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CHARACTERIZATION AND POTENTIAL APPLICATIONS OF A RECOMBINANT ANTIFREEZE PROTEIN FROM AN ANTARCTIC YEAST *Glaciozyma antarctica* PRODUCED IN *Pichia pastoris*

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ABSTRACT

Ice recrystallization during thawing post-cryopreservation results in extensive cellular damage and ultimately leads to cell death and reduced cell viability. Antifreeze proteins (AFPs) are a group of proteins that allow organisms to survive in sub-zero environments. These proteins have thermal hysteresis and ice recrystallization inhibitory activities. In this present study, we demonstrated the efficiency of a recombinant antifreeze protein from the Antarctic yeast, *Glaciozyma antarctica*, as a recrystallization inhibitor (RI) of ice growth and assessed its application as a cryopreservative of the fungal cutinase enzyme against freeze-thaw cycles. Recombinant Afp1 from *G. antarctica*, a psychrophilic yeast, has been produced in a methylotrophic yeast, *Pichia pastoris*, system that results in the expression of a hyper-glycoprotein (~55 kDa). Recombinant Afp1 exhibits antifreeze functions: thermal hysteresis (TH) and recrystallization inhibition where the highest TH values recorded for ~0.5°C at 10 mg/mL. The cryoprotective effects of Afp1 on purified recombinant cutinase showed that Afp1 can retain enzymatic activity up to ~20% when subjected to several cycles of freeze thawing. These findings indicate that Afp1 might act as a cryoprotective agent and thus, has great potential in biotechnology applications.

Key words: antifreeze protein, *Glaciozyma antarctica,* recrystallization inhibition, cryoprotective

INTRODUCTION

Antifreeze proteins (AFPs) have evolved in cold-adapted organisms to control ice crystal growth upon exposure to sub-zero temperatures. It has been suggested that the effect of these proteins results in small sizes of ice crystals, which mitigates mechanical damage to frozen tissues and cells that can be caused by large ice crystals. These proteins directly interact with ice surfaces and act to depress the freezing point of body fluids, inhibit ice recrystallization, or promote ice nucleation (Barrett, 2001). Several hypotheses have been advanced to describe the mechanism of AFP binding to ice, which occurs because of the diversity of structural folds in this protein. Based on studies of insects and fish AFPs, these include adsorption-inhibition, hydrogen bond interactions and roles of hydrophobic amino acids (Raymond & DeVries, 1977; Knight *et al*., 1993). The delicate control over ice growth makes these proteins applicable to any field that requires control of ice growth. AFPs from fish (Types I–IV and antifreeze glycopeptide) have been recognized as having the potential to be widely used in most applications because they are hypoallergenic (Kim *et al.,* 2015). AFPs can be applied in various foods and medical uses (Christner, 2010). They have been shown to improve the texture of ice cream (Regand & Goff, 2006), increase the quality and half-life of yeasts in frozen dough (Zhang *et al.,* 2007) and help to preserve meat (Griffith & Ewart, 1995). AFPs have also been used in cryosurgery and in blood and organ preservation (Amir *et al.,* 2003; Venkatesh & Dayananda, 2008).

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Glaciozyma antarctica, a psychrophilic yeast isolated from sea ice near Casey Research Station, Antarctica, has been shown to produce AFP (Hashim *et al.,* 2013). The thermal hysteresis (TH) value recorded from *G. antarctica* culture filtrate was 0.1°C and the Afp also exhibited recrystallization inhibitory activity. Previously, recombinant expression of *G. antarctica* Afp1 in *Escherichia coli* was shown to result in the formation of inactive inclusion bodies, which required further manipulation to achieve biological activity. Refolded recombinant Afp1 produced in *E. coli* exhibited antifreeze activity that was lower than that of the native form (TH=0.08°C) (Hashim *et al.,* 2013). However, because of poor yields obtained from prokaryotic expression systems, a eukaryotic expression system was chosen to overproduce this protein. In this present study, we report on the expression of *G. antarctica* Afp1 in methylotrophic yeast, *Pichia pastoris*. This methylotrophic yeast was used to express recombinant Afp1 because of its ability to generate large amounts of properly folded protein, ease of isolation and potential to be produced as an extracellular protein (Cregg *et al*., 1993). Additionally, the application of the recombinant Afp1 recrystallization inhibition (RI) activity as a cryoprotective agent against other enzymes undergoing freeze-thawing was explored.

MATERIALS AND METHODS

Micro-organisms and plasmids

The methylotrophic yeast *Pichia pastoris* X-33 and expression vector pPICZαC used to express Afp1 were purchased from Invitrogen (Carlsbad, CA, USA). Plasmid pAFP1, harbouring a full-length *AFP1* sequence, was obtained from the Molecular Mycology Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, UKM (Hashim *et al.,* 2013).

Construction of pPICZαC_Afp1 and *P. pastoris* transformation

The full sequence of *AFP1* was amplified using specific primers, AFP1_ClaI_F: 5'-TCA CCA TCG ATG GCC ACC GCC ATC GA-3' and AFP1_XbaI_ R: 5'-GAA TTC ACT TCT AGA AAC CCA GGC GCG-3', whereby the *Xba*I and *Cla*I restriction sites were introduced at the 5'- and 3'-ends of the sequence for cloning purposes. The 50 µL PCR reactions contained the following contents: 2 µL pAFP1 harbouring full-length *AFP1*, 1× PCR buffer, 0.2 mM dNTP, 20 pmol/µL forward and reverse primers, 1.5 mM $MgCl_2$, and 0.5 U/µL *Taq* polymerase (Invitrogen, Carlsbad, CA, USA). PCR was conducted using the following thermocycling conditions: initial denaturation 94°C for 5 min; 29 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 30 sec and elongation at 72°C for 1 min and a final extension at 72°C for 15 min. PCR products were purified, cloned into linearized pPICZαC and transformed into *E. coli* DH5α and trans formants were then verified via PCR, restriction enzyme analysis and sequencing.

To transform *P. pastoris*, a total of 5 µg pPIC_Afp1 was linearized using *Pme*I, followed by transformation into *P. pastoris* X-33 by electroporation with an Electroporator System 2510 (Eppendorf, AG, Hamburg, Germany). Transformants were plated onto YPD medium that contained 100 µg/mL zeocin and was incubated at 30°C for 2 days. To assess correct integration, colonies were screened by PCR using 5' and 3' *AOX1* primers according to the manufacturer's instructions. Positive transformants were plated onto YPD plates that contained zeocin at different concentrations; 1 and 2 mg/mL were used to select multi-copy integrants.

Expression of recombinant Afp1

In brief, recombinant *P. pastoris* strain X33 cells that had been transformed by pPICZαC harbouring the mature *AFP1* gene were grown in Buffered Minimal Glycerol Complex Medium (BMGY) that contained (per litre) 10 g yeast extract, 20 g peptone, 1 M potassium phosphate (pH 6.0), 13.4 g yeast nitrogen base, 400 µg biotin, and 10 mL glycerol. Minimal medium (MM) consisted of (per litre) 10 mL 10×yeast nitrogen base, 10 mL 1× methanol and 0.2 mL 500× biotin as an expression medium; expression was induced daily by the addition of 5 mL methanol at 28°C for 3 days. Culture supernatants were confirmed by sodium dodecyl sulphate-polyacrylamide (SDS) gel electrophoresis (SDS-PAGE) and western blot analyses.

Protein purification

Crude protein was applied to an affinity chromatography (Ni-NTA) column using 20 mM Tris–HCl (pH 8), 150 mM NaCl and 10 mM imidazole as binding buffer and with 20 mM Tris–HCl (pH 8), 150 mM NaCl, and 500 mM imidazole as elution buffer. Proteins were further purified by size exclusion chromatography using a Superdex S200 10/300 column (GE Healthcare, USA). All purification steps were carried out using an AKTA purifier (GE Healthcare, USA).

Antifreeze protein assay and cryoprotective effects

Recrystallization inhibition (RI) and the thermal hysteresis assay (TH) using method described by Kawahara *et al* (2007) were carried out using a temperature-controlled freezing stage (Model THM 600, Linkham Scientific Instrument, UK) with a temperature controller programming unit (Model

TMS 94, Linkham Scientific Instrument, UK). The RI assay was carried out by the addition of 1 mg/mL recombinant Afp1 to a mixture that contained recombinant *Glomerella cingulata* cutinase (1 mg/mL) (Wan Seman *et al.,* 2014) to monitor the ability of Afp1 to inhibit ice recrystallization. The cryoprotective effects of Afp1 were further tested by incubating Afp1 with recombinant protein at a ratio of 1:1. Samples were maintained at -20°C for 3 h and then were thawed at room temperature; the experiment was repeated for five cycles. After five cycles of freeze-thawing, samples were tested for specific activity according to the protocol of Seman *et al* (2014). Samples were then maintained at -20°C and the freeze thaw cycles were then performed every 12 h for two days.

RESULTS AND DISCUSSION

The cloning of a mature sequence of *AFP1* and transformation into *P. pastoris* was successfully conducted. Figure 1 shows that recombinant Afp1 was expressed as a secreted protein. However, recombinant Afp1 was produced at ~55 kDa, which was larger than the predicted size of ~15 kDa. Expression was confirmed by western blot analysis using anti-His antibodies for which signals were detected at ~55 kDa. This observation might indicate this protein had been glycosylated. Our previous findings also showed that native Afp1 from *G. antarctica* was glycosylated when probed using anti-Afp1 antibodies (Hashim *et al.,* 2013).

The activity of purified recombinant Afp1 was measured by observing changes in ice crystal morphology and in the inhibition of ice recrystallization. Generally, in the presence of antifreeze proteins, ice crystal growth is inhibited, resulting in an irregular ice crystal shape. This occurs because the antifreeze protein can bind to specific ice planes depending upon the complementary of its binding site with ice planes (Kawahara, 2002). In contrast to samples without antifreeze proteins, ice can expand when no inhibitors are present in the solution, which results in ice growth as round shaped crystals. Based on our observations, the presence of recombinant Afp1-changed ice morphology into a "flowery" shape (Figure 2A) compared with solutions treated with Proteinase K, in which round shape ice crystals formed (Figure 2B & 2C).

Ice crystal formation was also modified by AFPs, as the AFPs reduced the freezing point of a solution without changing the melting point. This process was defined as thermal hysteresis (TH) (Barrett, 2001). The TH value could be measured as the difference between the temperature when single ice crystals formed and the temperature for which the ice started to change. The TH value recorded for solutions that contained recombinant Afp1 was 0.5°C compared with solutions that contained Proteinase K (0°C). The TH value was higher

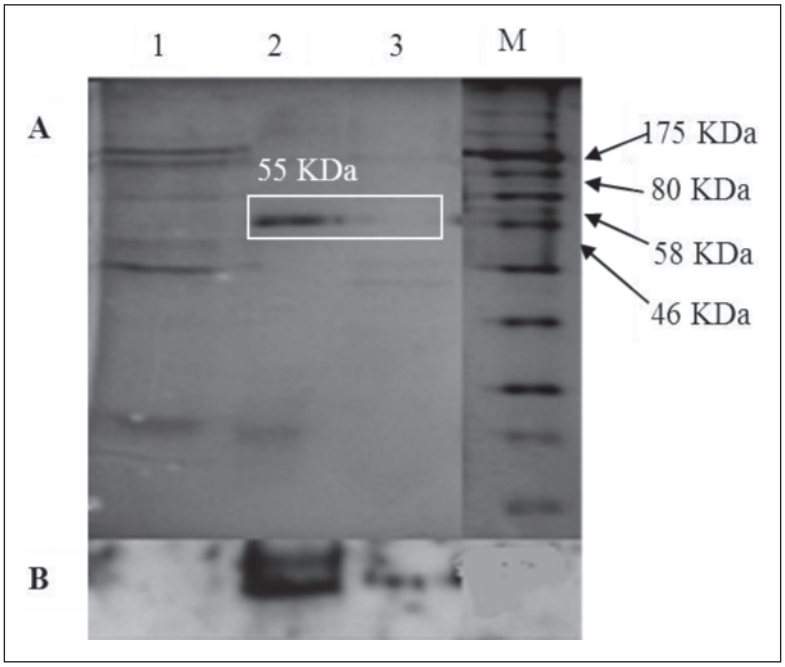

Fig. 1. SDS-PAGE (A) and western blot analysis (B) of the expression of Afp1 in *P. pastoris.* 1: negative control (host *Pichia pastoris*); 2: Afp1 clone induce with methanol; 3: Afp1 clone without induction. M: Prestained protein markers (New England Biolabs, UK).

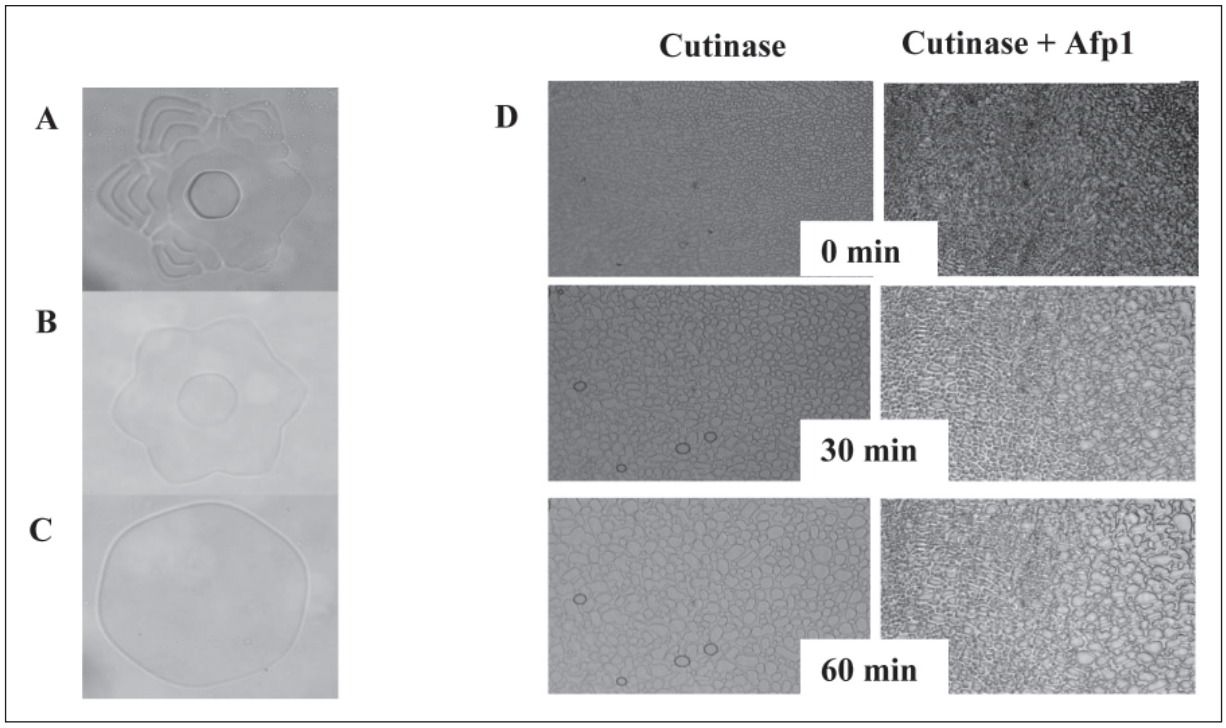

Fig. 2. Assays for ice crystal morphology and recrystallization inhibition of recombinant Afp1. The addition of proteinase K inhibits Afp1 activity, which indicates ice crystal morphology. (**A**) Afp1 before treatment with proteinase K (**B**) Afp1 and Proteinase K before incubation of (**C**) Afp1 after treatment with Proteinase K. (**D**) Recrystallization inhibition assay of cutinase enzyme or cutinase enzyme with Afp1.

compared with recombinant rLeIBP from *Leucosporidium* sp by 0.1°C (Lee *et al.,* 2010), and recombinant Afp1 produced in an *E. coli* system (TH value=0.1°C) (Hashim *et al.,* 2013). We hypothesized that these observations might be a consequence of hyperglycosylation activity on Afp1 that was produced in *P. pastoris*, which could not be performed in a bacterial system. For *Pseudomonas putida* AFP, the effects of its glycans towards AFPs function has been observed when the activity was reduced after glycans were removed from the protein (Xu *et al.,* 1998). To demonstrate that antifreeze activity was associated with ice crystal morphology, a series of experiments were carried out to determine whether protease activity towards Afp1 affected the morphology of the ice crystals. The ice crystals grown in a solution that contained untreated Afp1 exhibited a hexagonal shape compared with Afp1 treated with Proteinase K, which had round shaped ice crystals. The loss of antifreeze activity by protease treatment indicated that the antifreeze activity of Afp1 could disrupt ice crystal morphology.

A RI activity assay was conducted to compare the activity of ice grains in two different conditions: a solution of cutinase enzyme without Afp1 and another solution with a mixture of cutinase and Afp1. Smaller ice grains were observed in solutions that contained Afp1 after 1 h incubation at -6°C compared with solutions that contained cutinase enzyme alone without Afp1 (Figure 2D). This observation established that Afp1 could inhibit ice recrystallization. Ice recrystallization represents the process of forming larger grains of ice upon expansion from smaller ones. Since this process can cause damage to cell membranes, the RI property can increase the survival of cells after freeze-thaw cycles (Raymond & Knight, 2003). These properties appear to be favourable for the cold-adaptation strategies of many polar organisms (Davies *et al.,* 2002; D'Amico *et al.,* 2006). Microbial AFPs are known to have superior activity for recrystallization inhibition compared with other organisms because microbes produce AFPs as part of their freezing tolerance strategy to protect against ice injury threats present at sub-zero temperatures (Lee *et al.,* 2010). Ice formation during preservation or cold storage processes represents a major problem as it causes nutrient loss, cell injury and denaturation (Kim *et al.,* 2015). We assessed the ability of Afp1 to function as a cryoprotective agent by incubating recombinant *G. cingulata* cutinase with Afp1 and subjecting it to several freeze–thaw cycles. Samples were tested for its specific activity to assess whether the activity was retained after a few cycles of freeze-thawing. Cutinase in the presence of Afp1 retained up to 20% of its activity after 2 days of incubation compared with the control sample (no addition of

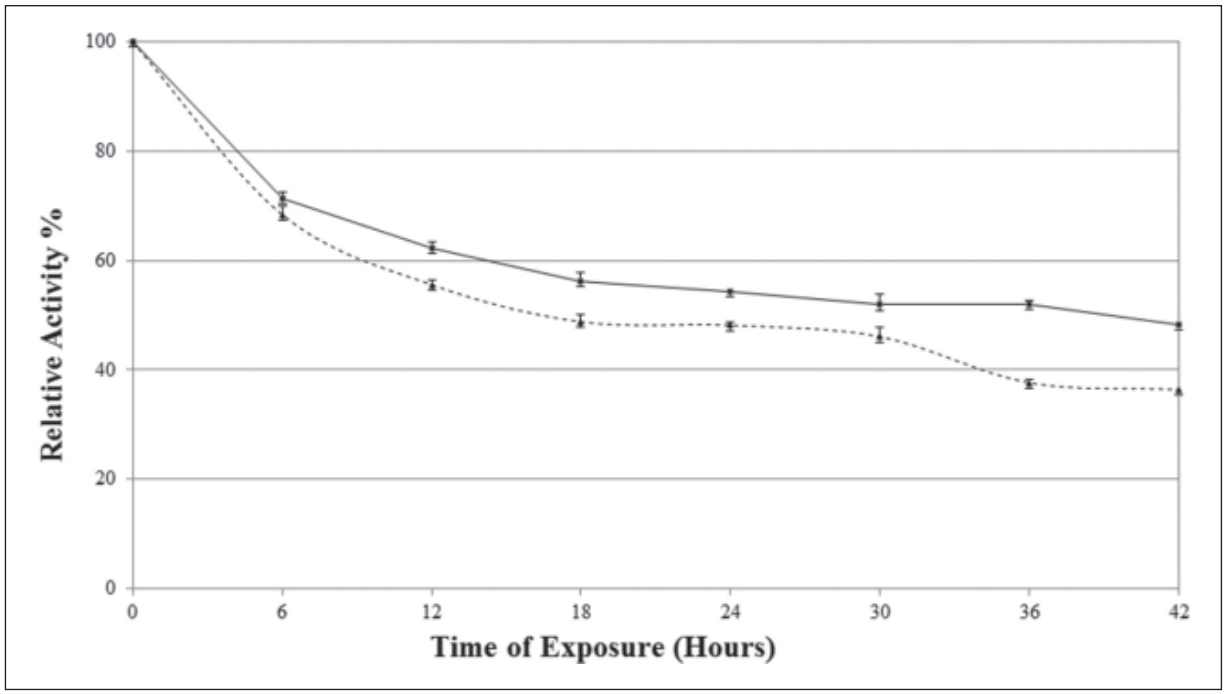

Fig. 3. Cryoprotective effects of Afp1 on cutinase activity after several freeze thaw cycles. Images showed assays with cutinase and Afp1 mixture (*single line*) or cutinase only (*dashed line*).

Afp1) (Figure 3). This revealed that Afp1 could protect cutinase against protein degradation during the freeze-thawing process. These observations also suggest the potential of Afp1 to be applied as a cryoprotective agent in industries such as food and medicine. Currently, ongoing studies are aimed at the production of large-scale recombinant Afp1 (i.e., in a bioreactor) and further assessments of its potential applications as a cryoprotective agent.

CONCLUSION

In this study, recombinant Afp1 was successfully produced via *P. pastoris* expression as glycoproteins with a size of ~57 kDa. Further study showed that recombinant Afp1 had the highest TH value at 0.5°C and could modify the morphology of ice crystals. Recombinant Afp1 lost its activity after treatment with proteinase K. The cryoprotective effects of Afp1 on other enzymes showed that Afp1 could improve enzyme stability after several rounds of freeze thaw cycles.

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