than 0.4 mg/ml LeIBP (Fig. 4).

Recrystallization Inhibition Assay

Each frozen solution containing 0, 0.4, and 0.8 mg/ml LeIBP in 30 % sucrose was warmed to −6 °C and maintained for 60 min to induce recrystallization in the presence of LeIBP, and no RBCs were used in this experiment. The recrystallization was obviously suppressed in 0.4 or 0.8 mg/ml LeIBP solution (Fig. 5). Similar results were obtained when 0.1 mg/ml bovine serum albumin was used as a negative control (data not shown).

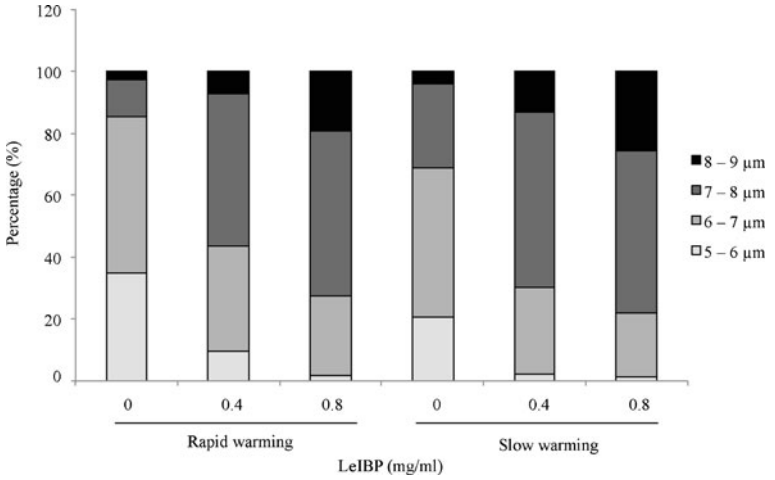


Fig. 4 Cell volume preservation effects of LeIBP. RBCs containing 0, 0.4, or 0.8 mg/ml LeIBP in 40 % glycerol were frozen in LN_2. The cryopreserved RBCs were thawed at either 45 °C (rapid warming) or 22 °C (slow warming). The cells were stained with Giemsa solution, and the cell size distribution of RBC was microscopically determined using TOMORO ScopeEye™ software (Saramsoft, South Korea). The cell size distribution is shown. The RBCs frozen in 40 % glycerol supplemented with 0.4 mg/ml or 0.8 mg/ml LeIBP displayed typical cell size distribution, even after thawing by either rapid or slow warming method

Discussion

In our present study, the potential properties of recombinant LeIBP, an ice-binding protein from the Arctic yeast *Leucosporidium* sp., as a cryoprotectant for RBC cryopreservation were investigated. We showed that LeIBP was effective in preventing cryoinjury of RBC on a small scale. Experiment with small volumes is considered a feasible laboratory-scale method to evaluate RBC preservation [45].

Influence of LeIBP on Hemolysis of Cryopreserved RBCs

Less hemolysis after prolonged storage periods has been one of the main goals in improving RBC storage systems [46, 47]. When blood was stored at 1 to 6 °C for 6 weeks in the presence of some additives (13 sugars, citrate, phosphate, adenine, and saline), hemolysis was only 0.4 % [32]. Although such additives prolong the storage period, alternative approaches are necessary to extend storage times. In general, all biochemical reactions are virtually suspended under frozen conditions at −196 °C. Indeed, we did not notice any difference in the results of short-term storage (1-day post-freezing) and long-term storage (1-month post-freezing) of RBCs in liquid nitrogen (data not shown). In the present study, almost 90 % hemolysis with the slow warming method was dramatically decreased to <16 % when 0.4 or 0.8 mg/ml LeIBP was added (Fig. 1). In a previous study, 77 μg/ml IBP from Antarctic sea ice diatom, which is similar in structure to LeIBP [26], reduced hemolysis up to 50 and 13 % at 0 and 45 °C thawing temperature, respectively [40]. Another research showed that 5–150 μg/ml fish AFP reduced hemolysis up to 50 % [39]. Since slow warming highly induces ice recrystallization that causes increased hemolysis [39, 40], the recrystallization inhibition activity of LeIBP (Fig. 5) may have caused our hemolysis result, possibly by suppressing extracellular ice crystal growth during the

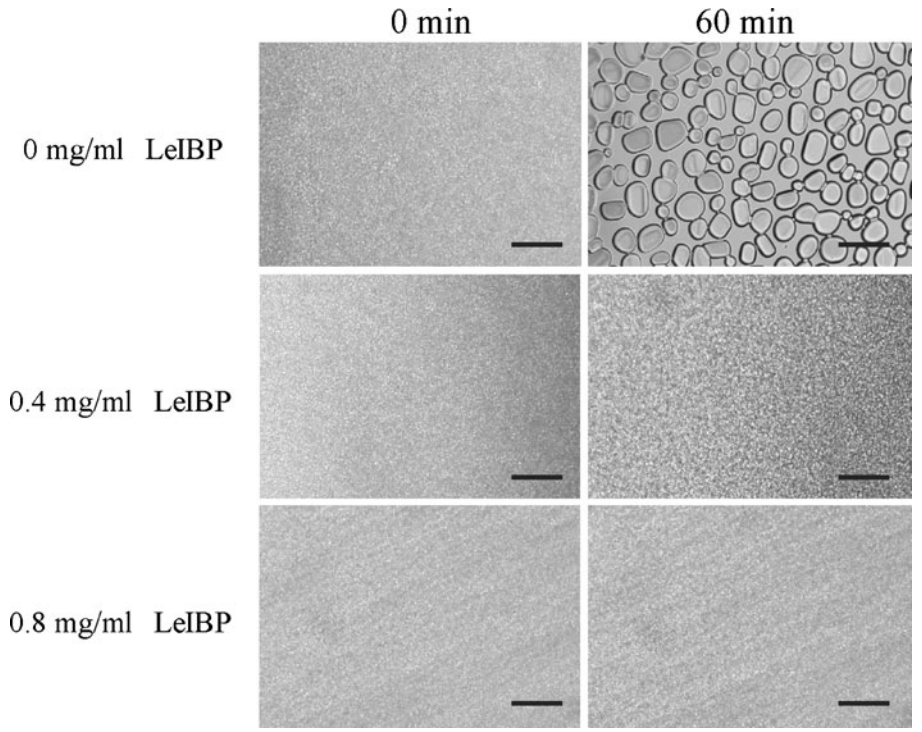


Fig. 5 Recrystallization inhibition activity of LeIBP. A 2-μl droplet of each solution was cooled at 90 °C/min to −80 °C and maintained for 1 min. Then, the sample was warmed at 90 °C/min to −6 °C and maintained for 60 min to allow ice recrystallization to occur. The resultant ice crystal images at 0 min (*left column*) and 60 min (*right column*) were observed under a microscope at ×200 magnification. *Scale bar*=50 μm

melt. The reason for the increased hemolysis at LeIBP concentrations of 1 mg/ml and higher (Fig. 1) is not clear. However, higher AFP concentrations (>1.54 mg/ml) were found to recruit massive and destructive ice growth around the cells, eventually inducing cell lysis [39].

RBC Cell Count Recovery After Cryopreservation Using LeIBP

The cell count recovery has been another issue in RBC storage systems [28, 46, 47]. In a non-freezing system, the best recovery of RBCs was 84 % after 6 weeks of storage [32]. However, recovery of RBCs in cryopreservation studies had not been reported. LeIBP increased the cell recovery of cryopreserved RBCs (Fig. 2). However, the results did not coincide with the hemolysis data, in particular, with the results obtained with slow warming method (Fig. 1). The hemolysis in 0.4 and 0.8 mg/ml LeIBP was similar to each other (Fig. 1), but the RBC cell recovery in 0.8 mg/ml LeIBP obtained from slow warming method was higher than 0.4 mg/ml (Fig. 2). This result explains why reduced hemolysis is not the sole factor used to evaluate the potential of cryoprotectants for RBC cryopreservation. Previous studies demonstrated that the solution effect was increased by supplementing the freezing solution with glycerol [33, 48]. This indirect cell damage may reoccur during the thawing process which is when ice recrystallization occurs. Once the frozen cells are thawed at a slow warming rate in the presence of AFP, the cells would be allowed to slowly

equilibrate to an isotonic condition. Consequently, slow warming would enhance RBC recovery when AFP is present in the freezing solution. The present result would possibly support this idea. A dramatic cell recovery was achieved with slow warming with LeIBP supplements (0.4 or 0.8 mg/ml; Fig. 2). The recovery with glycerol treatment in rapid warming was already high before the application of LeIBP, so the enhancement was not very noticeable.

Morphological Assessment and Cell Size Distribution of Cryopreserved RBCs

In general, the effect of additives on RBC cryopreservation has been evaluated by measuring post-thawing hemolysis. However, if cryoinjured cells are harvested along with normal cells after cryopreservation, the damaged cells may eventually cause potential problems after transfusion, although cryoprotectants may protect the membrane from complete destruction. Recrystallized extracellular ice may induce mechanical damage in membrane proteins. If cytoskeletal proteins are cryodamaged, the RBC cannot recover its normal shape and cell size, even when the cells are in isotonic conditions. Indeed, most of the outer cell boundary was rough, and shrunken cells were dominant in the non-LeIBP-containing RBCs (Fig. 3). Moreover, a considerable portion of irregular-sized RBCs was observed in non-LeIBP-containing RBCs (Fig. 4). In contrast, interestingly, the cryopreserved cells in the presence of LeIBP displayed intact cell size distribution. These results indicate that inhibition of ice recrystallization by LeIBP helps the RBCs avoid critically damaging electrolyte concentrations and the solution effects during melting. Therefore, we conclude that examining cell size distribution after cryopreservation would further evaluate potential cell damage, although this would not still completely evaluate the cryopreserved cells.

Further studies are still needed to confirm that the method works on a large-scale sample, up to several hundreds of milliliters of blood (i.e., a typical donation). Once the cryoprotective ability of LeIBP is fully evaluated in RBC cryopreservation, it would be extended to other blood cells, such as thrombocytes, lymphocytes, monocytes, and hematopoietic progenitor cells, to enhance the efficiency of cryopreservation. In conclusion, recombinant LeIBP expressed in *P. pastoris* clearly reduced the freeze–thaw-driven hemolysis and increased post-thaw cell recovery. The recovered RBCs revealed a cell size distribution almost the same to that of intact cells. Considering all these data, recombinant LeIBP can be a key component in improving the efficiency of RBC cryopreservation.

Acknowledgments The authors wish to thank So Ra Yoon for helping in blood collection. This work was supported by grants from Korea Polar Research Institute (PE11100) and Korea Research Council of Fundamental Science and Technology (PG11010 and PG12010).

References

1. Davies, P. L., Baardsnes, J., Kuiper, M. J., & Walker, V. K. (2002). Structure and function of antifreeze proteins. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 357*, 927–935.
2. Jia, Z., & Davies, P. L. (2002). Antifreeze proteins: an unusual receptor–ligand interaction. *Trends in Biochemical Sciences, 27*, 101–106.
3. Knight, C. A., & Duman, J. G. (1986). Inhibition of recrystallization of ice by insect thermal hysteresis proteins: a possible cryoprotective role. *Cryobiology, 23*, 256–262.
4. Knight, C. A., DeVries, A. L., & Oolman, L. D. (1984). Fish antifreeze protein and the freezing and recrystallization of ice. *Nature, 308*, 295–296.

5. Duman, J., & Horwath, K. (1983). The role of hemolymph proteins in the cold tolerance of insects. *Annual Review of Physiology, 45*, 261–270.
6. Urrutia, M. E., Duman, J. G., & Knight, C. A. (1992). Plant thermal hysteresis proteins. *Biochimica et Biophysica Acta, 1121*, 199–206.
7. DeVries, A. L. (1983). Antifreeze peptides and glycopeptides in cold-water fishes. *Annual Review of Physiology, 45*, 245–260.
8. Davies, P. L., & Hew, C. L. (1990). Biochemistry of fish antifreeze proteins. *FASEB Journal, 4*, 2460–2468.
9. DeVries, A. L., & Cheng, C. H. C. (1992). *The role of antifreeze glycopropeptides and peptides in the survival of cold-water fishes*. Berlin: Springer.
10. Yu, S. O., Brown, A., Middleton, A. J., Tomczak, M. M., Walker, V. K., & Davies, P. L. (2010). Ice restructuring inhibition activities in antifreeze proteins with distinct differences in thermal hysteresis. *Cryobiology, 61*, 327–334.
11. Kristiansen, E., & Zachariassen, K. E. (2005). The mechanism by which fish antifreeze proteins cause thermal hysteresis. *Cryobiology, 51*, 262–280.
12. DeVries, A. L., & Wohlschlag, D. E. (1969). Freezing resistance in some Antarctic fishes. *Science, 163*, 1073–1075.
13. Chen, G., & Jia, Z. (1999). Ice-binding surface of fish type III antifreeze. *Biophysical Journal, 77*, 1602–1608.
14. Low, W. K., Lin, Q., Stathakis, C., Miao, M., Fletcher, G. L., & Hew, C. L. (2001). Isolation and characterization of skin-type, type I antifreeze polypeptides from the longhorn sculpin, *Myoxocephalus octodecemspinosus*. *Journal of Biological Chemistry, 276*, 11582–11589.
15. Deng, G., & Laursen, R. A. (1998). Isolation and characterization of an antifreeze protein from the longhorn sculpin, *Myoxocephalus octodecimspinosis*. *Biochimica et Biophysica Acta, 1388*, 305–314.
16. Hew, C. L., Fletcher, G. L., & Ananthanarayanan, V. S. (1980). Antifreeze proteins from the shorthorn sculpin, *Myoxocephalus scorpius*: isolation and characterization. *Canadian Journal of Biochemistry, 58*, 377–383.
17. Hew, C. L., Wang, N. C., Yan, S., Cai, H., Sclater, A., & Fletcher, G. L. (1986). Biosynthesis of antifreeze polypeptides in the winter flounder. Characterization and seasonal occurrence of precursor polypeptides. *European Journal of Biochemistry, 160*, 267–272.
18. Yamashita, Y., Miura, R., Takemoto, Y., Tsuda, S., Kawahara, H., & Obata, H. (2003). Type II antifreeze protein from a mid-latitude freshwater fish, Japanese smelt (*Hypomesus nipponensis*). *Bioscience, Biotechnology, and Biochemistry, 67*, 461–466.
19. Atici, O., & Nalbantoglu, B. (2003). Antifreeze proteins in higher plants. *Phytochemistry, 64*, 1187–1196.
20. Kawahara, H., Iwanaka, Y., Higa, S., Muryoi, N., Sato, M., Honda, M., et al. (2007). A novel, intracellular antifreeze protein in an antarctic bacterium, *Flavobacterium xanthum*. *Cryo Letters, 28*, 39–49.
21. Raymond, J. A., Fritsen, C., & Shen, K. (2007). An ice-binding protein from an Antarctic sea ice bacterium. *FEMS Microbiology Ecology, 61*, 214–221.
22. Gilbert, J. A., Davies, P. L., & Laybourn-Parry, J. (2005). A hyperactive, Ca2+-dependent antifreeze protein in an Antarctic bacterium. *FEMS Microbiology Letters, 245*, 67–72.
23. Xiao, N., Suzuki, K., Nishimiya, Y., Kondo, H., Miura, A., Tsuda, S., et al. (2010). Comparison of functional properties of two fungal antifreeze proteins from *Antarctomyces psychrotrophicus* and *Typhula ishikariensis*. *FEBS Journal, 277*, 394–403.
24. Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annual Review of Physiology, 63*, 327–357.
25. Graether, S. P., & Sykes, B. D. (2004). Cold survival in freeze-intolerant insects: the structure and function of beta-helical antifreeze proteins. *European Journal of Biochemistry, 271*, 3285–3296.
26. Lee, J. K., Park, K. S., Park, S., Park, H., Song, Y. H., Kang, S. H., et al. (2010). An extracellular ice-binding glycoprotein from an Arctic psychrophilic yeast. *Cryobiology, 60*, 222–228.
27. Rous, P., & Turner, J. R. (1916). The preservation of living red blood cells in vitro: I. Methods of preservation. *Journal of Experimental Medicine, 23*, 219–237.
28. Hess, J. R. (2006). An update on solutions for red cell storage. *Vox Sanguinis, 91*, 13–19.
29. Hess, J. R., & Greenwalt, T. G. (2002). Storage of red blood cells: new approaches. *Transfusion Medicine Reviews, 16*, 283–295.
30. Hill, H. R., Oliver, C. K., Lippert, L. E., Greenwalt, T. J., & Hess, J. R. (2001). The effects of polyvinyl chloride and polyolefin blood bags on red blood cells stored in a new additive solution. *Vox Sanguinis, 81*, 161–166.
31. Heaton, W. A., Holme, S., Smith, K., Brecher, M. E., Pineda, A., AuBuchon, J. P., et al. (1994). Effects of 3-5 log10 pre-storage leucocyte depletion on red cell storage and metabolism. *British Journal of Haematology, 87*, 363–368.
32. Dumont, L. J., & AuBuchon, J. P. (2008). Evaluation of proposed FDA criteria for the evaluation of radiolabeled red cell recovery trials. *Transfusion, 48*, 1053–1060.

33. Pegg, D. E. (1987). *Mechanisms of freezing damage*. Cambridge: The Company of Biologists Ltd.
34. Pegg, D. E. (2007). *Cryopreservation and freeze-drying protocols* (2nd ed.). Totowa: Humana Press.
35. Smith, A. U. (1950). Prevention of haemolysis during freezing and thawing of red blood-cells. *Lancet, 2*, 910–911.
36. Meryman, H. T., & Hornblower, M. (1972). A method for freezing and washing red blood cells using a high glycerol concentration. *Transfusion, 12*, 145–156.
37. Rowe, A. W., Eyster, E., & Kellner, A. (1968). Liquid nitrogen preservation of red blood cells for transfusion; a low glycerol-rapid freeze procedure. *Cryobiology, 5*, 119–128.
38. Chao, H., Davies, P. L., & Carpenter, J. F. (1996). Effects of antifreeze proteins on red blood cell survival during cryopreservation. *Journal of Experimental Biology, 199*, 2071–2076.
39. Carpenter, J. F., & Hansen, T. N. (1992). Antifreeze protein modulates cell survival during cryopreservation: mediation through influence on ice crystal growth. *Proceedings of the National Academy of Sciences of the United States of America, 89*, 8953–8957.
40. Kang, J. S., & Raymond, J. A. (2004). Reduction of freeze-thaw-induced hemolysis of red blood cells by an algal ice-binding protein. *Cryo Letters, 25*, 307–310.
41. Park, K. S., Lee, J. H., Park, S. I., Do, H., Kim, E. J., Kang, S. H. & Kim, H. J. (2012) Characterization of a homodimeric ice-binding protein from *Leucosporidium* sp. *Cryobiology, 64*, 286–296.
42. Drabkin, D. L., & Austin, J. H. (1935). Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. *Journal of Biological Chemistry, 112*, 51–65.
43. Runck, A. H., Valeri, C. R., & Sampson, W. T. (1968). Comparison of the effects of ionic and non-ionic solutions on the volume and intracellular potassium of frozen and non-frozen human red cells. *Transfusion, 8*, 9–18.
44. Smallwood, M., Worrall, D., Byass, L., Elias, L., Ashford, D., Doucet, C. J., et al. (1999). Isolation and characterization of a novel antifreeze protein from carrot (*Daucus carota*). *Biochemical Journal, 340*(Pt 2), 385–391.
45. Schmid, P., Huvard, M. J., Lee-Stroka, A. H., Lee, J. Y., Byrne, K. M., & Flegel, W. A. (2011). Red blood cell preservation by droplet freezing with polyvinylpyrrolidone or sucrose-dextrose and by bulk freezing with glycerol. *Transfusion, 51*(12), 2703–2708.
46. Hess, J. R. (2010). Red cell storage. *Journal of Proteomics, 73*, 368–373.
47. Zimrin, A. B., & Hess, J. R. (2009). Current issues relating to the transfusion of stored red blood cells. *Vox Sanguinis, 96*, 93–103.
48. Lovelock, J. E. (1953). The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. *Biochimica et Biophysica Acta, 11*, 28–36.