

Research Article

Methods in Biosynthesis and Characterization of the Antifreeze Protein (AFP) for Potential Blood Cryopreservation

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The cryopreservation of red blood cells (RBCs) is very important to modern medicine. Cryoprotectants (CPAs) such as dimethyl sulfoxide (DMSO), proline, trehalose, and polyvinyl alcohol (PVA) have been used in the cryopreservation of RBCs, but the results are not satisfactory. *Marinomonas primoryensis* antifreeze protein (MpAFP) is a Ca^{2+} -dependent AFP derived from Antarctic bacteria, which can prevent bacteria from freezing under extremely cold conditions and may be suitable for cryopreservation of RBCs. The active region of MpAFP is located in region IV and is called MPAFP_RIV. In this paper, the gene of region IV of MpAFP is introduced into BL21 (DE3) competent cells, and MpAFP_RIV is obtained after culture, separation, and purification. The improved splat assay is proposed, and this method first proves that MpAFP_RIV has strong ice recrystallization inhibition (IRI) activity without Ca^{2+} . The improved splat assay is easier to operate and lower the cost compared to the traditional one, and the results are consistent with the classic sucrose sandwich assay, proving that this method can accurately detect IRI activity. The feasibility of MPAFP_RIV combined with classical CPA for cryopreservation of RBCs and the methods to increase the yield of MPAFP_RIV are proposed.

1. Introduction

Cryopreservation refers to the method of preserving cells [1], tissues [2], and organs [3] at extremely low temperatures. Modern biology, medicine, physiology, and so forth are inseparable from cryopreservation [4, 5]. At low temperatures, large ice crystals formed by ice recrystallization can easily pierce cell membranes and cause severe mechanical damage to cells [6]. In addition, a large amount of freezing of the extracellular solution can increase the osmotic pressure and cause osmotic dehydration to cells [7]. To reduce the damage in cryopreservation, many cryoprotectants (CPAs) have been developed [8]. The cryopreservation of red blood cells (RBCs) is the basis of blood transport [9], which plays an important role in treating traumatic blood loss, leukemia, and hemolytic anemia [10–12]. More than thirty million units of blood are consumed every year in the United States,

and a patient sometimes needs to transfuse as much as 57 liters of blood [13]. However, the current preservation time of RBCs is short, and even the most advanced techniques can only make RBCs survive for less than 42 days [14, 15]. As a result, as many as 1.7 million red blood cells were discarded in the United States in 2011, resulting in huge waste [9]. The current golden standard CPA for cryopreservation of cells is dimethyl sulfoxide (DMSO) [16]. DMSO can permeate into cells and adjust osmotic pressure, reducing the osmotic dehydration of cells during cryopreservation [17]. However, there are some disadvantages to DMSO. DMSO is unable to inhibit ice recrystallization, so it is unable to reduce the harm caused by large ice. In addition, DMSO is a toxic organic solvent that is believed to be associated with developmental disorders and apoptosis of cells [18, 19]. Therefore, DMSO needs to be quickly removed after thawing. The removing process requires specialized instruments

and complicated steps [20, 21] and may lead to osmotic shock that is harmful to the survival rate of RBCs [22]. Trehalose [23], proline [7], and PVA [13] have also been used in cryopreservation of RBCs, but the efficiency is limited.

Antifreeze proteins (AFPs) are classical CPAs, which are the key for fish [24], bacteria [25], insects [26], plants [27], and others to survive in extremely cold conditions. AFPs have strong ice recrystallization inhibition (IRI) activity because AFPs can be adsorbed on the ice surface and inhibit the flow of water among ice crystals. Therefore, the growth of ice is restricted [28]. IRI activity allows organisms to tolerate damage from smaller ice rather than being fatal. In addition, AFPs have thermal hysteresis (TH) activity, which can significantly reduce the freezing point of the solution [29]. TH activity can prevent organisms from freezing at subzero temperatures and avoid the damage from ice. At present, the methods to obtain AFPs include extraction from freeze-resistant plants [30], expression in transgenic plants [31], chemical synthesis [32], and expression in transgenic bacteria [33]. Currently, there are few studies on the use of AFPs in the cryopreservation of RBCs.

Marinomonas primoryensis AFP (MpAFP) comes from Antarctic bacteria with a molecular weight greater than 1.5 MDa. The antifreeze active site of MpAFP is in region IV (MpAFP_RIV) with a size of 322 amino acids [34]. Studies have shown that the TH activity of MpAFP_RIV strongly depends on the concentration of Ca^{2+} . When the concentration of Ca^{2+} is appropriate, the TH activity can reach about 2°C [35]. In this way, bacteria can avoid damage from freezing at subzero temperatures. When the concentration of Ca^{2+} is low, the TH activity will decrease rapidly [36]. There are few studies on the IRI activity of MpAFP_RIV currently.

The main methods to characterize IRI activity are splat assay [37] and sucrose sandwich assay [30]. In splat assay, a small amount of sample is dropped from a high place to a glass slide precooled on dry ice, making the sample instantly disperse and freeze. Then, the glass slide is quickly placed on the cold stage and annealed at -8°C for 30 minutes. This step is to allow the ice to fully recrystallize. Ice crystals' size is observed under a polarizing microscope, and IRI activity is inversely proportional to it. Mean grain size (MGS) [38] and mean largest grain size (MLGS) [13] of all ice in the field of view are indicators for quantitatively characterizing IRI activity. MGS (MLSG) is inversely proportional to IRI activity. The traditional splat assay has some drawbacks. First of all, dry ice is expensive and volatile, increasing the cost greatly; second, the glass slide is easy to frost on the dry ice, which seriously affects the observation of the sample; finally, it is difficult to accurately drop the sample from the high altitude on the glass slide. Sucrose sandwich assay needs to add a sufficient amount of sucrose to the sample. Sucrose can increase the viscosity of the sample, inhibit water flow and ice crystal coalescence, and facilitate the observation of ice crystal growth behavior. Then place a small amount of sample between two glass slides. The glass slides are immersed in liquid nitrogen for quick freezing and annealed on a cold stage at -6°C for 1 h. During this period, the growth of ice crystals is observed and the ice growth rate is calculated

[39]. The ice growth rate is inversely proportional to IRI activity. Sucrose sandwich assay and splat assay characterize IRI activity from different perspectives and are usually used together.

In this study, MpAFP_RIV was obtained by biosynthesis, and its IRI activity in the absence of Ca^{2+} was determined by improved splat assay and sucrose sandwich assay. The feasibility of MpAFP_RIV for cryopreservation of RBCs was discussed, and the optimized synthetic route for industrial production was proposed, as shown in Figure 1, which can obtain MpAFP_RIV cheaply and promote the development of cryopreservation.

2. Materials and Methods

2.1. Transfection. Referring to previous reports [36], PET-24a plasmid with MPAFP_RIV gene was provided by Shanghai Sangon Biotechnology Co., Ltd., and the C terminal of MPAFP_RIV was labeled by six histidines. BL21 (DE3) competent cells were taken from -80°C and thawed on ice. After thawing, the plasmid was added to competent cells and put on an ice bath for 30 min. Then, the competent cells were heated in 42°C water for 60 seconds and immediately inserted back into the ice for 2 minutes. $500\ \mu\text{L}$ of Luria-Bertani medium with kanamycin was added to competent cells and resuscitated on a shaker at 37°C and 200 rpm for 1 h. After the treatment, the bacteria suspension was coated on Luria-Bertani medium containing kanamycin and placed upside down in an incubator at 37°C overnight. After the incubation, pick a small number of colonies on the medium and place them in the Luria-Bertani culture medium containing kanamycin and cultivate overnight at 37°C .

2.2. Agarose Electrophoresis. Two primers were designed to determine whether the transfection was successful. The forward primer is $5'$ -TAATGCCAGCGGTAAAGTGC- $3'$, and the reverse primer is $5'$ -AGAACATCGTCTCCTGTCCC- $3'$. Five groups of bacterial suspension with different volumes (5, 10, 15, 20, and $25\ \mu\text{L}$, respectively) and $10\ \mu\text{L}$ PET-24a plasmid were mixed with primers for PCR amplification. The 1% gel was prepared from agarose and Tris-acetate buffer (TAE) and heated in a microwave oven to melt it. After cooling to 50 - 60°C , added gel red and mixed well, then poured it into the electrolytic cell, and inserted the comb. After coagulation, the comb was taken out and the samples were loaded, and electrophoresis was performed at 80 V for 40 minutes. Then the gel was placed in the imager (Chemidoc Imaging Systems, Bio-Rad), and the bands of Marker and samples were observed to judge whether the transfection is successful.

2.3. Expression and Purification of His₆-Tagged MpAFP_RIV. The bacteria suspension was placed in Luria-Bertani medium containing kanamycin and routinely cultured at 37°C until OD_{600} reached 0.6. Isopropyl β -D-thiogalactoside (IPTG) was added to 1 mM, and the culture was continued for 4-5 h. The bacteria suspension was centrifuged at 4000 g at 4°C for 20 min. The supernatant was discarded, and the precipitation was collected. The nondenatured lysate

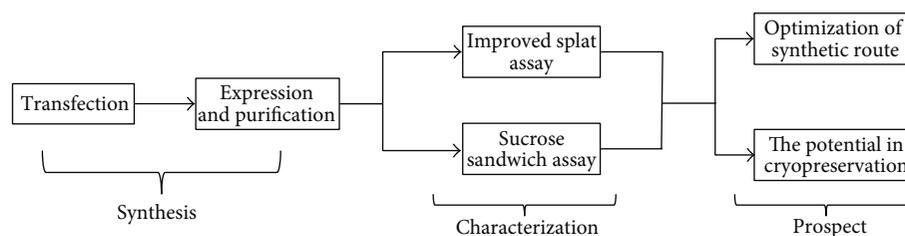


FIGURE 1: Chart of the synthesis, characterization, and potential for cryopreservation of RBCs of MpAFP_RIV.

[50 mM NaH₂PO₄, 300 mM NaCl, pH = 8] was added to the precipitation, and phenylmethylsulfonyl fluoride (PMSF) was added as a protease inhibitor. Lysozyme was added to the concentration of 1 mg/mL, and then the bacteria were lysed by ultrasonic on ice. The bacterial lysate was repeatedly sucked with a thin syringe needle several times to cut the sticky DNA fragments. After centrifugation at 4°C, 10000 g for 30 min, the collected supernatant and the His-tag purification resin (P2218, Beyotime) were evenly mixed in the shaker and loaded into the chromatographic column. The purified MpAFP_RIV could be obtained by washing 3 times with nondenaturing washing solution [50 mM NaH₂PO₄, 300 mM NaCl, 2 mM imidazole] and eluting 3 times with nondenaturing eluent [50 mM NaH₂PO₄, 50 mM NaH₂PO₄, 50 mM imidazole].

2.4. Sucrose Sandwich Assay. A sufficient amount of sucrose was added to the MpAFP_RIV sample (1×10^{-4} M) to make its concentration reach 60%wt. Take 6 μ L sample between two glass slides. The glass slides were immersed in liquid nitrogen for quick freezing so that a large number of small ice crystals were produced. Then moved the glass slides into the cold stage at -8°C and annealed for 60 min. Photographs were taken every two minutes under a polarizing microscope. The length of a single ice crystal was measured, and the graph of ice crystal size relative to time was made. The slope of ice crystal size and time was calculated by linear regression, and the ice growth rate was determined. The experiment was repeated with blank eluent as the control group.

2.5. Improved Splat Assay. 6 μ L MpAFP_RIV sample (1×10^{-4} M) was taken and quickly spread on the glass slide that precooled in liquid nitrogen as evenly and dispersedly as possible so that the sample was instantly frozen into a thin ice layer. The glass slide was immersed into liquid nitrogen again to prevent the ice from melting. Then, the slide was annealed in a cold stage at -8°C for 30 minutes to make the sample recrystallized. The size of ice crystals was observed under a polarizing microscope. The average length of the longest axis of the ten largest ice crystals in the field of view was calculated, which refers to the MLGS. The experiment was repeated with the blank eluent as the control group.

2.6. Cryopreservation of Red Blood Cells. 10% DMSO (XiLong Scientific Co., Ltd.), 8% proline (Sinopharm Chemical Reagent Co., Ltd.), and 1 mg/mL PVA (Kuraray Co., Ltd.) were prepared, and 5 mL of each sample was taken in a test tube as CPA. 5 mL 0.01 M PBS (Codow Co., Ltd) solution

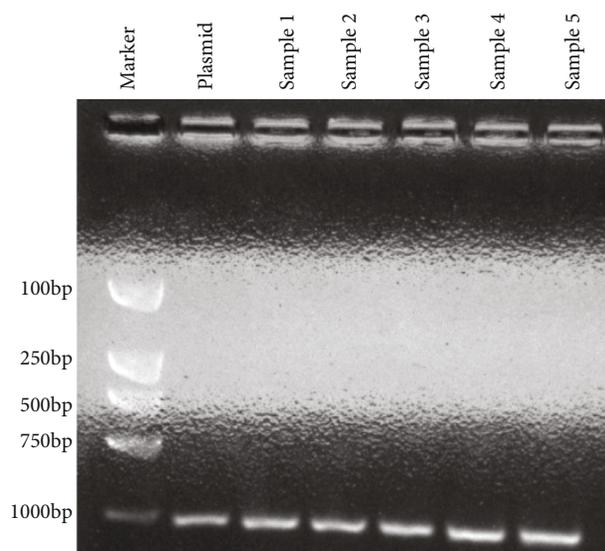


FIGURE 2: Agarose electrophoresis of bacterial suspension and plasmid. Samples 1, 2, 3, 4, and 5 were bacterial suspension of different volumes (5, 10, 15, 20, and 25 μ L, respectively). After PCR amplification, there were clear bands at about 1000bp in plasmid and all the 5 groups samples, which was consistent with the MPAFP_RIV gene.

was taken into the test tube as blank control. Fresh sheep RBCs (Guangzhou Hongquan Biotechnology Co., Ltd.) were washed with PBS solution 3 times, and the supernatant was removed. 100 μ L RBC precipitates were transferred into each test tube with CPAs and PBS, incubated for 30 min, and immersed in liquid nitrogen to be frozen completely. The RBCs were placed in water at 37°C until thawed, then centrifuged at 4000 rpm, and the supernatant was taken to measure the absorbance at 450 nm [40]. RBCs is dissolved in deionized water as a positive control (100% hemolysis) and placed in PBS as a negative control (0% hemolysis). The survival rate of RBCs is calculated by the formula [9]:

$$RBC \text{ survival rate}\% = \left(1 - \frac{A - A_0}{A_1 - A_0}\right) \times 100\%, \quad (1)$$

where A is the absorbance of the measured sample, A_0 is the absorbance of the RBCs in PBS, and A_1 is the absorbance of RBCs in deionized water.

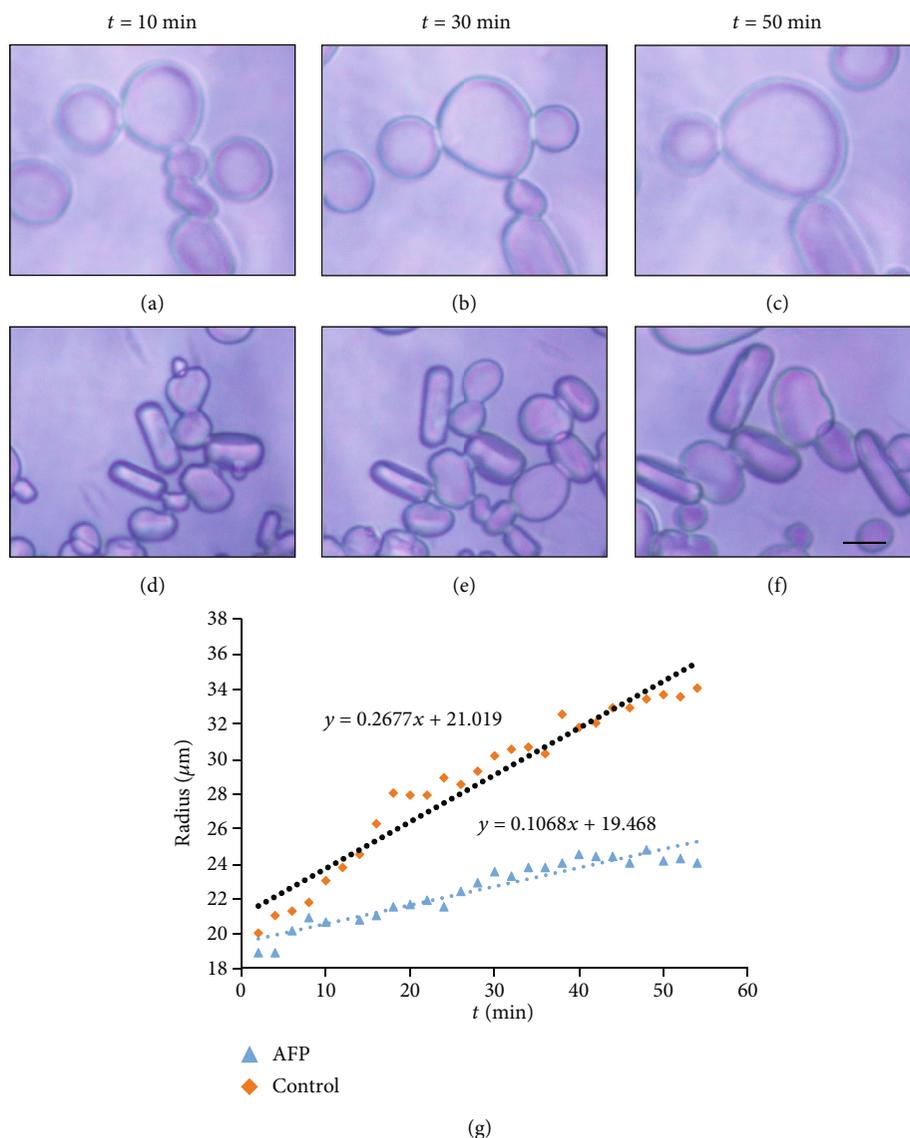


FIGURE 3: The ice growth rate of MpAFP_RIV (1×10^{-4} M) and blank eluate detected by sucrose sandwich assay. (a–c) are blank eluate, (d–f) are MpAFP_RIV. (g) is the ice crystal size changed with time. The slope is the ice growth rate. Scale bar = $20 \mu\text{m}$.

3. Results and Discussion

3.1. Transfection. Transfection is a process in which foreign DNA fragments are actively or passively introduced into eukaryotic cells to obtain a new phenotype [41], and it is the key to the successful expression of proteins in bacteria. In this study, PET-24a plasmid with MpAFP_RIV gene was introduced into BL21 (DE3) competent cells to express MpAFP_RIV. Agarose electrophoresis was performed on the bacterial suspension and plasmid after PCR amplification, and the results are shown in Figure 2. It can be seen that after PCR amplification, there were clear bands at about 1000 bp for both bacterial suspension of different volumes and plasmid, which was consistent with the MpAFP_RIV gene, indicating that the plasmid with the gene of MpAFP_RIV has been introduced into BL21(DE3) competent cells successfully.

3.2. Sucrose Sandwich Assay. Sucrose sandwich assay is a classic method to detect ice growth rate. This method adds sufficient sucrose to the sample to achieve a concentration of about 60%wt. A large amount of sucrose in the solution inhibits water flow among ice, which is convenient for observing the behavior of ice growth [42]. Figure 3 shows the ice growth in MpAFP_RIV and blank eluate.

In the blank eluate group, the ice grew very fast, and the ice crystals were close to each other and gradually fused. However, the ice growth rate slows down greatly, and the ice volume does not increase obviously in the MpAFP_RIV group. After 50 minutes of annealing, the ice crystal size of the blank eluate group was significantly larger than that of the MpAFP_RIV group. The ice growth rate of the blank eluate was $0.2677 \mu\text{m}/\text{min}$, while the MpAFP_RIV was $0.1068 \mu\text{m}/\text{min}$, which was only 39.9% of the control group. In the process of thawing, small ice crystals will disappear and large

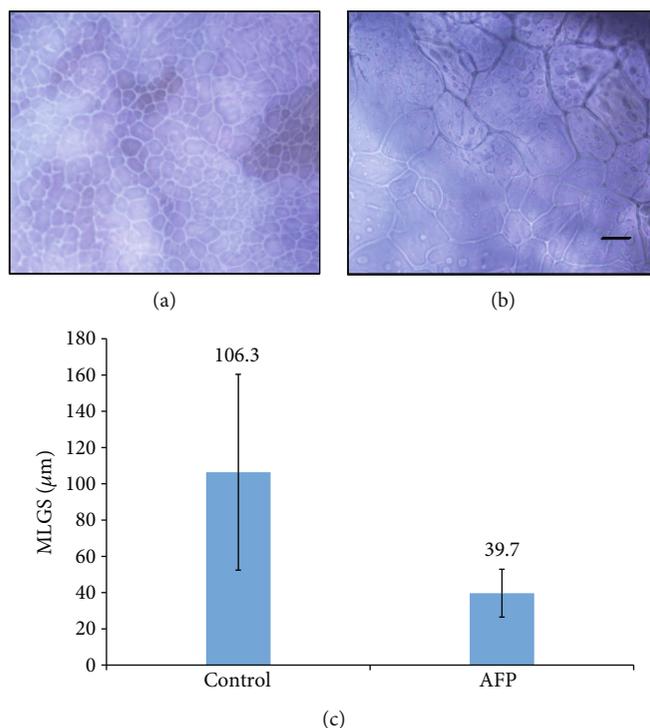


FIGURE 4: IRI activity of control group and MPAFP_RIV (1×10^{-4} M) detected by improved splat assay. (a) is MPAFP_RIV, (b) is the control group (blank eluent). (c) MLGS: mean largest grain size, which means the average size of the ten largest ice crystals in the field of view. Scale bar = $40 \mu\text{m}$. Error bars represent the standard deviation of three trials.

ice crystals will grow up. This phenomenon is called ice recrystallization, which is in line with the Ostwald ripening process. Large ice crystals formed by ice recrystallization can easily pierce cell membranes and cause fatal mechanical damage to cells. AFP is a powerful inhibitor of ice recrystallization. During the thawing process, AFP will be adsorbed on the ice surface, bend the ice surface, and inhibit the flow of water among ice crystals [28]. In this way, ice recrystallization is suppressed. MpAFP_RIV is the effective region of MpAFP. The results prove that MpAFP_RIV has the same strong IRI activity as classic AFP and can significantly reduce the ice growth rate. This can greatly reduce the mechanical damage from large ice that the cells receive during cryopreservation.

3.3. Improved Splat Assay. Traditional splat assay in detecting IRI activity has many disadvantages, such as difficulties in operation, high cost, and inconvenience in observation. The IRI activity of MPAFP_RIV was tested by improved splat assay, and the results are shown in Figure 4. The MLGS of the blank eluent was $106.3 \mu\text{m}$, while the MPAFP_RIV was $39.7 \mu\text{m}$, which was only 37.3% of the control group. It proves that MPAFP_RIV has strong IRI activity. In cryopreservation, sharp ice crystals can pierce cell membrane, and the excessive freezing of extracellular solutions can lead to osmotic dehydration. MPAFP_RIV can effectively inhibit the growth of ice caused and reduce the damage by recrystallization, indicating the potential for cryopreservation.

According to previous reports, the TH activity of MpAFP_RIV is strongly dependent on Ca^{2+} . When the con-

centration of Ca^{2+} is suitable, the TH activity can reach 2°C . However, if there is no Ca^{2+} , the TH activity will decrease rapidly below 1°C [36]. In our study, MpAFP_RIV still has strong IRI activity in the absence of Ca^{2+} . This shows that MpAFP_RIV can be directly used in the cryopreservation of cells by its IRI activity without the need for additional Ca^{2+} .

In this experiment, the results of the improved splat assay and sucrose sandwich assay were consistent, which proved that MPAFP_RIV had strong IRI activity from two perspectives. Also, the consistency of the conclusions of the two methods proved that the improved splat assay is very accurate in detecting IRI activity. Compared with the traditional one, the improved splat assay avoids the use of expensive dry ice, simplifies the operation process, and avoids the frosting of glass slides, which may affect the observation. It provides a new way of characterizing IRI activity.

3.4. Cryopreservation of Red Blood Cells. The cryopreservation of RBCs is critical to modern medicine, biology, and physiology. In this article, 10% DMSO, 8% proline, and 1 mg/mL PVA are used as CPAs to explore their effects on the cryopreservation of RBCs. The result is shown in Figure 5.

It can be seen that DMSO, Pro, and PVA can all improve the cryopreservation efficiency of RBCs. However, the survival rate of the Pro and PVA groups was only 56.6% and 62.1%, respectively, which was only about 30% higher than the control group. This is far from the clinical requirement for a large number of RBCs. 10% DMSO is currently the

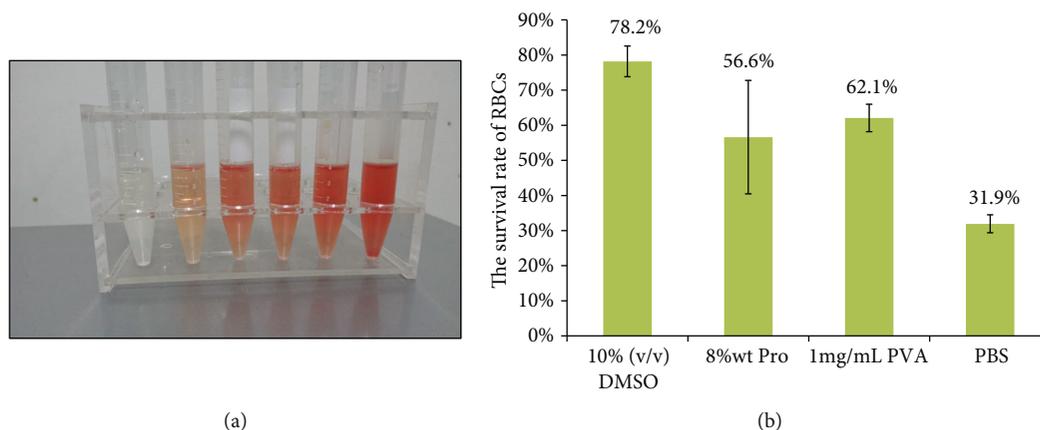


FIGURE 5: (a) RBCs after freezing and thawing. From left to right: positive control (RBCs are placed to PBS without freezing and regarded as 0% hemolysis), 10% *v/v* DMSO, 8%wt Pro, 1 mg/mL PVA, PBS (blank control), and negative control (RBCs are dissolved in deionized water and regarded as 100% hemolysis). (b) Survival rate of RBCs after freezing and thawing. Error bars represent the standard deviation of three trials.

golden standard CPA for cell cryopreservation. When used on RBCs, the survival rate can be increased to 78.2%. However, DMSO is a toxic organic solvent, which can cause cell development disorders and impaired enzyme function and may induce abnormal differentiation of stem cells [43].

MPAFP_RIV has excellent IRI activity, which can significantly inhibit ice recrystallization and reduce ice size. Therefore, MPAFP_RIV may have synergistic effects with Pro, PVA, and DMSO, improving the efficiency of cryopreservation of RBCs comprehensively by reducing ice crystal size, regulating the osmotic pressure, and reducing the number of organic solvents. MPAFP_RIV works mainly outside the cell, thus, eliminating the cumbersome washing process. Moreover, the synthesis of MPAFP_RIV is simple and high-yielding, and it works greatly even at low concentrations. These advantages further illustrate the great prospect of MPAFP_RIV in the cryopreservation of RBCs.

It must be noted that when AFP is adsorbed on the ice surface, sharp needle-like ice will be formed [44]. These needle-like ice may pierce cell membranes easier, resulting in only a half success rate when AFP is used for cell cryopreservation [45]. MpAFP_RIV is the effective region of MpAFP, whether MpAFP_RIV can cause the production of needle-like ice and whether it is suitable for cell cryopreservation to remain to be further tested.

4. Conclusions

In summary, MpAFP_RIV could be obtained through biosynthesis. The TH activity of MpAFP_RIV is positively correlated with Ca^{2+} . The improved splat assay and sucrose sandwich assay proved that MpAFP_RIV also had strong IRI activity in the absence of Ca^{2+} . By comparing the results of different groups, it was found that the MLGS of the MPAFP_RIV group decreased to 37.3% of the blank control group, and the ice growth rate decreased to 39.9%. Compared with the traditional splat assay, the improved one is easier to

operate, lower cost, and the effect is also accurate. The strong IRI activity of MpAFP_RIV may have a synergistic effect with classic CPAs such as DMSO and glycerol, improving the efficiency of cryopreservation of RBCs. In order to expand the production of MpAFP_RIV to meet the needs of industrial applications, we propose some methods to improve the synthetic route. First, BL21 (DE3) is a universal competent cell for protein expression. In mass production, it can be replaced with bacteria which are high-yielding, endotoxin-free, and tolerant to MpAFP_RIV. In addition, the His-tag purification resin contains Ni^{2+} , which has a strong affinity for His-tagged proteins, so the obtained MPAFP_RIV is in high purity. However, this resin is expensive and only suitable for laboratory production. In industrial applications, it can be separated by molecular sieves. This method slightly reduces the purity, but can greatly reduce the cost. Last but not least, the cells can begin to express protein only after the promoter is induced by IPTG in this article. This is to avoid the adverse effects of MPAFP_RIV on bacterial growth and reproduction. Therefore, IPTG was not added until the bacteria had fully multiplied. However, the addition of IPTG will increase costs. In industrial production, bacteria that are tolerant to MpAFP_RIV can be used, and the promoter on the plasmid can be replaced with one that does not require induction, allowing the bacteria to express proteins while multiplying, saving time, and reducing costs.

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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