Expression of a Winter Flounder Antifreeze Protein in *E. coli* and its Effect on Ice Nucleation

by

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ABSTRACT

Ice-binding proteins are known to interact with ice to depress freezing point non-colligatively or to inhibit ice recrystallization; however, their effects on ice nucleation are not fully understood. The antifreeze proteins of fish are among the best-studied ice-binding proteins, and AFP6 found in the winter flounder plasma is a small alpha-helical antifreeze protein that depresses the freezing point. In this study, recombinant AFP6 was expressed intracellularly in *E.coli* as fusion protein, then cleaved to produce monomeric active AFP6. A mutant AFP6 without antifreeze activity was produced in the same manner. AFP6 and its fusion protein precursor exhibited similar antifreeze activity, whereas the mutant AFP had no measurable antifreeze activity. AFP6 and its fusion protein precursor were both found to inhibit silver iodide-induced ice nucleation. Conversely, AFP6 was observed to promote uninduced ice nucleation at lower temperatures, while its fusion protein precursor and the AFP6 mutant did not.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AFGP	antifreeze glycoprotein
AFP	antifreeze protein
Da	Dalton
g	gravity
HPLC	high performance liquid chromatography
IBPs	ice binding proteins
INP	ice nucleating protein
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
TH	thermal hysteresis

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CHAPTER 1: INTRODUCTION

The history life on Earth is one of adapting to new and changing conditions. Low-temperature environments are prominent among those to which many species have adapted, and these environments bring the additional risk of freezing. Water is the universal solvent that is involved in all cellular processes, and the freezing of water involves the transition of water from liquid to solid state. In general, ice is incompatible with life, as cellular processes require the mobility of liquid water, and the solidification of water by freezing could be lethal to living cells. All organisms consist of cells, and when cells are subjected to freezing temperature, the formation of intracellular crystals could cause damage (Acker & McGann, 2001). Also, the formation of extracellular ice can cause an increase in extracellular solute concentration, thereby removing water osmotically from the cell and resulting in dehydration; moreover, the formation of extracellular ice can directly damage the cells, tissues, and organs (Mazur, 1984). Many species in cold environments have ways to avoid freezing, and the production of ice-binding proteins (IBPs) is key for the survival of organisms in cold environments with the presence of ice (Białkowska et al., 2020).

1.1 Ice-Binding Proteins

1.1.1 Ice-Binding Proteins and Their Diversity

IBPs are diverse class of proteins that are widespread across biological kingdoms, and they bind to ice surfaces to control ice growth, thereby allowing organisms avoid damage

from uncontrolled freezing (Chasnitsky & Braslavsky, 2019; Dolev, Braslavsky, & Davies, 2016). There are a variety of proteins that function as IBPs in a wide variety of organisms, including bacteria, yeasts, microalgae, diatoms, plants, fish and insects (Dolev, Braslavsky, et al., 2016). The three known forms of IBPs includes ice adhesion proteins, ice-nucleating proteins (INPs), and antifreeze proteins (AFPs) (Cid et al., 2016; Guo et al., 2012).

1.1.2 Biological Roles of Ice-Binding Proteins

The ice-adhesion proteins are the newest member of the known IBPs. They were discovered in an Antarctic bacterium, *Marinomonas primoryensis*, which is a Gram-negative, aerobic, psychrophilic, halophilic, and motile species found in marine coastal ice (Dolev & Braslavsky, 2017; Gilbert, Davies, & Laybourn-Parry, 2005; Romanenko et al., 2003). The 1.5 MDa ice-adhesion protein consists of five different domains, and the 34 kDa fourth domain is the only part that contributes to ice binding. Ice-adhesion proteins serve as adhesins that attach the bacteria to the lower surface of floating ice, where oxygen and nutrients are available (Dolev, Bernheim, et al., 2016). Another related ice-adhesion protein from *Shewanella frigidimarinas*, named *Sf*IBP, was found to be alter ice crystal growth. These two proteins share general structures such as tandem domains as extenders, a C-terminal domain that binds to ice, and a N-terminal domain that attaches the protein to the outer membrane of the cells (Vance, Graham, & Davies, 2018).

Ice-nucleation proteins (INPs) are a family of proteins that promote nucleation of ice

at relatively high temperatures as warm as -5 °C and they are found in several Gram-negative bacteria such as *Erwinia uredovora*, *Pseudomonas syringae*, or *Xanthomonas campestris* that are ubiquitous on the surfaces of frost-sensitive plants (Cid et al., 2016; Gurian-Sherman & Lindow, 1993). INPs are anchored to the outer cell membrane of ice nucleation active bacteria, and they can initiate ice formation that results in frost injury in order to attack plants (Lindow, Arny, & Upper, 1982). Most INPs are hydrophobic, especially in the large repetitive regions that are active in ice nucleation, while the N- and C-terminal domains are responsible for membrane-anchoring and protein folding, respectively (Morris, Georgakopoulos, & Sands, 2004). The highly repetitive domain that comprises the central core of INPs consist of 48-residue repeats that each contain 3 repeats of a 16-amino acid motif (Warren, 2013). The repetitive domain has been proposed to be responsible for aligning water molecules to form a stable seed crystal, which is the rate-limiting step in solution freezing (Warren, 2013). Models of ice nucleation proteins propose that monomers of INPs can aggregate to form a nucleation site, and they can closely complement ice crystal faces by the means of planar arrays formed by hydrogen binding groups (Gurian-Sherman & Lindow, 1993).

AFPs are the most studied and best understood IBPs. These relatively small proteins typically adhere to the ice surface through flat hydrophobic surfaces formed by particular side chain arrangements (Hudait et al., 2019; Wang et al., 2017). The binding of AFPs to the surface of ice forces the addition of new water molecules to form an energetically unfavorable curve in between the bound AFPs, which allows the directional inhibition of

growth and shaping of ice; as a result of the addition of new water molecules being energetically unfavorable, the binding depresses the solution freezing temperature and prevents freezing of the organism (Dolev & Braslavsky, 2017; Salvay et al., 2010). This freezing point depression is non-colligative, resulting in a thermal hysteresis (TH) between the melting and freezing points. The extent of freezing depression, termed antifreeze activity, is measured by the TH. The presence of AFPs in the blood of marine fish depresses the freezing point, which allows fish species that expresses AFPs to inhabit cold seawater below the colligative freezing point of their blood (Kim et al., 2017). Other than freezing point depression, AFPs can also provide freeze-tolerance by inhibiting ice recrystallization, which is a process in which large ice crystals grow at the expense of smaller ones. Ice recrystallization occurs in a partially frozen solution, such as in partially frozen freeze-tolerant organisms, and it is inhibited by AFPs (Tomczak et al., 2003). The formation of intracellular or extracellular ice during freezing causes cell damage, and many freeze-tolerant organisms live in sub-zero environments produce large quantities of AFPs or small-molecule recrystallization inhibitors to protect themselves from the development of large ice crystals (Chantelle, Doshi, & Robert., 2013). For example, Antarctic bacteria are found to possess AFPs that are known to have low antifreeze activity of less than 0.1 °C, and it is widely believed that their AFPs provide freeze-tolerance by ice recrystallization inhibition rather than freeze avoidance by freezing point depression (Chattopadhyay, 2008).

1.1.3. Roles and Distributions of AFPs

The flat and relatively hydrophobic ice binding sites of the AFPs are thought to organize water into an ice-like arrangement that merges and freezes with the quasi-liquid layer just above the ice lattice (Davies, 2014). Although all AFPs have ice binding sites that interact with ice surfaces, they can have different antifreeze activities or structures that serve different roles in a variety of species according to their natural habitats.

Polar microalgae can live and thrive in sea ice by having AFPs that controls the growth of ice crystals. Most AFPs from microalgae are active extracellularly, and it has been suggested that the AFPs are accumulated within the extracellular polysaccharide substances secreted by the microalgae (Bayer-Giraldi, Jin, & Wilson, 2014).

Many insects in freezing regions are known to produce “hyperactive” AFPs, because those insects are often exposed to much lower terrestrial temperatures which are often below -30 °C, and having AFPs with much higher antifreeze activity is necessary for their survival (Graham et al., 1997; Kristiansen et al., 2012; Kuiper et al., 2015).

AFPs has also been found in many overwintering plants. When the temperature drops below 0 °C, ice formation can be lethal to plant cells. The formation of intracellular ice can destabilize the plant cell membrane by rupturing the plasma membrane, and the formation of extracellular ice can result in water loss and protein denaturation (Lin et al., 2005). Some overwintering plants have evolved to counter the cold temperature by expressing AFPs that modify the growth of ice and depress ice recrystallization. The antifreeze activity generated by plant AFPs is generally modest, between 0.2 °C to 0.5 °C,

and it was suggested that the main role of plant AFPs is the inhibition of ice recrystallization rather the prevention of ice formation (Lin et al., 2005).

AFPs found Antarctic fish species are known to cause antifreeze activity of more than 1 °C by interacting directly with ice surfaces, and the activity of fish AFPs is generally between 0.7 °C to 1.5 °C. Other than the inhibition of ice formation, fish AFPs were also suggested to protect the structural integrity of cells and tissues to prevent the leakage of membranes under hypothermic conditions (Venketesh & Dayananda, 2008). However, some fish AFPs were shown to destroy membrane integrity during freezing (Tomczak et al., 2001).

1.1.4 Types of Fish AFPs

There are five structurally distinct types of AFP among polar and north temperate fish species (Davies, 2014). Type I AFPs are amphiphilic proteins with alanine-rich alpha-helix. The helicity is mostly maintained by the high alanine content, and the alpha-helical structure presents a flat ice-binding platform consisting of alanine and threonine, while threonine is responsible for binding to ice through hydrogen bond and hydrophobic interactions to inhibit the growth of ice (Harding, Ward, & Haymet, 1999; Hudait et al., 2019; Sicheri & Yang, 1995). Type I AFPs are found in fish inhabiting north temperate oceans such as winter flounder (*Pseudopleuronectes americanus*), cunner (*Tautogolabrus adspersus*), and sculpins, including *Myoxocephalus scorpius*, at concentrations of 10-15

mg/mL (Fletcher, Hew, & Davies, 2003; Hobbs et al., 2011). The type I AFPs appear to have arisen from convergent and independent evolution, since winter flounder and sculpin diverged long before the Arctic glaciation (Scott, Fletcher, & Davies, 2011). Type II AFPs are found in herring (*Clupea harengus*), smelts such as *Osmerus mordax*, and the sea raven (*Hemitripterus americanus*). They are 130- to 150-residue globular proteins containing five disulfide bonds (Fletcher et al., 2003; Graham et al., 2012; Yamashita et al., 2003). Type II AFPs are homologous to the carbohydrate recognition domain of C-type lectins and their folds are very similar (Ewart, Rubinsky, & Fletcher, 1992; Liu et al., 2007; Logsdon & Doolittle, 1997; Wolfram Gronwald et al., 1998). Type III AFPs are approximately 7 kDa and they are found in Arctic and Antarctic eelpouts. (Crevel, Fedyk, & Spurgeon, 2002). Type III AFPs were found to be homologous to the C-terminal region of mammalian sialic acid synthase through multiple sequence alignments, especially the protein core and the flat ice-binding region in both sequence and structure, which provides evidence that type III AFP evolved from the gene of sialic acid synthase (Baardsnes & Davies, 2001). Type IV AFP is closely related to a helix-bundle serum apolipoprotein. It was discovered in the blood plasma of the longhorn sculpin (*Myoxocephalus octodecemspinosus*) (Deng, Andrews, & Laursen, 1997). Nonetheless, the plasma antifreeze activity of different longhorn sculpin species has been found to be extremely low and the type IV AFP is not expressed in a sufficient quantity in fish to protect them from freezing (Gauthier et al., 2008).

1.2 Winter Flounder Type I AFP

1.2.1 Life History of the Winter Flounder (*P. americanus*)

Winter flounder is the most common flounder species found in waters from Newfoundland to Massachusetts Bay, living in deep water away from the shore during summer when temperatures increase above 15 °C, and then returning to shallow nearshore waters during winter (Pereira, 1999). Winter flounder can grow to reach a length of 64 cm and weight of 3.6 kg, their eyes are located on the right site of their body (Hoornbeek, Sawyer, & Sawyer, 1982). These fish can live up to 15 years, reaching maturity at 6-7 years, and mature winter flounder can lay up to 3.3 million adhesive eggs that hatch within 2-3 weeks depending on the temperature (Roberts, 2018). The larvae transit from planktonic to bottom-oriented lifestyle over time. Meanwhile, their left eye begins to migrate to the right side of the body around 5-6 weeks after hatching, and the migration completes at around 8 weeks when the larvae are 8-9 mm (Pereira, 1999). There are two forms of AFP in winter flounder. They are small proteins with a length shorter than 40 residues, and they are encoded by many gene copies and expressed throughout the fish (Gong et al., 1996). Multiple serum AFP isoforms were isolated, with the major forms initially referred to as HPLC6 and HPLC8 (now AFP6 and AFP8), and several skin AFP isoforms were identified as well (Fourney et al., 2011).

The plasma of winter flounder also contains a hyperactive antifreeze protein that is a dimer of long hair-pinned alpha helices, with an antifreeze activity 10- to 100-fold higher than type I AFP, which makes it comparable in antifreeze activity to insect AFPs (Marshall,

Chakrabartty, & Davies, 2005). At a concentration of 0.1 mg/mL, the hyperactive antifreeze protein can provide thermal hysteresis of 1.1 °C, while type I AFP and other fish AFPs provide less than 0.1 °C of thermal hysteresis at the same concentration. However, the hyperactive AFP is scarce, with about 0.2 mg/mL circulating concentration, which is 50-fold less than the smaller type I AFP. The hyperactive AFP is also extremely unstable; it denatures irreversibly at room temperature and at low pH (Marshall, Fletcher, & Davies, 2004). Different types of AFPs are structurally distinct, but they share the ability of inhibiting ice growth by binding to ice surface; thereby decreasing the freezing temperature (Gong et al., 1996).

1.2.2 Expression of Diverse AFP Forms in the Winter Flounder

Winter flounders produce several AFPs including skin antifreeze protein and liver antifreeze proteins which are found in their skin and serum, respectively (Davies & Hew, 1980; Fletcher et al., 2003; Gong et al., 1996). The presence of AFPs in serum provides winter flounder freezing avoidance to the freezing point of sea water; therefore, winter flounders can survive in sea water with the temperature as low as -1.9 °C. AFPs bind to the ice crystals to inhibit their growth, and the binding also shapes the morphology of the ice crystals in a concentration dependent manner as illustrated in Figure 1 (Rahman et al., 2019).

Serum AFPs are synthesized in the liver as large preproAFPs of 82 amino acids; the

presequences are cotranslationally processed and the prosequence is cleaved off in the blood (Davies, Hew, & Fletcher, 1988). Winter flounder liver AFPs include several isoforms but have two main components being AFP6 and AFP8 (Gong et al., 1996). AFP6 is the major serum isoform of winter flounder, and AFP8 is abundant in winter flounder serum as well. The major amino acid component for AFP6 and AFP8 in primary structure is alanine, they both contain 11-residue ice-binding motifs and have 37 residues, and they are different by two residues (Pickett et al., 1984).

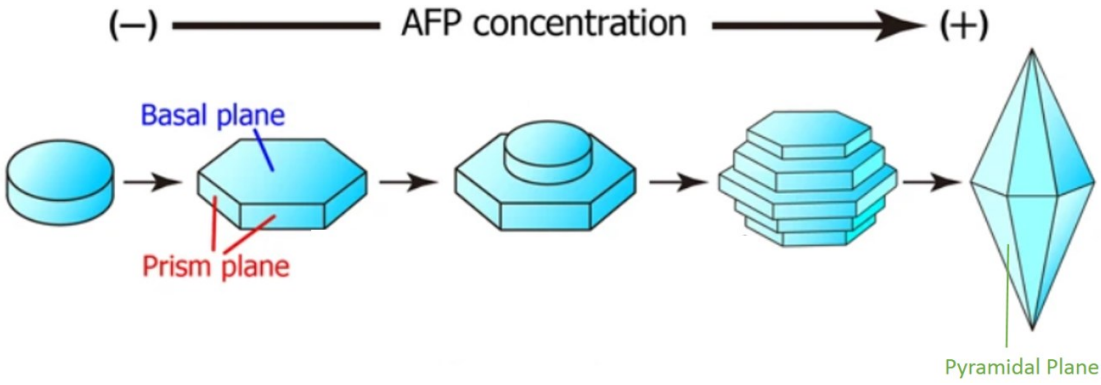


Figure 1. Concentration dependent ice shaping of AFP. The ice crystal forms an ice disk without AFP. The presence of AFP can shape the ice crystal into different morphologies depending on the AFP concentration, and the absorption of enough AFP can eventually shape the ice crystals into the bipyramidal structure. (Open-access image modified from Rahman, 2019)

Skin AFPs are produced as mature polypeptides, the absence of both signal and pro sequences suggests that they function intracellularly, and it was found that the genes of skin AFPs are distinct from the genes encoding liver AFPs (Gong et al., 1996). Skin AFPs are also alanine-rich and contain similar threonine 11-amino acid repeats, but they are less active than serum AFPs. Although the antifreeze activity of the skin AFPs are in the range of general fish AFP activities, they are not as active as liver AFPs because skin AFPs lack several residues that are known to contribute to antifreeze activity in serum AFPs (Fletcher, Kao, & Fourney, 2011; Gong et al., 1996). Although both skin AFPs and liver AFPs form helical repeats that provide flat ice-binding surface, skin AFP only have Thr as ice interacting sites, while liver AFPs also have Asn or Asp other than Thr as ice interacting sites (Sicheri & Yang, 1995; Stevens et al., 2021). The lower antifreeze activity of skin AFP may be due to the single Thr residues composition, because single Thr residues are incomplete motifs that binds to ice weakly (Gong et al., 1996).

1.2.3 Structure and Function of the Plasma AFP6

The canonical type I AFP that has been carefully studied is a major serum isoform currently named AFP6, and previously named HPLC6 and wflAFP-6. (Dubé, Leggiadro, & Ewart, 2016). The protein is monomeric in solution, and the crystal structure showed that the 37 amino acid chain fold into a single amphiphilic alpha helix (Sicheri & Yang, 1995; Yang et al., 1988). AFP6 consists of several amino acids, including Asp, Thr, Ala, Ser, Leu, and Glu, with Ala being predominant (~65%) as shown in Figure 2, the

abundance of this residue is likely to be responsible for stabilization of the helical structure (Cheng, & Mer, 1997; Rohl, Fiori, & Baldwin, 1999). It is known that three alanine residues and an adjacent threonine residue in AFP6 form a surface that promotes ice binding, and threonine residues within the ice-binding motif are located at nearly identical distances throughout the helical structure; by replacing Thr with Ser or Val, it was also found by that the ice growth inhibition of AFP6 is contributed by the hydrophobic interaction between the methyl group of Thr and ice surface (Baardsnes et al., 1999; Cheng et al., 1997; Davies et al., 2002).

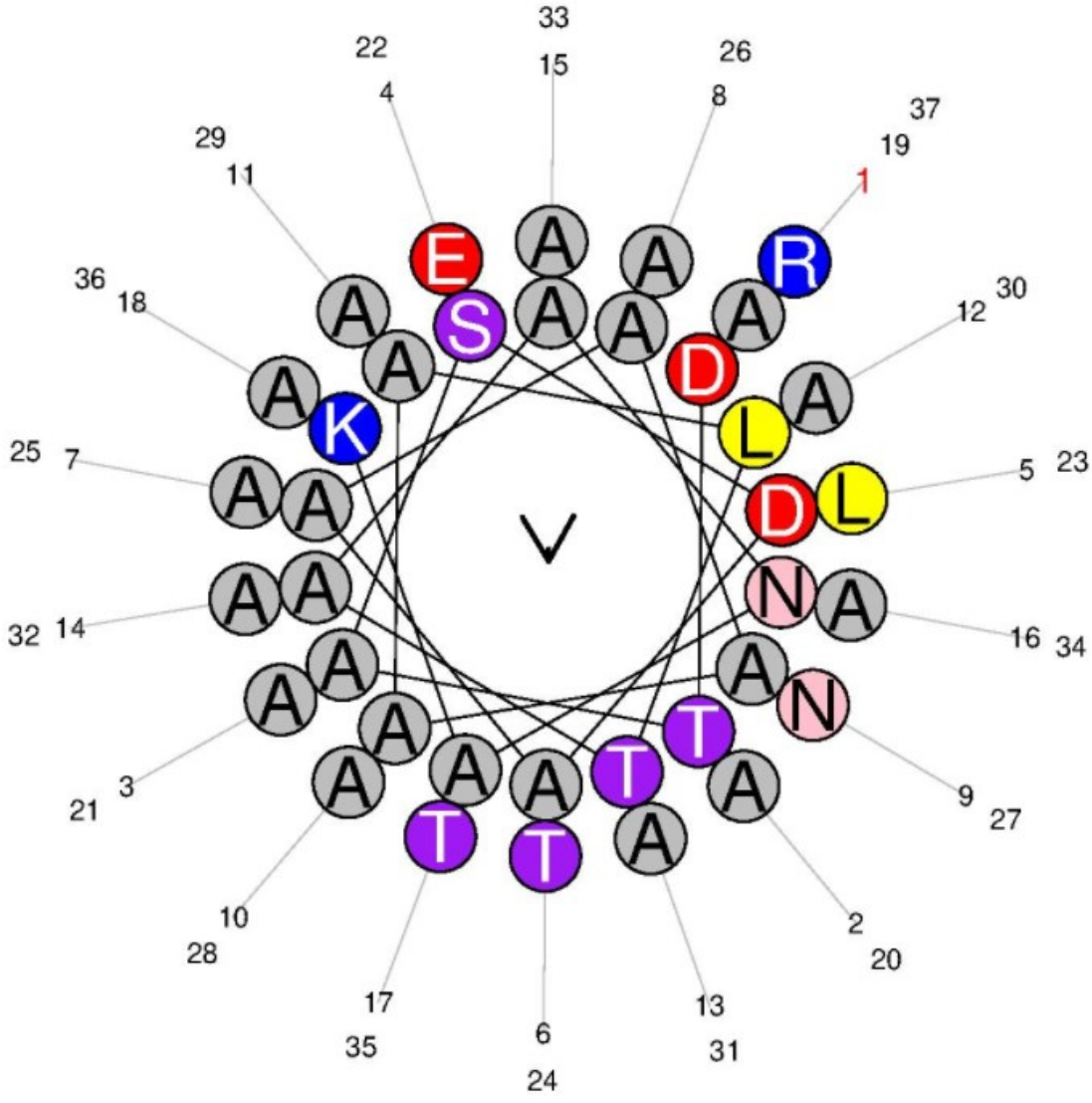


Figure 2. Helical wheel of the winter flounder AFP6. The arrowhead in the middle points toward the ice-binding face of the alpha helix. The image was generated using HeliQuest. (Gautier et al., 2008).

AFP6 prevents the growth of ice through an absorption-inhibition mechanism by adsorbing on the pyramidal plane of ice crystals to make the addition of water molecules to ice restricted to the exposed surface between protein molecules (Knight et al., 1991;

Raymond & DeVries, 1977). Therefore, the ice crystals are forced to grow in less thermodynamically favored curved surface to lower the freezing point (Knight et al., 1991; Raymond & DeVries, 1977). The antifreeze activity of AFP6 is also correlated with the stability of the helix structure. It was found that the introduction of two additional salt bridges that enhance the helix content also enhances the antifreeze activity of the AFP, and the AFP was able to be active at seven-to eight-fold lower concentration (Chakrabartty & Hew, 1991). The helix content of AFP is negatively correlated with the energy required for confrontational transition when AFP binds to ice; therefore, the higher the helix content, the higher the antifreeze activity. Most structural studies of AFP6 involve the use of chemically synthesized proteins, because full activity requires a native N-terminal aspartate and an amidated C-terminus (Patel & Graether, 2010). Amidation is a terminal peptide modification from a carboxyl group to amide group by cleaving a terminal glycine, and amidated peptides have longer half-life and greater resistance to proteolytic degradation (Kumar, Eipper, & Mains, 2014). It has been reported that the antifreeze activity of a non-amidated AFP6 is approximately 35% lower compared to amidated AFP6, and the higher TH activity was caused by higher rigidity provided by the amidated C-terminus (Kumar et al., 2014). It was suggested by the same study that the increase of AFP flexibility prevents AFP from binding strongly to the ice surface and causes the AFP to have decreased activity.

1.3 Production of Single Isoform of AFP6

1.3.1 Traditional Sources of AFP6

AFP6 is one of the two major AFPs in the blood plasma of winter flounder and it is the most intensely studied form. AFP6 is also the best-understood IBP, but the detailed mechanisms of its antifreeze activity still remain unclear (Tong et al., 2000). There are still several aspects of small alpha-helical AFP structure and function that remain to be studied, namely, more specific understanding of the features that allow interaction between AFP6 and ice surface (Davies et al., 2002). Further study of AFP6 is also of interest because it has been found to form amyloid upon freezing and thawing (Graether, Slupsky, & Sykes, 2003). This appears to result from direct AFP-ice interaction (Dubé et al., 2016) and yet the mechanism by which this transition takes place is unknown. Thus, there are many aspects of AFP6 that require further investigation, and this will require substantial amounts of pure AFP6. Given the myriad isoforms of AFP in the plasma of flounder, it is difficult to separate a particular isoform of AFP from the mixture, and purification of individual isoforms in quantities sufficient for study is impractical (Fourney, Fletcher, & Hew, 1984). Therefore, given the short 37-residue length of AFP6, most studies have employed peptide synthesis to generate study material (Baardsnes et al., 1999; Dubé et al., 2016; Graether et al., 2003). Another advantage of synthetic AFP6 is that it can be C-terminal amidated, as the native AFP6 is. However, commercially synthesized AFP6 is often low yield and refractory to solubilization, and the high cost makes the synthesis of large amount of AFP6 prohibitive (Sands, 2020). The same study also reported inconsistency in synthetic AFP6

solubility from different manufacturers, as well as limited quality and quantity. Therefore, although peptide synthesis is widely available, synthetic AFP6 is not ideal for many studies due to the variation in products from different manufacturers.

1.3.2 Approaches for the Heterologous Expression of AFP6

The alternative approach of obtaining AFP6 is by expression from a single sequence in a heterologous host, and this has been achieved in yeast and bacteria (Liyanage, Jayawardana, & Kodithuwakku, 2013). Yeast species are effective hosts of the production of functional recombinant proteins due to their fast growth, high protein yield, and proper posttranslational modifications (Baghban et al., 2019). A study reported the expression and secretion of AFP6 in yeast as tandem repeats, which were linked by short sequences that allow the peptide to be processed by protease Xa to yield AFP6 monomers (Driedonks et al., 1995). The expression level of the multimers approached 100 mg/L; however, the proteolytic processing was incomplete, generating a heterogeneous mixture of AFP6 monomers and longer AFP6 tandem repeats (Driedonks et al., 1995). Therefore, a substantial separation process was required to yield pure AFP6 (Driedonks et al., 1995). Other than yeast AFP6 expression, AFP6 has also been expressed in bacteria in different studies (Solomon & Appels, 1999; Tong et al., 2000). *E. coli* is one of the most widely used host for expression of heterogeneous proteins, and its genetics are well studied and better characterized than other microorganisms (Fakruddin et al., 2013). *E. coli* is a

preferred host for its relative simplicity, inexpensive, and fast high-density cultivation. Although *E. coli* has many advantages in terms of expressing heterogeneous proteins, expression of proteins is not always successful due to many factors such as codon bias, protein degradation, strength of promoter, stability of vector, protein solubility, and protein toxicity (Rosano & Ceccarelli, 2014). AFP6 is a small protein with the size of 3.3 kDa. The small size and the lack of globular tertiary structure of AFP6 may render this protein more prone to degradation by proteases in bacterial cells (Mueller et al., 1991). Large-scale production of small type I AFPs has been hampered by their instability when expressed in heterologous systems, and the common approaches for expressing small AFPs include expressing AFPs as fusion proteins, multimers, or secretory proteins. To overcome the difficulties associated with the small molecular size of AFP6, it was expressed in *E. coli* as a multimer and designed to be secreted into the medium to avoid degradation (Tong et al., 2000). The gene of AFP6 can be engineered to have more ice-binding motifs which results in a longer version of AFP6 that has decreased solubility compare to shorter natural AFP6, so it can be accumulated in inclusion bodies of *E. coli* that is resistant to protease degradation (Solomon & Appels, 1999). Small type I skin AFPs have also been expressed in *E. coli* as a secretory protein to avoid degradation (Lin et al., 1999).

1.3.3 Fusion Proteins for the Expression of Type I AFPs in *E. coli*

Expressing AFP6 as a fusion protein could be used to overcome the drawbacks of its small molecular size. In the study by Mueller et al. (1991, above), the AFP-encoding sequence was fused in frame with the C-terminus of staphylococcal protein A (Mueller et al., 1991). Fusion proteins are proteins created through the joining of two or more ORFs of separate proteins, and the expression of the fusion gene can generate polypeptides with functional properties from each original protein (Riggs, 2001). Gene fusion has been widely used for improving protein expression, solubility, or purification in *E. coli*, it can also be used to simplify protein refolding and increase its efficiency, and to prevent proteolysis (Costa et al., 2014; Kosobokova, Skrypnik, & Kosorukov, 2016). An ideal fusion tag for AFP6 expression and purification should provide AFP6 degradation resistance while leaving the properties of AFP6 intact, and the expressed fusion protein should be easily retrievable from cell lysates, which can be accomplished by polyhistidine tag. The polyhistidine tag consists of six consecutive histidines, which form a Ni^{2+} ion binding site. The imidazole side chain of histidine has a specific binding affinity towards nickel metal ion; therefore, proteins with polyhistidine tags can be affinity-purified using nickel (Spriestersbach et al., 2015). The DNA sequence encoding the polyhistidine can be added at either end of the DNA sequence that encodes the target protein, so the target protein can include polyhistidine at its N or C terminus.

Small ubiquitin-like modifier (SUMO) proteins belong to the ubiquitin-like protein family. They can be covalently attached to and removed from other proteins to modify

their function in cells, and SUMOylation is a reversible post-translational modification that is involved many cellular processes (Hay, 2005; Peroutka III et al., 2011). It has been reported that the attachment of SUMO to the N-terminus of under-expressed proteins dramatically enhances the protein expression in *E. coli*, and it was also reported that the addition of a SUMO tag decreases proteolytic degradation (Butt et al., 2005; Cartier et al., 2019; Malakhov et al., 2004; Marblestone et al., 2006; Wang et al., 2011; Zuo et al., 2005). A SUMO fusion system joins the gene of SUMO to the genes of other proteins to express a fusion protein with SUMO on its N-terminal end, and SUMO on the expressed protein can be cleaved by SUMO protease through their endopeptidase activity (Panavas, Sanders, & Butt, 2009).

A fusion protein consists of polyhistidine tag (His), SUMO tag (SUMO), and AFP6 from N- to C- terminus could be ideal for the expression and purification of AFP6, since the His-SUMO-target protein construct was reported to have successful expression in *E. coli* intracellularly (Xu et al., 2012). When the fusion proteins were expressed, the polyhistidine-tag can be used to collect the fusion proteins. The SUMO tag can be cleaved from the fusion protein with the addition of SUMO protease to generate AFP6, and the polyhistidine-SUMO tags that are cleaved off can be collected by immobilization on a Ni^{2+} column.

1.4 The Modulation of Ice Nucleation by Antifreeze Proteins

1.4.1 Effects of Ice on Cells and Tissues

The modulation of ice nucleation has an impact on cryopreservation applications such as the cryopreservation of food, cells, or organs. The fact that cellular metabolism in living cells dramatically diminishes at low temperature allows for the long-term preservation of living cells and tissues, but nucleation of ice is the most significant uncontrolled variable in cryopreservation that leads to variation in cell recovery and viability (Gao & Critser, 2000; John Morris & Acton, 2013). In order to reduce ice nucleation variation in cryopreservation, a number of methods are used to introduce controlled ice nucleation, including seeding by small ice crystals, electrofreezing, mechanical shaking, introducing cold spot, or using chemical nucleators (Petersen et al., 2006). In addition, modulation of nucleation also helps with cell survival during cryopreservation, because the challenge to cell cryopreservation is not long-term storage at ultra-low temperature, but the -15 to -60°C temperature zone the cells need to travel through. Within this temperature range, ice forms in the external medium, while intracellular content remains supercooled, which can cause the supercooled water in the cell flows out of the cells osmotically (Mazur, 1984; Prickett et al., 2015). If the cooling is too rapid, insufficient cell water is removed to eliminate supercooling, which causes cells to freeze intracellularly, and intracellular ice formation has been linked to death of cells (Mazur, 1984; Prickett et al., 2015). However, if cells are cooled too slowly, the cells would experience volume shrinkage and expose to high solute concentration caused by water loss, and both could lead to cell damage (Mazur,

1984; Prickett et al., 2015). High subzero nucleation results in larger extracellular ice crystals and cell dehydration, while low subzero nucleation temperatures resulted in smaller ice crystals and intracellular ice formation; therefore, with the modulation of ice nucleation during cryopreservation, intracellular ice formation could be efficiently reduced to help with the survival of cells (Lauterboeck et al., 2015). When it comes to cells types such as embryos or oocytes that are sensitive to freezing injury, the control of ice nucleation is more important with these cells types or multicellular structures containing those cells (Diener et al., 1993). Also, multiple studies discovered that inducing extracellular ice formation at relatively warm supercooled temperatures is beneficial to the post-thaw viability of the cells as well (Lauterboeck et al., 2015; Prickett et al., 2015; Wolkers et al., 2007).

1.4.2 Ice Nucleation

Ice formation is one of the most common processes on earth, and nucleation is the initial event required for the formation of ice from liquid water. In liquid water, molecules move freely, and they are constantly mobile in a temperature-dependent fashion. In contrast, while in ice, water molecules are aligned to face the same direction in a rigid lattice. The mobility of water molecules diminishes as temperature decreases, but the spontaneous arrangement of water molecules into the correct pattern for ice formation below 0 °C is still improbable (Sosso et al., 2016). Lowering the temperature slows down

the movement of water molecules, but the slow-down is not sufficient for pure water to readily form ice until the temperatures drops to -40 °C, because water molecules do not tend to associate into a stable crystalline pattern that would constitute an ice nucleus (Fitzner et al., 2019; Zhang & Liu, 2018). In other words, ice nucleation is the initial step of freezing, and pure water cooled below freezing point would normally remain supercooled until a far lower temperature is reached. Nucleation requires small groups of molecules to arrange into order ice-like clusters, and this cluster acts as the nucleus to form ice crystals (Mathews et al., 1972; Sosso et al., 2016). The size of the nucleus is important for initiating freezing; a small nucleus is likely to be dissolved, while a large nucleus can cause more water molecules to join the arrangement to form ice crystals (Mathews et al., 1972; Sosso et al., 2016).

Ice nucleation can occur in two ways: homogeneous ice nucleation and heterogeneous ice nucleation. Homogeneous ice nucleation can occur in pure water, as it is the formation of stable ice seeds by the association of water molecules. Homogenous nucleation does not require any exogenous ice template but it requires a very low temperature, because ice nucleation is a stochastic probability-defined event, and the probability of homogeneous ice nucleation is associated with the lowering of the temperature (Lu et al., 2020). Almost all ice nucleation occurs as a result of heterogenous nucleation, which is the formation of ice-like templates from non-water molecules and subsequent growth of ice from water on these templates. Although the initiation of homogenous nucleation is improbable until the temperatures falls to around -40°C, heterogeneous nucleation can occur at a higher sub-

zero temperature due to the templating effect of non-water nucleation agents (Sosso et al., 2016). Many small molecules have been shown to be effective ice nucleation agents, including for example graphite, silver iodide, kaolinite and dust components (Metya & Singh, 2018; Perkins et al., 2019; Zielke, Bertram, & Patey, 2015). Silver iodide triggers ice nucleation, and this is thought to occur because the crystal lattice geometry of AgI corresponds to that of ice (Vonnegut, 2004). AgI is therefore used widely in cloud seeding; it can trigger ice nucleation in water at temperatures as high as -3 °C.

1.4.3 Ice Nucleation and Ice-Nucleating Proteins

Non-water exogenous ice nucleating particles act as ice nuclei to trigger nucleation due to their specific ice-like surface properties, which reduce the energy barrier for freezing (Ling et al., 2018). There are a large variety of molecules that can trigger nucleation, biomolecules such as proteins can also catalyze or depress the heterogeneous ice nucleation by providing an site that stabilizes the formation of ice nucleus or leads to disordered structure (Eickhoff et al., 2019; Liu et al., 2016). It is well known that small antifreeze proteins adsorb to the surface of ice crystals to inhibit their growth, while larger ice-nucleating proteins (INPs) can trigger the formation of new ice crystals at temperatures much higher than the homogeneous ice nucleation temperature (Eickhoff et al., 2019). INPs appear to work by acting as a template to assemble the water molecules to act as an ice nucleus, while the smaller antifreeze proteins accumulate on the ice surface to inhibit

the growth of the ice crystals (Kobashigawa et al., 2005).

Ice-nucleation active bacteria can promote the growth of ice at temperatures just below the freezing point to induce frost damage to plants, and this feature comes from their specialized INPs, which are known to induce nucleation at temperatures close to 0 °C (Lindow et al., 1982; Morris et al., 2004). Although the mechanisms of protein-induced ice nucleation are still unclear, there are studies suggesting that INPs can effectively order water at the surface of the bacteria (Kassmannhuber et al., 2020; Pandey et al., 2016). It was suggested that ice-binding in the repetitive domains of the INPs might be responsible for aligning water molecules in the seed crystal, which leads to the sudden crystallization of the surrounding liquid (Warren, 2013). The ice nucleating ability of INPs can be affected by several factors. Experimentation with truncated versions INPs revealed that the central repetitive region adopts a beta-helical structure that reorients at low temperature to order the water molecules and promote ice nucleation by increasing contact with water molecules (Roeters et al., 2021). Other studies observed that aggregates of INPs have increased ice nucleating activity, suggesting the ice nucleation activity of INPs could also be size-related (Burke & Lindow, 1990; Govindarajan & Lindow, 1988). The connection of INP size and ice nucleation activity was also supported by another study that reported truncated versions of a bacterial INP have decreased in-nucleating ability compare to the original INP (Ling et al., 2018).

1.4.4 Antifreeze Proteins and Ice Nucleation

It is known that the presence of AFP can inhibit the growth of macroscopic ice crystals, but the effect of AFP on ice nucleation is not as well studied as its ice growth inhibition activity, and conflicting results regarding the effect of AFP on ice nucleation has been reported. A study reported that the presence of antifreeze glycopeptides from the Antarctic cod lowered the heterogeneous nucleation temperature (Parody-Morreale et al., 1988), while another study also reported the presence of antifreeze glycopeptides from the Antarctic notothenioid inhibited the ice-nucleating activity of the bacterium *Erwinia herbicola* and it was hypothesized that AFPs inhibit heterogeneous ice nucleation by binding to mask the ice nucleation sites (Parody-Morreale et al., 1988; Wilson & Leader, 1995). A study regarding the effect of AFP on homogenous ice nucleation found that antifreeze glycoproteins isolated from some species of polar fish and overwintering insects were not able to depress homogenous ice nucleation any more than polyvinyl pyrrolidone, which is a polymer without thermal hysteresis activity (Franks et al., 1987). It was also reported that removing carbohydrates from an antifreeze glycoprotein did not noticeably alter the antifreeze activity, but caused diminished ice-nucleation activity, suggesting that antifreeze activity could be unrelated to the ice-nucleation activity of AFPs (Xu et al., 1998). Overall, the results of the studies described above suggest that the AFPs can inhibit heterogeneous ice nucleation, but the AFPs has little to no effect when it comes to homogenous ice nucleation.

A more recent study suggested that different sites of AFP can have distinct effects on

ice nucleation. When the surface of substrates are coated by the active site of AFP, the ice nucleation activity was enhanced compared to substrates that are coated with the inactive site of the AFP, suggesting that it is the active site of the AFP that is responsible for ice nucleation activity (Charpentier et al., 2013; Liu et al., 2016). Another study researching a portion of an INP also reported that the fragment of INP exhibited ice binding properties to shape an ice crystal, a concentration-dependent ice growth inhibition activity was also detected from this INP fragments (Kobashigawa et al., 2005). Furthermore, partially purified antifreeze protein isolated from the Antarctic notothenioid enhanced ice nucleation rather than inhibiting it at above 8 mg/mL concentration, suggesting that AFP with high enough concentration could act similarly to an INP (Wilson et al., 2010). The results from the studies described above provide supporting evidence that although AFPs and INPs have distinct biological roles, the mechanism behind their activities could be connected.

1.5 Research Objectives

1.5.1 Evaluation of the Effects of AFP6 on Ice Nucleation

Although the freezing point depression resulting from AFP6 binding to ice has been well studied, the effect of this protein on ice nucleation remains unclear. Therefore, the objective of this study was to express AFP6 and related proteins in *E. coli* and purify them to study the factors of AFP6 that are important in modulating ice nucleation. AFP6 was expressed a series of fusion- and sequence-modified variants and ice nucleation effects

were examined. This study aimed to investigate the important factors of AFP6 in ice nucleation by studying the difference of ice nucleation activity of AFP6, AFP6 mutated to have no antifreeze activity, AFP6 fusion protein (His-SUMO-AFP), and the fusion tag control (His-SUMO). The results may contribute to the application of AFP in ice nucleation modulation, as well as providing structural and mechanistic insight into the modulation of ice nucleation by AFPs.

CHAPTER 2: EXPRESSION AND CHARACTERIZATION OF AFP6 AND EXPERIMENTAL VARIANTS

2.1 Introduction

Winter flounder AFP6 is known to decrease the freezing point of water by absorption inhibition to ice crystals, but its effect on ice nucleation is not well studied, because many studies were conducted using semi-pure preparations of AFP (Schwidetzky et al., 2020; Wilson et al., 2010). In order to study the effect of AFP on ice nucleation, it is essential to obtain a purified form of AFP and AFP related proteins. It is impractical to obtain a large amount of pure AFP6 directly from the serum of winter flounder due to the presence of myriad isoforms, and synthetic AFP6 made in different companies are reported to behave inconsistently; therefore, expressing AFP6 in a heterologous system would appear to be a better approach (Fourney et al., 1984; Sands, 2020).

Although there are a wide range of expression systems and host species to be considered for AFP6 expression, *E. coli* was considered to be an ideal expression host in this project because it is relatively straightforward and inexpensive. AFP6 is a small protein and therefore expression as a fusion protein allowing clean N-terminal cleavage was sought in order to avoid interference in the native protein. In addition, a series of controls and variants, including fusion proteins and mutants, were required. Therefore, the AFP6 was expressed as a fusion with an N-terminal polyhistidine tag and a small ubiquitin-like modifier (SUMO) protein, which allowed a more complete analysis of the determinants of ice nucleation modulation.

2.2 Materials and Methods

2.2.1 Design of Expression Constructs for the Expression of AFP6 and Related Proteins

A sequence encoding SUMO and the AFP6 was designed and codon-optimized for expression in *E. coli* (Figure 3A). Synthetic double-stranded DNA with this sequence was manufactured (Bio-Basic) and cloned into the BamH1 site of the pET-15b plasmid vector by Bio-Basic (Figure 3B). A further expression construct of was designed based upon the above, but with a set of four mutations (A17L, T2S, T24S, T35S) chosen to eliminate thermal hysteresis activity while preserving the pI and molecular mass of the protein. The protein sequences of AFP6 and the mutant, designated MutAFP are aligned in Figure 4, as well as their helical net representations. The triple T to S mutant replaces the crucial amino acids responsible for ice binding, and the mutations inactivate its thermal hysteresis activity. The A17L mutation also inactivates the thermal hysteresis activity by hindering the interaction of ice binding side to ice crystals (Zhang & Laursen, 1998). These mutations were combined to produce the MutAFP, which has the same pI and MW as the wild type AFP (Baardsnes et al., 1999). A further control construct consisting only of the polyhis-SUMO, was also expressed. The variety of expression constructs and products are shown schematically in Figure 5. For clarity, shortened notation was adopted for each of the proteins and fusions: recombinant AFP6 (rAFP6) is referred to as AFP, mutated rAFP6 is denoted MutAFP and the polyhistidine tag is shortened to His.

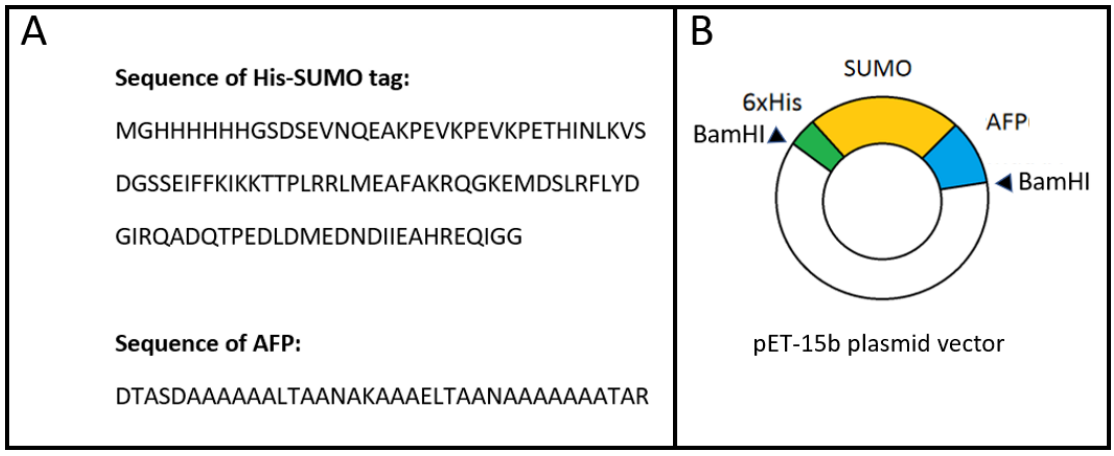


Figure 3. Sequence and plasmid construct of His-SUMO-AFP. (A) Protein sequence of His-SUMO and AFP6. The sequences were codon-optimized for expression in *E. coli*. (B) Schematic diagram of the plasmid construct for His-SUMO-AFP constructed by BioBasic.

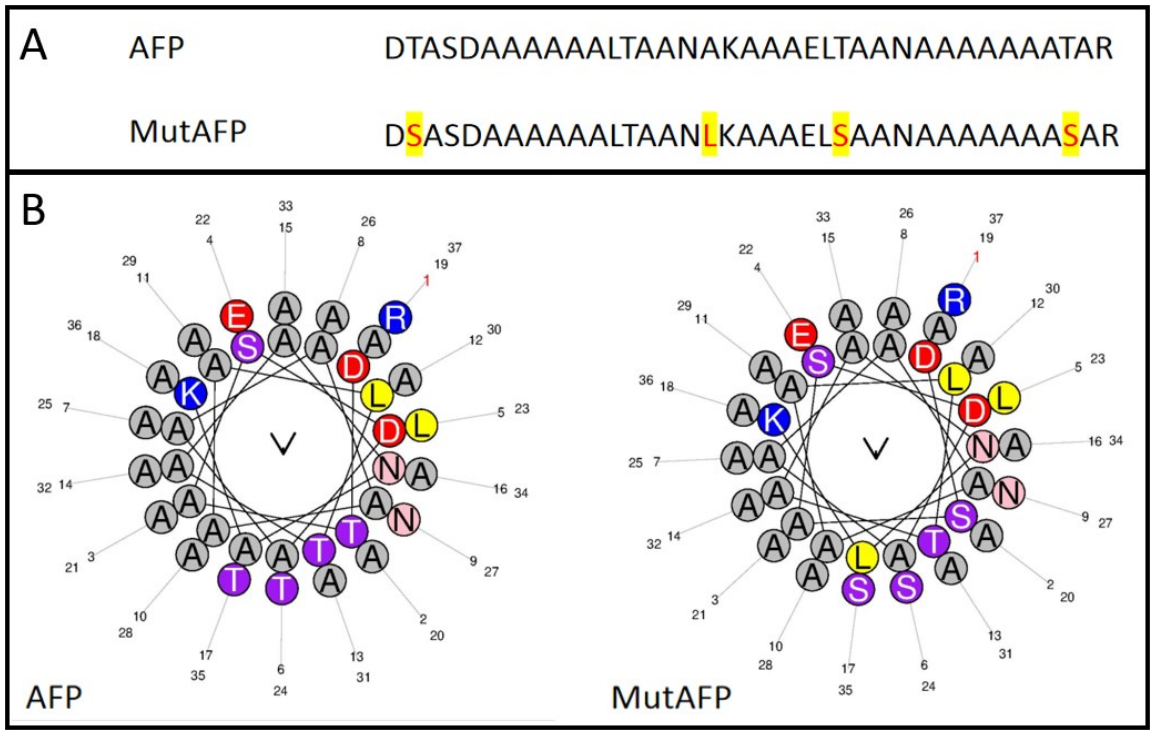


Figure 4. The aligned sequences and helical wheel of AFP6 and MutAFP. (A) Alignment of the sequences of AFP6 and MutAFP Sequences are shown in single letter code with substituted residues in MutAFP shown in red letters with yellow highlight. (B) Helical wheel of AFP6 and MutAFP. The arrowhead in the middle points toward the ice-binding face of the alpha helix. The image was generated using HeliQuest (Gautier et al., 2008).

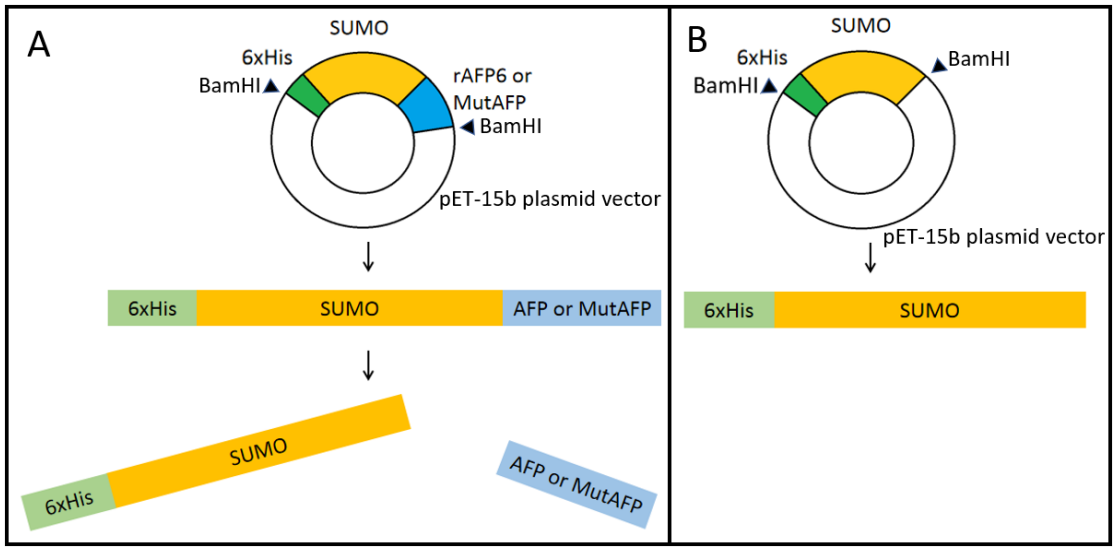


Figure 5. Schematic representation of the expression of His-SUMO, His-SUMO-MutAFP, and His-SUMO-AFP. (A) The plasmid constructs for His-SUMO-AFP or His-SUMO-MutAFP constructed by BioBasic. Expression of the encoded sequence in *E. coli* resulted in the production of His-SUMO-AFP or His-SUMO-MutAFP fusion protein. The N-terminal His-tag was used to collect protein, while the mid SUMO portion can be cleaved to yield AFP or MutAFP. Upon the addition of SUMO protease that cleaves off the His-Sumo tag, AFP or MutAFP can be released. (B) The plasmid constructs for His-SUMO made by BioBasic. Expression of this plasmid results in the production of His-SUMO. This protein does not carry any AFP or related protein on the C-terminus, and this protein was used as a control.

2.2.2 Expression of AFP6 and Related Proteins

The expression plasmids were transformed into *E. coli* BL21 using standard heat shock protocols. Cells were grown at 37 °C in LB medium containing ampicillin (50 μg/mL) for 14 h, then transfered to large volume of LB medium containing ampicillin (50 μg/mL) with a 1:100 ratio (v/v). The cells were grown to mid-log phase (OD600 of 0.8 to 1.2) and then induced by 0.8 mM IPTG at 37°C for 3 h for the expression of the His-SUMO-AFP fusion protein.

2.2.3 Purification of His-SUMO and His-SUMO-AFP

Cells were harvested at centrifugation 9000*g* for 5 minutes at 4°C, then resuspended in lysis buffer (50 mM $NaHPO_4$, 300 mM NaCl, pH 7.5). The resuspended cells were lysed using a French Pressure Cell Press (American Instrument Company), then subjected to centrifugation at 20,000*g* for 60 minutes at 4°C to remove insoluble material. The supernatant was collected and loaded onto a non-denaturing column packed with Ni-NTA Sepharose (Qiagen). The column was washed using lysis buffer (50 mM $NaHPO_4$, 300 mM NaCl, pH 7.5) before the bound fusion proteins were eluted in an elution buffer (300 mM imidazole, 50 mM $NaHPO_4$, 300 mM NaCl, pH 7.5).

2.2.4 Purification of AFP and MutAFP

The purification of His-SUMO-AFP and His-SUMO-MutAFP was conducted according to the protocol described above. The purified fusion proteins of His-SUMO-AFP and His-SUMO-MutAFP were treated with His-tagged SUMO protease (expressed intracellularly in *E. coli* and purified according to the protocol described above in 2.2.3) to remove the His-SUMO tag. The SUMO protease was mixed with the fusion protein in a 1:500 ratio (mole/mole) and dialyzed against water at 4°C overnight to remove imidazole from the reaction solution. The solution was passed through a column of Ni-NTA Sepharose (Qiagen), allowing the tag-free recombinant AFP to flowed through the column and was collected as purified protein, while other proteins (His-SUMO tag, SUMO protease, and uncleaved protein) with His-tag remaining bound to the column. The purified AFP was dialyzed against deionized water for 5 cycles with 4 hours per cycle, then subjected to lyophilization. The purified AFP or MutAFP were dissolved in 10 mM ammonium bicarbonate (Thermo Fisher Scientific), pH 8.3, before use. This buffer reaches a pH of 8.3 without adjustment.

2.2.5 SDS-PAGE of AFP6 and Related Proteins

Standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were made by mixing acrylamide (10%, Thermo Fisher Scientific) with ammonium persulfate and tetramethylethylenediamine (Thermo Fisher Scientific) with a 50:5:1 ratio.

The stacking gel was made by mixing 6% acrylamide with ammonium persulfate and tetramethylethylenediamine by a 1000:10:1 ratio. The gels were resolved under constant voltage at 200 V until the dye front reached the end of the gel. Following SDS-PAGE and staining by Coomassie Brilliant Blue R-250 dissolved in 45% methanol, 45% H_2O, and 10% acetic acid (2.5g/L), the gel was destained by 30% methanol, 60% H_2O, and 10% acetic acid.

For the visualization of antifreeze proteins, Pre-cast RunBlue^{TM} TEO-Tricine SDS-PAGE gels with an acrylamide gradient of 4-20% were used with manufacturer-formulated RunBlue^{TM} SDS running buffer and RunBlue^{TM} LDS sample buffer. The gels were run according to the manufacturer's instructions. The gels were resolved under constant voltage at 100 V until the dye front reached the end of the gel. The gel was then stained by curcumin (Alfa Aesar) dissolved in deionized water (5mg/mL).

2.2.6 Mass Spectrometry of AFP and MutAFP

Lyophilized AFP and MutAFP were dissolved in 10 mM ammonium bicarbonate with the concentration of 5 μg/μl, protein concentration was measured using a small-volume spectrophotometer (DeNovix), and protein samples were sent to the Mass Spectrometry Laboratory (Department of Chemistry, Dalhousie University) for electrospray ionization (ESI) mass spectrometry analysis (Bruker Daltonics Compact QTOF system).

2.2.7 Circular Dichroism Spectroscopy

The secondary structures of AFP6 and MutAFP were examined by circular dichroism (CD), and the measurements were made using an OLIS DSM20 spectropolarimeter using a 0.2 cm cuvette. Solutions of 12.5 μM AFP6 and MutAFP were prepared in 10 mM ammonium bicarbonate. All samples and the buffer control were read at 4 ºC with 40 increments between 200-250 nm. Each spectrum was collected three times and data were averaged by the system software. A buffer blank was analyzed in the same manner and subtracted from each protein spectrum. Then, unit conversion was conducted based on the known path length and protein concentrations used.

2.2.8 Thermal Hysteresis Measurement and Ice Crystal Morphology

Solutions of 1.5 mM AFP6, MutAFP, His-SUMO, and His-SUMO-AFP6 were made in 10 mM ammonium bicarbonate. Each solution was then diluted to appropriate concentrations before subjecting to thermal hysteresis measurements using a Clifton Nanolitre Osmometer and a compound microscope (Olympus). Thermal hysteresis was determined by observing the increase in size of a single ice crystal (freezing point) and the decrease in size of the same crystal (melting point). Hysteresis values were calculated as the means of triplicate measurements on separate crystals in separate sample wells. The temperature interval between these points was the thermal hysteresis. Buffer blank measurements were performed in the same manner and the resulting background hysteresis

was subtracted from each above measurement. To evaluate ice morphology, images of representative ice crystals formed in the presence of each protein were recorded using a microscope camera (AmScope). Measurements and imaging were carried out by Dr. Kathryn Vanya Ewart.

2.3 Results

2.3.1 Expression and Purification of Fusion Proteins and Derived AFPs

The His-SUMO control protein was expressed in *E. coli* and purified. Samples of the expression culture and purification fractions were resolved by SDS-PAGE (Figure 6) The majority of the fusion protein was soluble, as it was present in the supernatant fraction (Sn, Figure 6). The His-SUMO was efficiently recovered in the elution buffer (E1, Figure 6), and the yield was 46 mg/L. The His-SUMO-MutAFP and His-SUMO-AFP were expressed in *E. coli* as shown in Figure 7, both had a band with an identical molecular mass with the target protein in the starter culture, and both proteins were expressed after induction.

The purification of His-SUMO-MutAFP followed the same protocol as His-SUMO and is shown in Figure 8. The impurities were efficiently removed in flow through and washes as well, and the His-SUMO-MutAFP was highly soluble. After the SUMO cleavage reaction, around 50% of the His-SUMO-MutAFP was cleaved to release His-SUMO and MutAFP, although the cleaving efficiency was not ideal, the subsequent reverse purification efficiently removed the uncleaved protein and the His-SUMO from the mixture to yield MutAFP with high purity, and the yield of MutAFP was 16 mg/L.

The purification of His-SUMO-AFP followed the same protocol as His-SUMO and His-SUMO-MutAFP (Figure 9A), with His-SUMO-AFP behaving similarly to His-SUMO-MutAFP. A portion of the His-SUMO-AFP was collected to be used in the subsequent experiments, the remainder of the His-SUMO-AFP was used for the purification of AFP as shown in Figure 9B, and the yield of AFP was 6 mg/L.

The presence of 2 M urea in the SUMO cleavage reaction mixture accelerated the reaction to release more AFP, as shown in Figure 10. It appeared that the longer incubation time caused little or no difference in the cleavage since the amount of His-SUMO-AFP in 24 h and 48 h were similar, while the cleavage efficiency was improved in the presence of 2 M urea, as shown by the noticeable difference between the amount of His-SUMO-AFP in 48h and 48h with 2 M urea. The increase in efficiency at this step increased the final yield of AFP6 to 10 mg/mL from the original 6 mg/mL.

Each of the purified proteins was lyophilized and dissolved in 10 mM ammonium bicarbonate when required. The purified His-SUMO, His-SUMO-MutAFP, His-SUMO-AFP, and AFP were run on a gradient gel and stained by curcumin as shown in Figure 11, and each of them showed a uniform band. Despite of having identical molecular weights, MutAFP and AFP showed different migration as MutAFP ran faster than AFP on the same gradient gel (Figure 11).

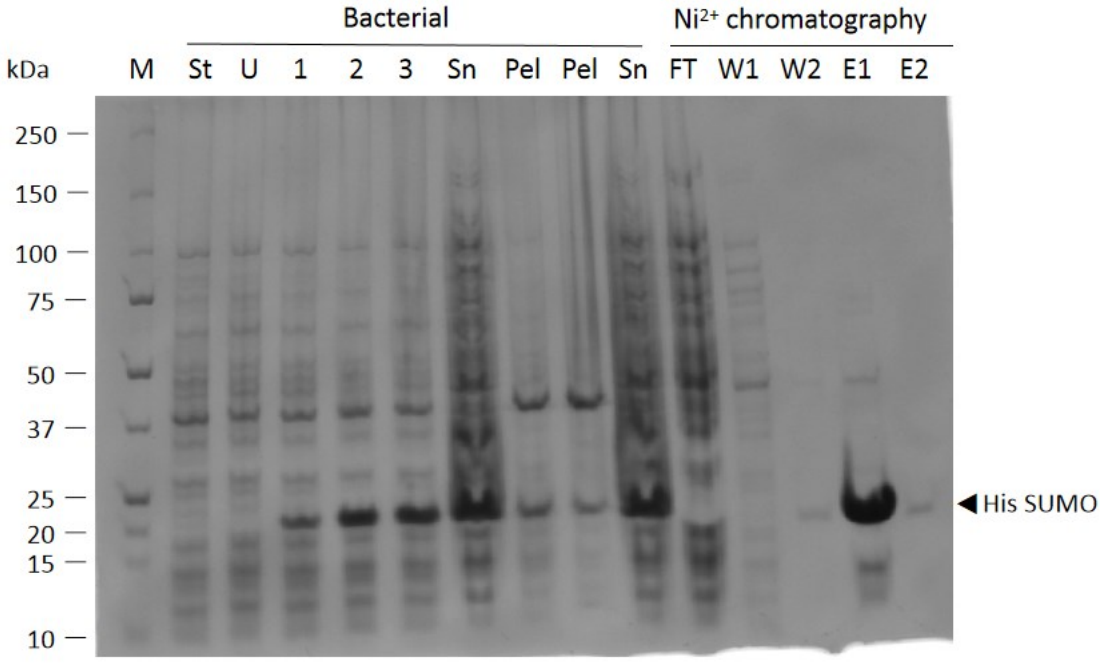


Figure 6. SDS-PAGE analysis of the expression and purification of His-SUMO. Expression was carried out as described in the Materials and Methods and products were resolved in a 10% polyacrylamide gel and stained with Coomassie blue. Lanes are: M, marker; St, starter culture; U, uninduced; 1, 1 hour after induction; 2, 2 hours after induction; 3, 3 hours after induction; Sn, cell lysis supernatant; Pel, cell lysis pellet; FT, flow through; W1, first wash;, W2, second wash; E1, first elution; E2, second elution.

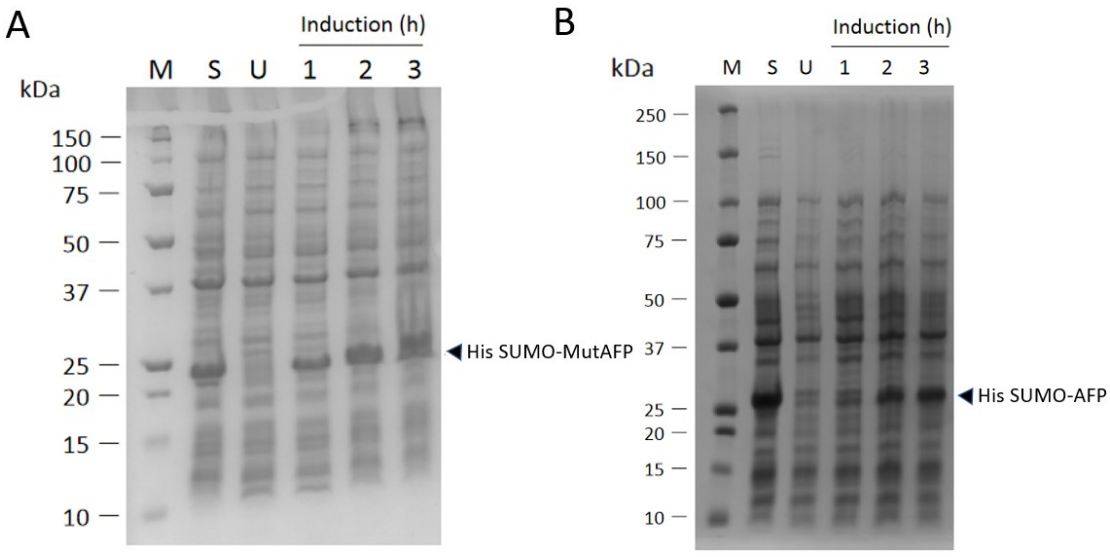


Figure 7. SDS-PAGE analysis of the expression of His-SUMO-MutAFP and His-SUMO-AFP. Expression was carried out as described in the Materials and Methods and products were resolved in a 10% polyacrylamide gel and stained with Coomassie blue. Panel A shows His-SUMO-MutAFP and Panel B shows His-SUMO-AFP. Lanes for both gels are: M, marker; St, starter culture; U, uninduced; 1, 1 hour after induction; 2, 2 hours after induction; 3, 3 hours after induction.

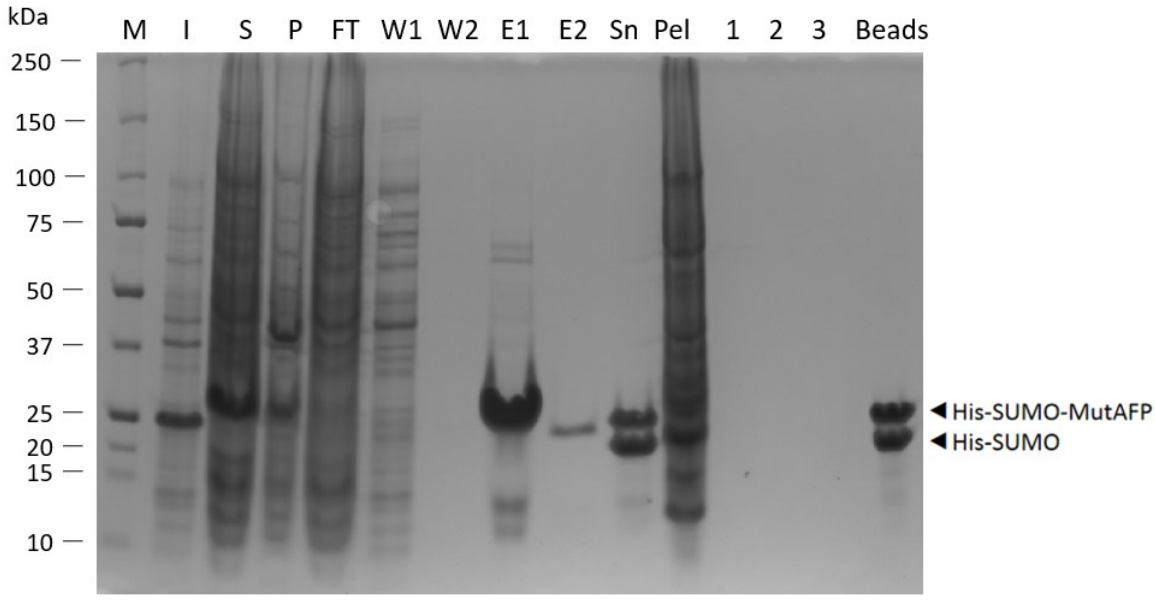


Figure 8. SDS-PAGE analysis of the purification of MutAFP. Purification was carried out as described in the Materials and Methods and products were resolved in a 10% polyacrylamide gel and stained with Coomassie blue. Lanes are: M, marker; I, after induction; S, cell lysis supernatant; P, cell lysis supernatant; FT, flow through; W1, first wash;, W2, second wash; E1, first elution; E2, second elution; Sn, supernatant after dialysis; Pel, pellet after dialysis; 1, first flow though; 2, second flow through; 3, third flow through; Beads, beads samples after flow through.

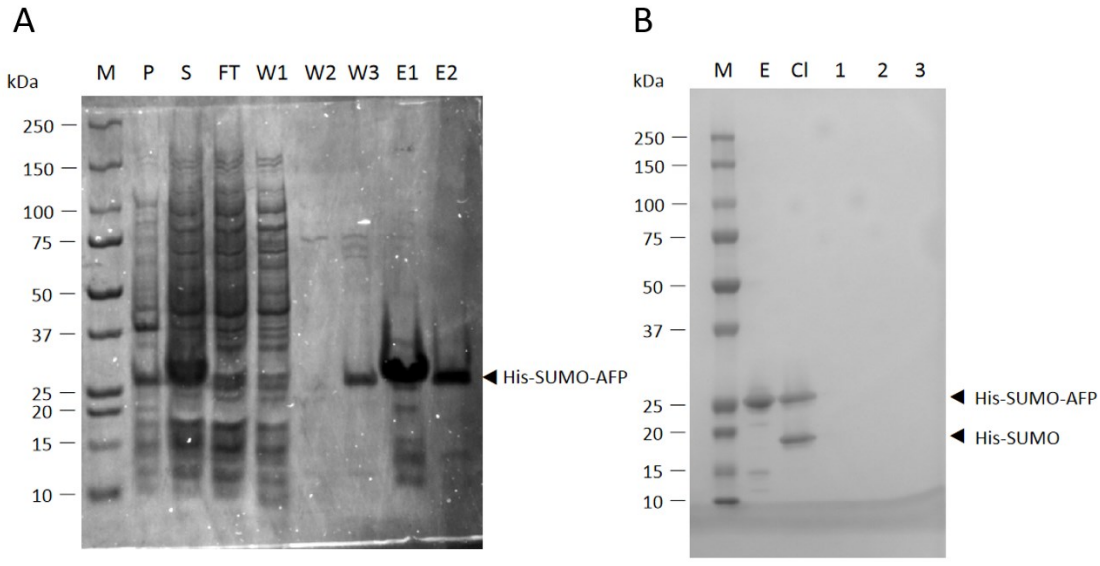


Figure 9. SDS-PAGE analysis of the purification of His-SUMO-AFP. Purification was carried out as described in the Materials and Methods and products were resolved in a 10% polyacrylamide gel and stained with Coomassie blue. Panel (a) Lanes are: M, marker; P, cell lysis pellet; S, cell lysis supernatant; FT, flow through; W1, first wash; W2, second wash; W3, third wash; E1, first elution; E2, second elution. (b) SDS-PAGE analysis of the purification of AFP. Purification was carried out as described in the Materials and Methods and products were resolved in a 10% polyacrylamide gel and stained with Coomassie blue. Lanes are: M, marker; E, elution; Cl, after SUMO-protease cleave; 1, first flow through; 2, second flow through; 3, third flow through.

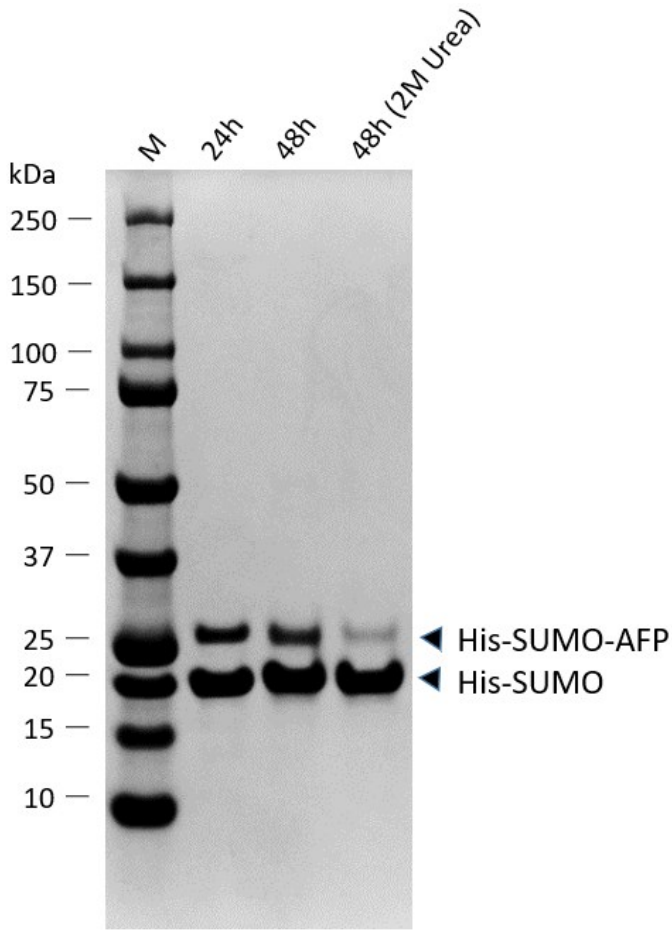


Figure 10. SDS-PAGE analysis of the SUMO-protease cleaving of His-SUMO-AFP. The protocol was carried out as described in the Materials and Methods and products were resolved in a 10% polyacrylamide gel and stained with Coomassie blue. Lanes are: M, marker; 24 h, 24 hours after incubating with SUMO-protease; 48 h, 48 hours after incubating with SUMO-protease; 48h (2 M urea), 48 hours after incubating with SUMO-protease with the presence of 2 M urea.

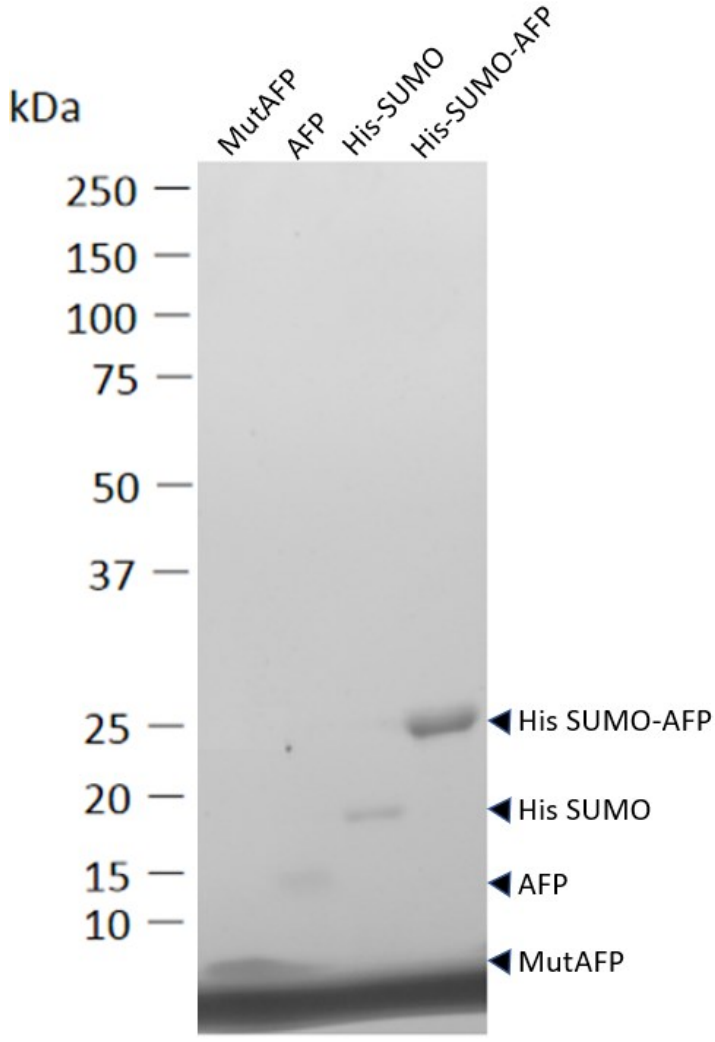


Figure 11. SDS-PAGE of purified MutAFP (10μg), AFP (10μg), His-SUMO (4μg), and His-SUMO-AFP (4μg) visualized by curcumin staining. Dissolved His-SUMO, MutAFP, AFP, and His-SUMO-AFP were run on a RunBlue^{TM} TEO-Tricine SDS-PAGE gels with an acrylamide gradient of 4-20% and stained using curcumin.

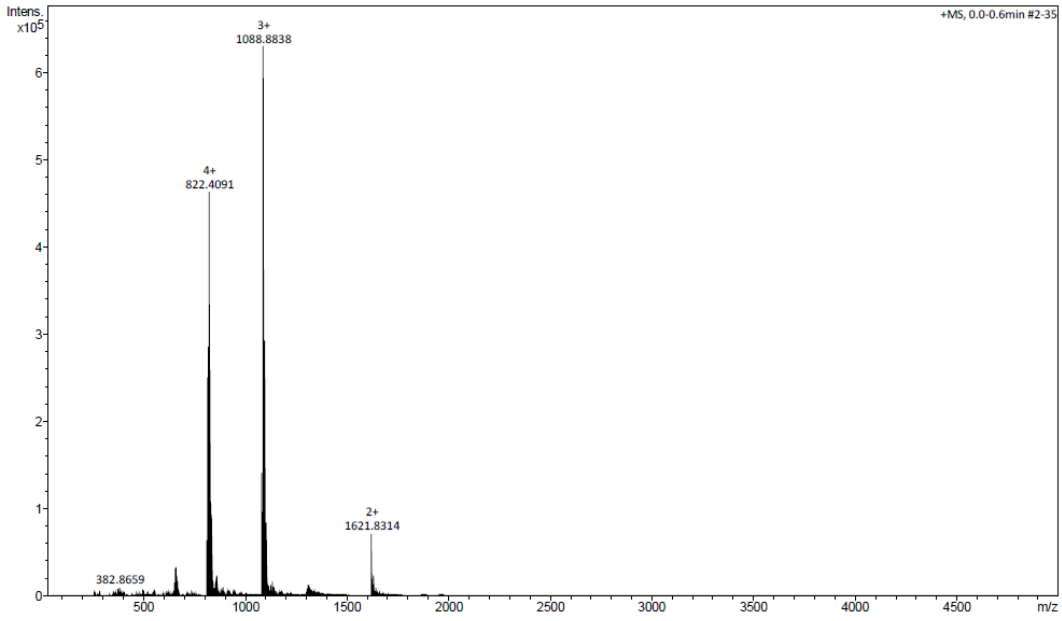
2.3.2 Mass Spectrometry

After visualizing MutAFP and AFP on a gradient gel, both proteins were subjected to mass spectrometry as shown in figure 12 and 13. The mass of MutAFP and AFP were consistent with their expected mass of 3.24 kDa, although the deconvoluted data exhibited multiple peaks with masses that corresponded to those of AFP and MutAFP. Further peaks at slightly higher masses were consistent with the presence of small ion adducts. Both MutAFP and AFP showed identical masses, consistent with their sequences.

2.3.3 Circular Dichroism Spectroscopy

The secondary structures of MutAFP and AFP6 were further compared using 12.5 μM protein samples, as shown in Figure 14. AFP6 showed a typical alpha-helical CD spectrum at 4 ºC with two strong minima at 208 and 222 nm, consistent with proper folding of the protein (Patel & Graether, 2010). The spectrum of MutAFP at 4 ºC corresponds to that of AFP, with the same strong minima. Nonetheless, the molar ellipticity of MutAFP is less negative compared to AFP6 in the vicinity of 208 nm, resulting in a higher $\theta_{222}/\theta_{208}$ ratio.

A



B

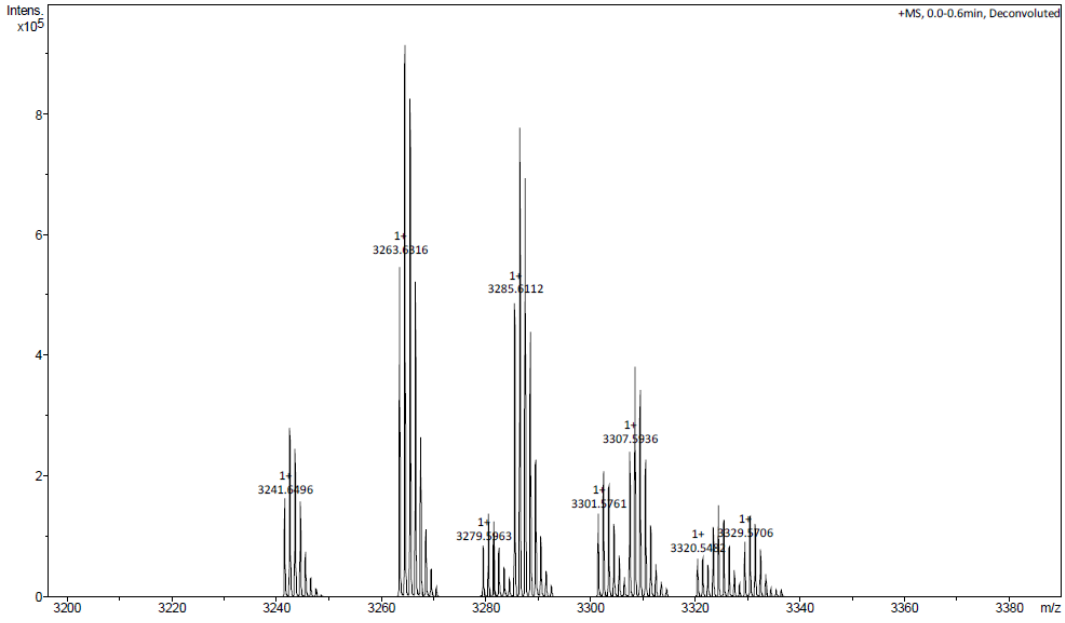
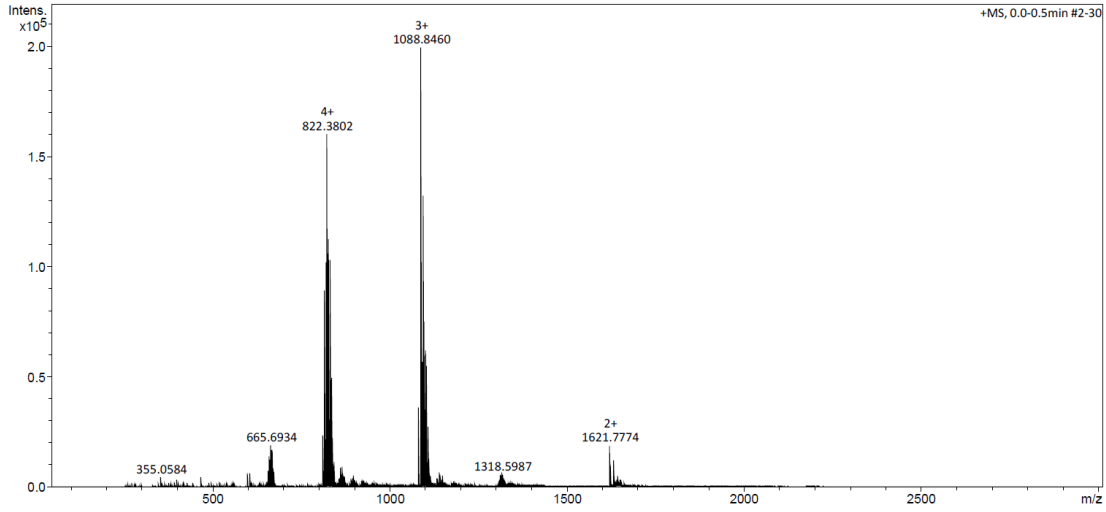


Figure 12. Mass spectrum of MutAFP. MutAFP protein was dissolved in 10 mM ammonium bicarbonate. Panel A: protein mass ion spectrum; panel B: deconvoluted spectrum.

A



B

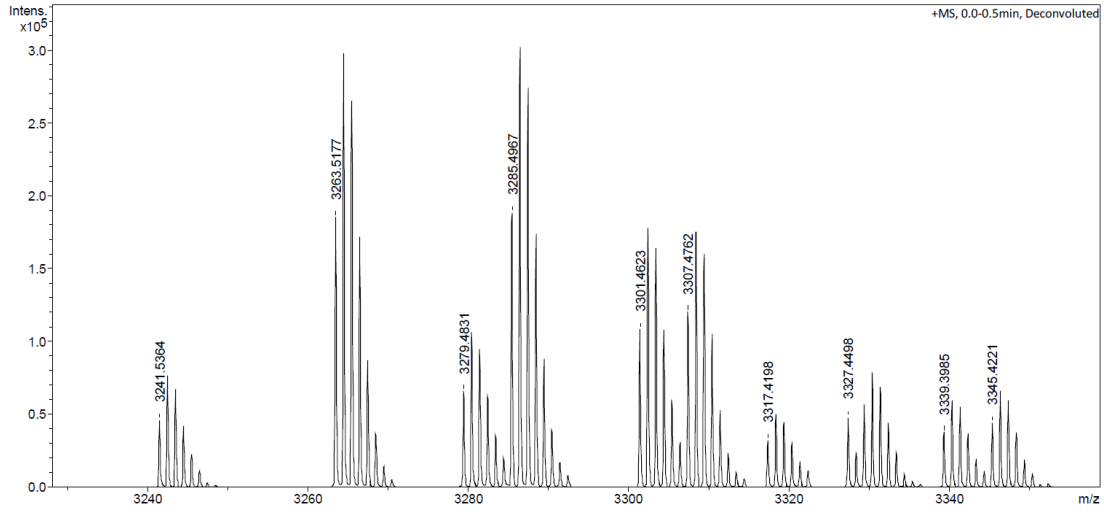


Figure 13. Mass spectrum of AFP. AFP protein powder was dissolved in 10 mM ammonium bicarbonate. Panel A: protein mass ion spectrum; panel B: deconvoluted spectrum.

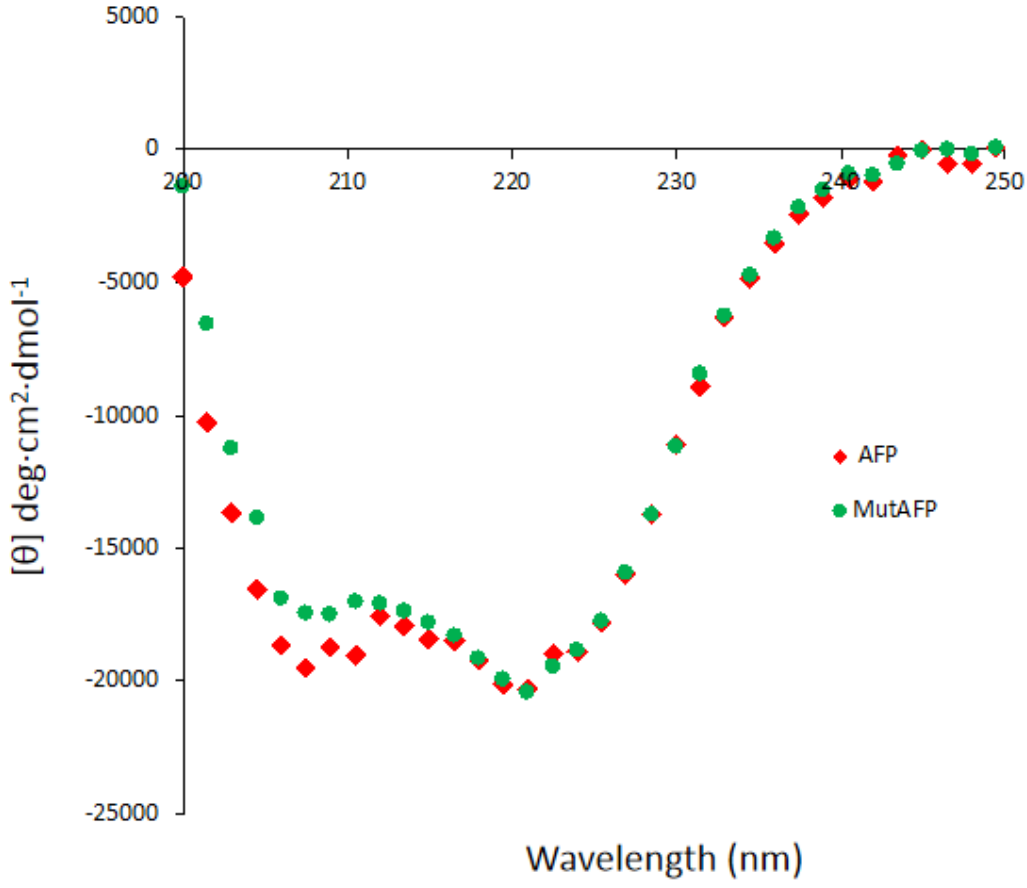


Figure 14. Circular dichroism spectroscopy of MutAFP and AFP. Each protein was prepared to a concentration of 12.5 µM in 10 mM ammonium bicarbonate.

2.3.4 Thermal Hysteresis

His-SUMO, MutAFP, AFP, and His-SUMO-AFP were subjected to thermal hysteresis analysis and the morphologies of ice crystals were evaluated in their presence. Ice crystals were formed in the presence of each protein or in the buffer control to study the thermal hysteresis, which is a separation of the freezing point and the melting point as described in the methods, at a series of concentrations of each protein were shown in Figure 15. Ice crystals grew smoothly with no expressed crystal faces in the presence of His-SUMO or buffer alone, resulting in rounded morphologies. In contrast, ice crystals were shaped into bipyramid structure in the presence of AFP6 or His-SUMO-AFP. The bipyramidal structure of ice crystals in the presence of AFP and His-SUMO-AFP are consistent with that seen at the same concentration of natural AFP6. In the presence of MutAFP, the ice crystal expressed hexagonal crystal faces consistent with the inhibition of growth normal to their surfaces. However, consistent with low or “trace” levels of antifreeze activity, no elongation of the ice crystal to a bipyramid was observed.

The concentration-dependent thermal hysteresis activities of His-SUMO, MutAFP, AFP, and His-SUMO-AFP were shown in Figure 16. MutAFP and His-SUMO showed little to no thermal hysteresis beyond background at any concentration. This is in agreement with the smooth crystals in the presence of His-SUMO and the hexagonal crystals in the presence of MutAFP. The interaction of latter is not strong enough to prevent ice growth. The His-SUMO-AFP and AFP both showed strong concentration dependence in their thermal hysteresis activity, with very similar activities on a concentration basis,

suggesting that the addition of the His-SUMO domain to AFP6 has little or no effect on its thermal hysteresis activities.

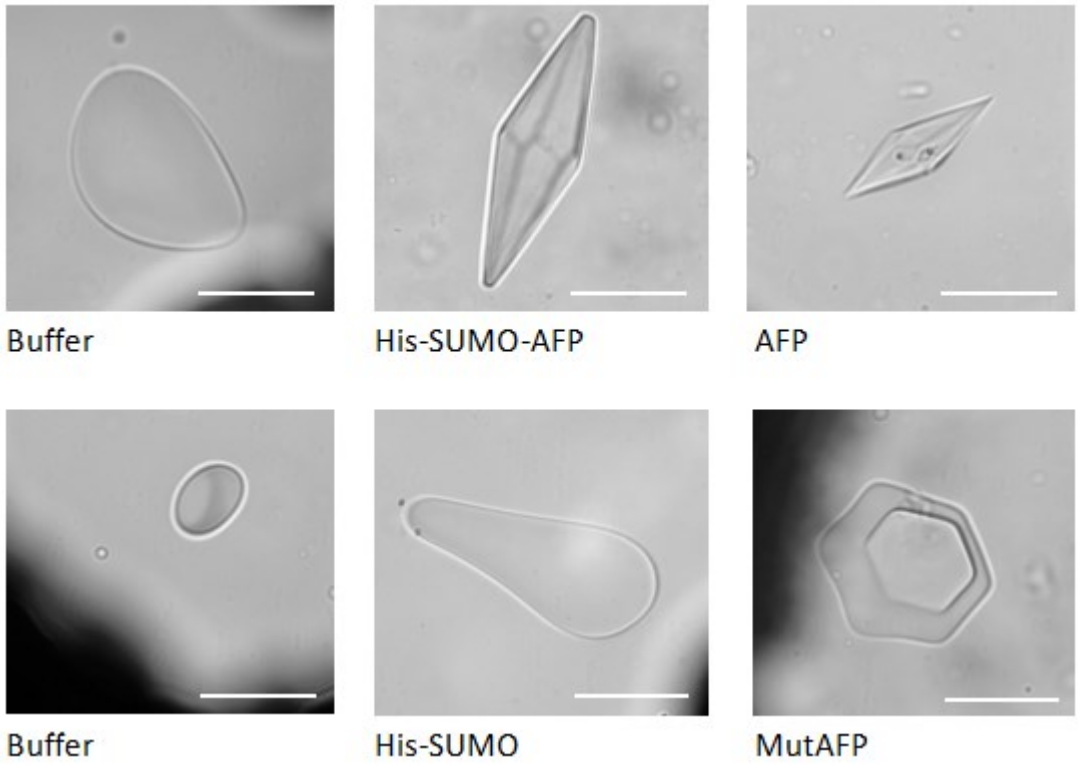


Figure 15. Ice crystals formed in the presence of 1.5 mM AFP and control constructs. The His-SUMO, MutAFP, AFP, His-SUMO-AFP proteins in 10 mM ammonium bicarbonate buffer and the buffer control were observed. Images are labeled with the protein name, or buffer for the buffer control. The white line in each image represents 50 microns. (These data were obtained by Dr. Kathryn Vanya Ewart.)

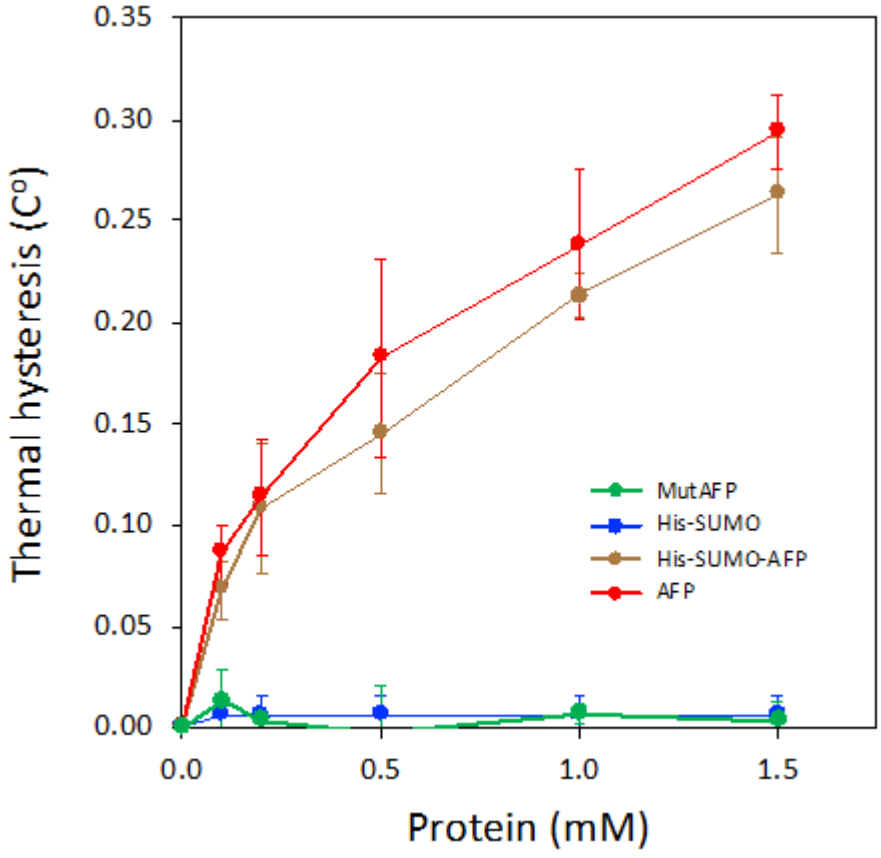


Figure 16. Concentration-dependent thermal hysteresis of His-SUMO, His-SUMO-AFP, MutAFP and AFP. Measurements were performed on each protein and background values for the buffer control were subtracted. Values shown are means ± SD for triplicate samples. (These data were obtained by Dr. Kathryn Vanya Ewart.)

2.4 Discussion

2.4.1 Optimizing the SUMO-Protease Cleaving Efficiency

AFP was expressed as fusion protein with SUMO and a removable protein tag for affinity purification, and the His-SUMO-AFP was successfully expressed in *E. coli* at relatively high yields in the vicinity of 50 mg/L. The release of AFP from the fusion proteins requires the presence of SUMO-protease to catalyze the cleaving via its endopeptidase activity, and the SDS-PAGE results from Figure 8 and 9 indicated a cleaving efficiency of approximately 50%. Following purification, desalting and lyophilization, this results in final yields of pure AFP of approximately 6 mg/L. It was unclear why the cleavage slowed down when approximately 50% of the His-SUMO-AFP was cleaved as shown in Figure 10, while there was little or no enzyme activity between 24h and 48h. It appeared unlikely that the cleaving was limited by the solubility of His-SUMO-AFP over time, since SUMO tag was reported to be soluble as well as the AFP (Dubé et al., 2016; Zuo et al., 2005). Nonetheless, in 2 M urea, SUMO cleavage efficiency increased, which raised the final yield of AFP to 10 mg/L. This finding might indicate that the slowed cleaving could due to masking of the cleavage site by native folding of His-SUMO-AFP, since urea is well known to promote protein unfolding, while SUMO-protease itself was reported to retain its functions in 2 M urea (Li et al., 2018). The presence of 2M urea improved the efficiency of obtaining AFP from His-SUMO-AFP, and the same method was applied for the purification of MutAFP from His-SUMO-MutAFP.

2.4.2 Characterizations of AFP and Related Proteins

As expected, MutAFP and AFP showed identical masses when analyzed by MS, since they were designed to have the same molecular mass. However, the SDS-PAGE results for these proteins revealed very different migration patterns. With a molecular weight of 3.3 kDa, AFP was expected to run ahead of the 10 kDa marker band, but the AFP ran well behind the 10 kDa band at a position expected for a far larger protein. In contrast, the MutAFP migrated faster than the 10 kDa band, consistent with its molecular weight. AFP was previously shown to migrate much slower than expected on SDS-PAGE (Fourney et al., 1984). Furthermore, inconsistency between molecular weight and protein migration in SDS-PAGE appears to be a common phenomenon for membrane proteins or small helical peptides, and increased alpha helicity appears to be associated with slower migration on SDS-PAGE (Rath et al., 2009). The substantial difference in migration between the highly similar AFP and MutAFP may be informative in term of identifying the sequence determinants of abnormal migration.

The greater $\theta_{222}/\theta_{208}$ ratio of MutAFP compared to AFP may indicate an increased helicity and likelihood of association into a coiled coil, although this would depend upon conditions (Crooks, Rao, & Mason, 2011; Kwok & Hodges, 2004). The inconsistency between the SDS-PAGE and mass spectrometry results of AFP could therefore be caused by differences in helicity compared to MutAFP, causing it to migrate slower than MutAFP on SDS-PAGE.

The absence of thermal hysteresis for MutAFP was consistent with the study that

reported moderate to complete loss of antifreeze activity caused by the substitution of Thr by Ser (Baardsnes et al., 1999; Zhang & Laursen, 1998). MutAFP showed no thermal hysteresis activity, and it exhibited no concentration dependence, although ice crystals formed in the presence of 1.5 mM MutAFP still exhibited straight edges, consistent with trace-level ice binding activity. However, the lack of thermal hysteresis activity of MutAFP indicated that the slight ice binding activity of MutAFP was insufficient to cause measurable thermal hysteresis. As expected, ice crystals formed in the presence of 1.5 mM AFP exhibited bipyramidal structures, which is an indication of the AFP binding altering the formation of the ice crystals as they grow (Drori et al., 2014). The concentration-dependent thermal hysteresis activity of AFP was also consistent with previous studies. The expression of AFP in *E. coli* results in a protein lacking the native C-amidation. The protein expressed here showed concentration-dependent thermal hysteresis activity consistent with that of the same non-amidated protein expressed in a similar way (Patel & Graether, 2010), but approximately half of that reported for the non-amidated AFP at 1.5 mM when expressed using a different system (Tong et al., 2000). Meanwhile, the ice crystal and thermal hysteresis data of His-SUMO-AFP were almost identical to AFP, suggesting nearly identical ice binding and thermal hysteresis activities between His-SUMO-AFP and AFP. The results also suggested that the comparatively large size of His-SUMO tag did not affect the antifreeze function of AFP, which could indicate that the His-SUMO tag and AFP are locationally independent from each other such that His-SUMO does not interfere with AFP functioning despite its comparatively larger size. On the other

hand, in studies using the unrelated Type III fish AFP, the addition of fusion proteins including maltose-binding protein and thioredoxin increased the thermal hysteresis (DeLuca, Comley, & Davies, 1998). Direct comparison is difficult because different fusion proteins and different AFPs were used. Nonetheless, it is unclear whether the SUMO has no appreciable effect on the activity of the AFP or whether it has both enhancing and reducing effects on activity that together result in no net change.

CHAPTER 3: EVALUATION OF THE EFFECTS OF AFP ON ICE NUCLEATION

3.1 Introduction

The modulation of ice nucleation is important for the survival of many organisms in different kingdoms. For example, as noted above, the INPs of ice-nucleating bacteria allow them to obtain nutrients by inducing frost damage on plants and some freeze-tolerant animal species also using INPs to trigger freezing at high sub-zero temperatures (Lindow et al., 1982). In the case of many freeze-tolerant plants and insects, the promotion of ice nucleation at high sub-zero temperatures prevents explosive freezing, and the slower freezing that occurs at higher sub-zero temperatures allows enough time for the equilibration of the osmotic pressure between freezing extracellular and unfrozen intracellular fluids (Lindow et al., 1982; Ramløy, 2000; Rozsypal, 2015). Modulation of ice nucleation can also be applied in the field of cryopreservation, since ice nucleation is the most significant uncontrolled variable in cryopreservation (John Morris & Acton, 2013; Petersen et al., 2006). It was also found that the inducing extracellular ice formation at relatively warm supercooled temperature is beneficial to the post-thaw viabilities of cells, and this requires precise control of ice nucleation (Lauterboeck et al., 2015; Prickett et al., 2015; Wolkers et al., 2007). Therefore, understanding of ice nucleation and its modulation is pertinent to understanding biological adaptations as well as to the development of new freezing technologies. Thus, the study of ice nucleation could provide the understanding necessary or technological improvement for its effective modulation.

Many of the nucleation-modulating agents in nature are known to be proteinaceous,

and ice nucleating proteins (INPs) that induce ice nucleation at high sub-zero temperature are well studied (Roeters et al., 2021). Many studies have reported related or opposing relationships between INPs and AFPs and among these are contrasting results. A fish AFGP (antifreeze glycoprotein) was found to counter the effects of bacterial ice nucleation proteins, presumably by binding directly to ice nucleators (Parody-Morreale et al., 1988). Yet, a more recent study has found that several other AFPs do not share this effect, and that the effect of Type I AFP might be due to bacterial surface binding instead of ice or INP interaction (Schwidetzky et al., 2020). The same study also suggested nucleation enhancement with AFP, but mixed isoform and semi-pure preparations appear to have been used and no account of protein purity was provided in these studies (Schwidetzky et al., 2020). In another study employing Type III fish AFP and a beetle AFP, contrasting effects on ice nucleation were attributed to roles of the non-ice binding face of the AFPs (Liu et al., 2016). A semi-pure preparation containing a mixture of Type I AFP isoforms and possibly other proteins appeared to inhibit nucleation at moderate concentrations but slightly induce it at higher levels; however, the role of proteins other than AFP was unclear (Wilson et al., 2010). Furthermore, an expressed fragment of an ice nucleating protein revealed antifreeze activity rather than nucleation (Kobashigawa et al., 2005). Therefore, the relationship between AFPs and ice nucleation remains unclear and different proteins, protein mixtures, purity levels and assay conditions have precluded comparison among results. For these reasons, it was of interest to examine aspects of AFP and ice nucleation using the simplest AFP in pure form and in a system allowing a variety of controls to be

used. This can provide important information regarding the connection between INPs and AFPs, as well as revealing factors that might allow precise control of nucleation modulation for technological applications.

3.2 Materials and Methods

3.2.1 Preparation of Buffers and Protein Samples

The 10 mM ammonium bicarbonate buffer was prepared as described in Chapter 2 and filtered through a 0.2 μm membrane (Avantor) for some experiments to reduce the level of background particulate nucleator presence for other experiments. AgI (99%, Sigma-Aldrich) was used in some experiments. Lyophilized preparations of recombinant AFP, MutAFP, His-SUMO, and His-SUMO-AFP were dissolved in 10 mM ammonium bicarbonate to give solutions with concentrations of 5 mM, where possible, and lower concentrations where proteins had more limited solubility. Further control proteins included bovine serum albumin (BSA, Sigma-Aldrich) and lysozyme (Worthington). The concentrations were measured by ultraviolet light absorbance using a small-volume spectrophotometer (DeNovix) following the manufacturer's instructions.

3.2.2 Ice Nucleation Analysis by the Droplet Assay

Protein solutions at a concentration of 5 mM were diluted to 1.5, 0.5, and 0.15 mM. A thin layer of Vaseline^{TM} (Unilever) was applied on the surface of a 6" x 1" aluminum

pie dish (No Name™, Loblaws). For each sample analysis, 20 droplets of 2 μL each were pipetted into a grid formation on the Vaseline™ surface. Buffer controls were analyzed in the same manner. Typical sets of droplet in ordered grid arrangements are shown in Figure 17A.

In some experiments, AgI was used as an ice nucleator in order to induce freezing. This was achieved by distributing a fine dusting of AgI powder on to the Vaseline by a spatula before applying the sample droplets on the layer of AgI power. A set of droplets on an AgI-powdered surface is shown in Figure 17B.

Once the droplets were applied, the dish was placed inside a refrigerated circulating bath with temperature stability of ± 0.1 ºC (VWR). The opening of the circulating chiller was covered by a layer of transparent plastic wrap to allow observation while preventing condensation on the cold surfaces. The temperature of the circulating bath was set to 0 ºC or another appropriate starting temperature and dropped by 0.5 ºC increments at 10 minute intervals. The number of frozen droplets was determined at each temperature until all 20 droplets were frozen or when the temperature reached -20 ºC , resulting in a cumulative freezing curve.

3.2.3 Data Analysis

For triplicate samples, mean freezing droplet numbers were determined at each temperature and a cumulative freezing curve was plotted. Data were fitted to a variable-

slope agonist-response curve and plotted with droplet freezing as a function of temperature in Prism (GraphPad).

Figure 17. Droplet assay for ice nucleation study. (A) Droplets in ordered grid for ice nucleation study at low sub-zero temperatures. Clear droplets are liquid and opaque droplets are frozen. (B) A set of droplets in the presence of AgI for high sub-zero temperature ice nucleation study. All droplets in this image are frozen.

3.3 Results

3.3.1 Effect of AFP Samples on AgI-Induced Ice Nucleation

MutAFP, AFP, and His-SUMO-AFP, were subjected to droplet assays along with His-SUMO, BSA, lysozyme, and buffer as controls, all at 1.5 mM, in the presence of the nucleator AgI (Figure 18). Lysozyme and BSA showed little or no inhibition of ice nucleation compared with buffer alone, as their cumulative freezing curves approached that of the buffer. Contrary to what was expected, the His-SUMO and MutAFP, which have no measurable antifreeze activity, delayed the onset of freezing by approximately a degree and the AFP and His-SUMO-AFP shifted it further. Freezing appeared to be most inhibited by His-SUMO-AFP, as the freezing onset was delayed and freezing was not complete until -7 °C, which was a full degree below complete freezing of the other samples (Figure. 18). The concentration dependence of the high sub-zero freezing profile of AFP was also measured (Figure 19). Ice nucleation inhibition by AFP exhibited strong concentration dependence, with greater inhibition at higher concentrations. The concentration-dependent freezing of other proteins were also measured (Figure 20). Nucleation inhibition in the presence of all concentrations of BSA was indistinguishable from buffer alone and that in lysozyme appeared to increase very slightly. All AFP-containing proteins showed some concentration dependence in ice nucleation suppression and the non-AFP construct, His-SUMO, showed some concentration-dependent effect as well. The results suggest that modest inhibition of high sub-zero temperature ice nucleation is a property shared to different extents by control proteins but that appears

greater for the AFP-containing proteins.

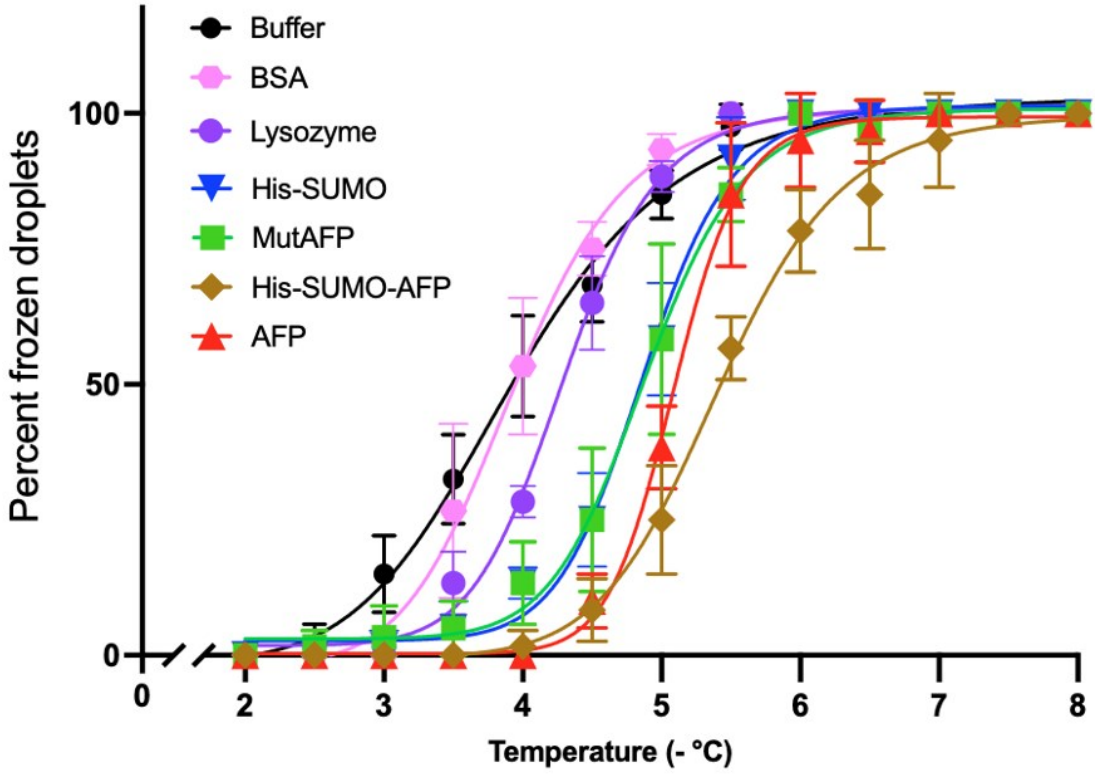


Figure 18. High subzero temperature freezing profile of proteins in the presence of AgI. 1.5 mM His-SUMO, MutAFP, AFP, lysozyme, BSA, His-SUMO-AFP in 10 mM ammonium bicarbonate buffer as well as buffer alone. Values shown are means ± SD for triplicate sample assays.

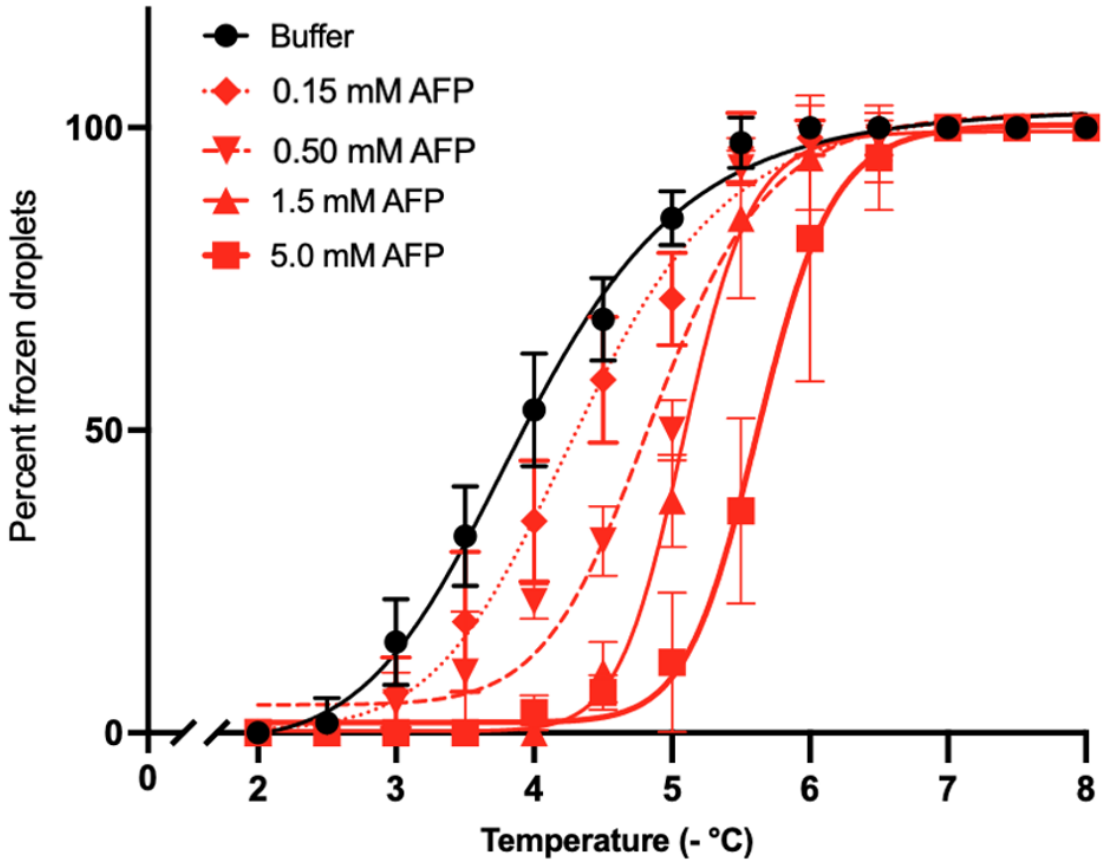


Figure 19. High sub-zero concentration-dependent freezing profile of AFP in the presence of AgI. Values shown are means ± SD for triplicate sample assays for the protein in 10 mM ammonium bicarbonate buffer and for buffer alone.

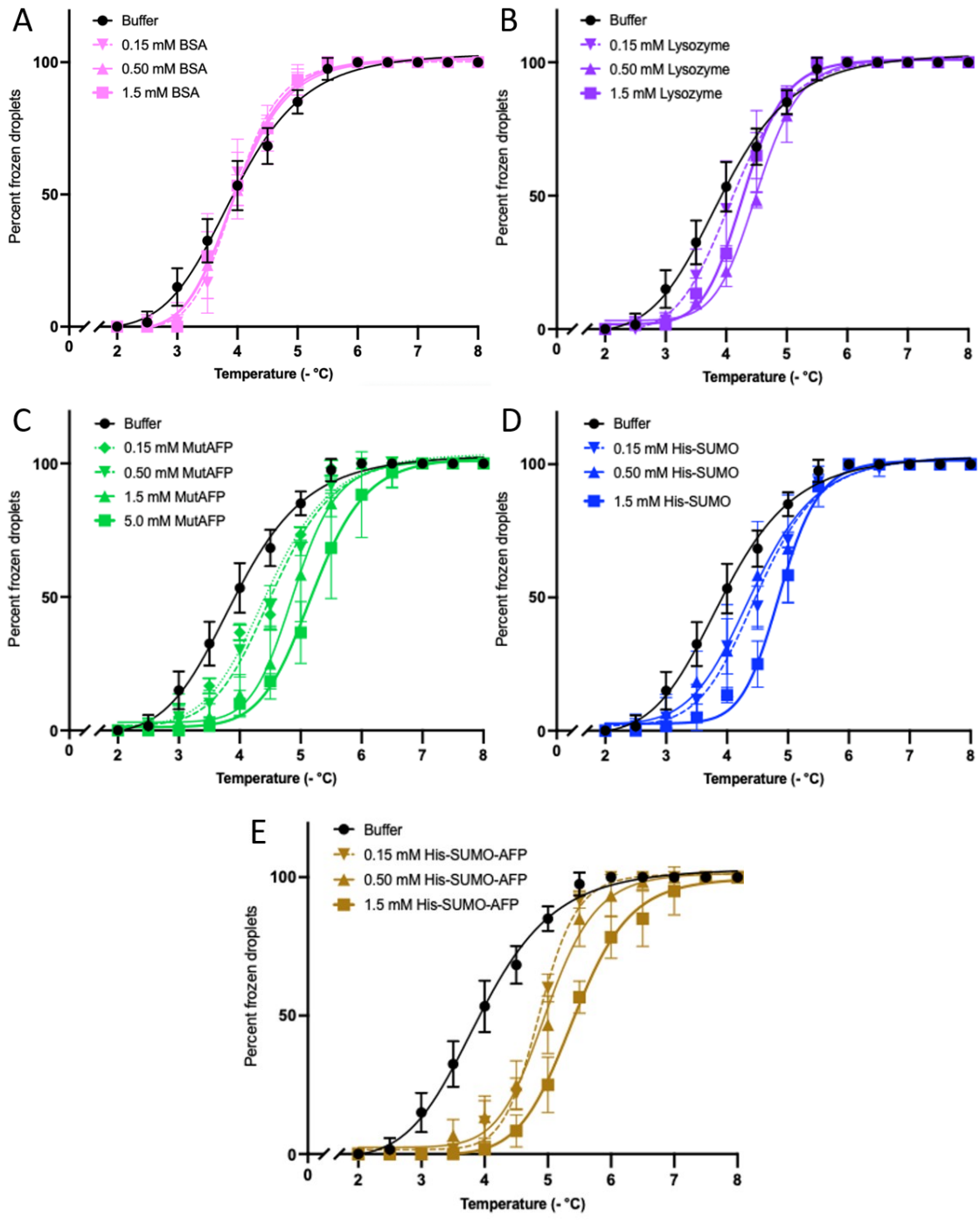


Figure 20. High sub-zero concentration-dependent freezing profile of AFP-related proteins and control proteins in the presence of AgI. Panels A-E are BSA, lysozyme, MutAFP, His-SUMO and His-SUMO-AFP, respectively. Values shown are means ± SD for triplicate sample assays for proteins in 10 mM ammonium bicarbonate buffer and for buffer alone.

3.3.2 Effect of AFP Samples on Natural Ice Nucleation

The low temperature droplet assay that studies the natural freezing profile of the samples was conducted in the absence of exogenous nucleators, and the assay was initially performed using unfiltered buffer. This was informative, as the moderate background nucleation allowed an assessment of the effects of AFPs and control proteins at lower temperatures. While all the proteins inhibited the onset of nucleation compared to buffer alone, the AFP then triggered rapid nucleation beginning at -17.5 °C and with all droplets frozen by -18.5 °C (Figure 21A).

In order to further examine this phenomenon, experiments were carried out in filtered buffer in order to reduce background nucleation. When the protein solutions prepared in filtered buffers were subjected to droplet assays, no nucleation was detected in any sample above -17 °C and it remained below 10% down to -20 °C for the buffer and all proteins except for the AFP. The AFP samples did not begin freezing until -17 °C, similarly to the other proteins; however, the AFP samples then froze readily, being half frozen at approximately -18 °C and fully frozen at -20 °C (Figure 21B). Thus, in the presence of AFP, nucleation did not begin at an elevated temperature, but it progressed far more rapidly than in any other sample. Following this experiment, nucleation by the AFP was assayed over a series of concentrations. Concentration dependence was shown, as nucleation increased between concentrations of 0.15 and 1.5 mM, but did not increase further at 5 mM AFP (Figure 22). Given this result, the other proteins were assayed at similar concentrations, within their solubility ranges and none appeared to show any

concentration-dependent effects on nucleation as shown in Figure 23.

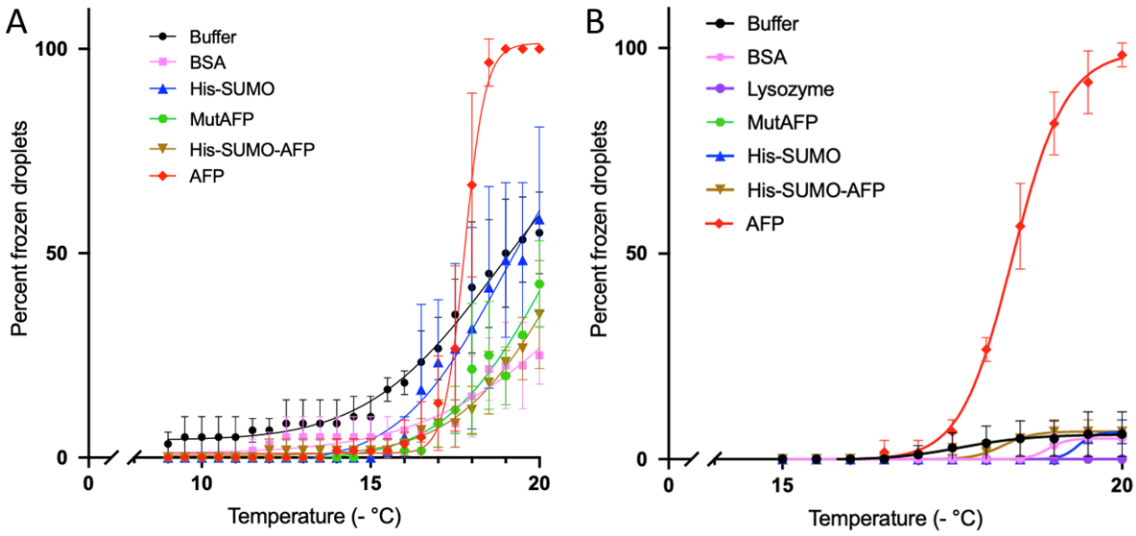


Figure 21. Low sub-zero concentration-dependent freezing profile of AFP, AFP-related proteins and control proteins in the absence of exogenous nucleators. (A) Freezing profile of 1.5 mM BSA, His-SUMO, MutAFP, His-SUMO-AFP, and AFP. (B) Freezing profile of 1.5 mM BSA, lysozyme, MutAFP, His-SUMO, His-SUMO-AFP, and AFP, and 10 mM ammonium bicarbonate buffer. Values shown are means ± SD for triplicate sample assays for proteins in 10 mM ammonium bicarbonate buffer and for buffer alone.

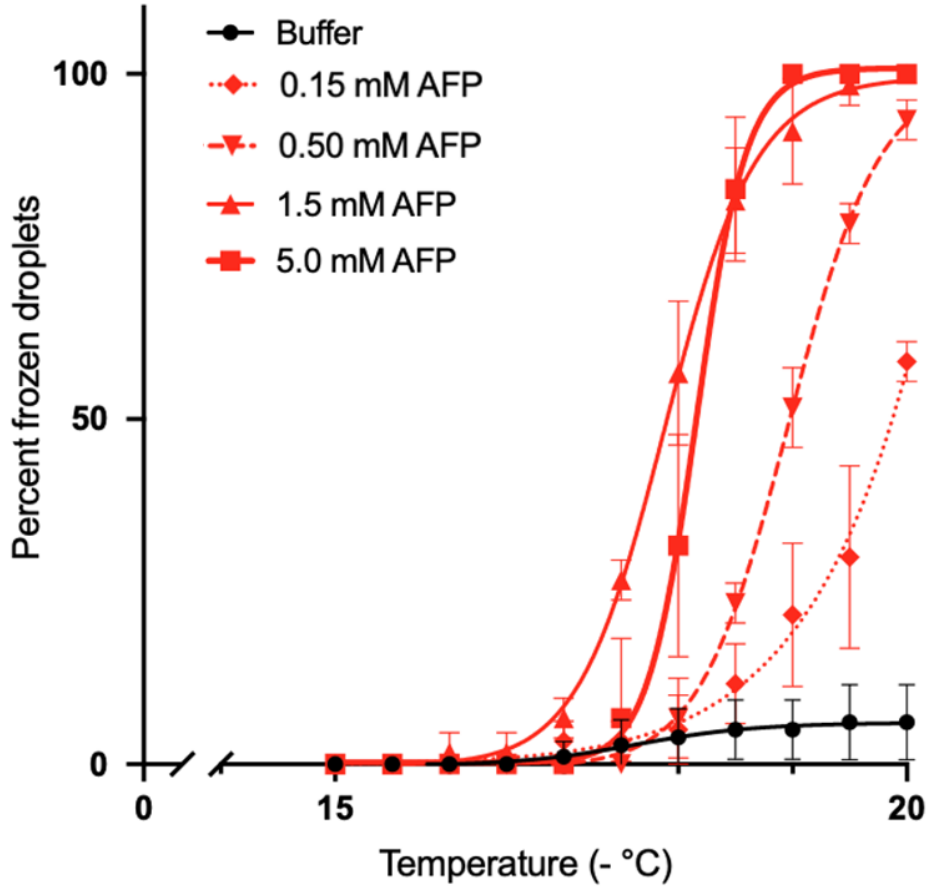


Figure 22. Low sub-zero concentration-dependent freezing profile of AFP in the absence of exogenous nucleators. Values shown are means ± SD for triplicate sample assays for proteins in 10 mM ammonium bicarbonate buffer and for buffer alone.

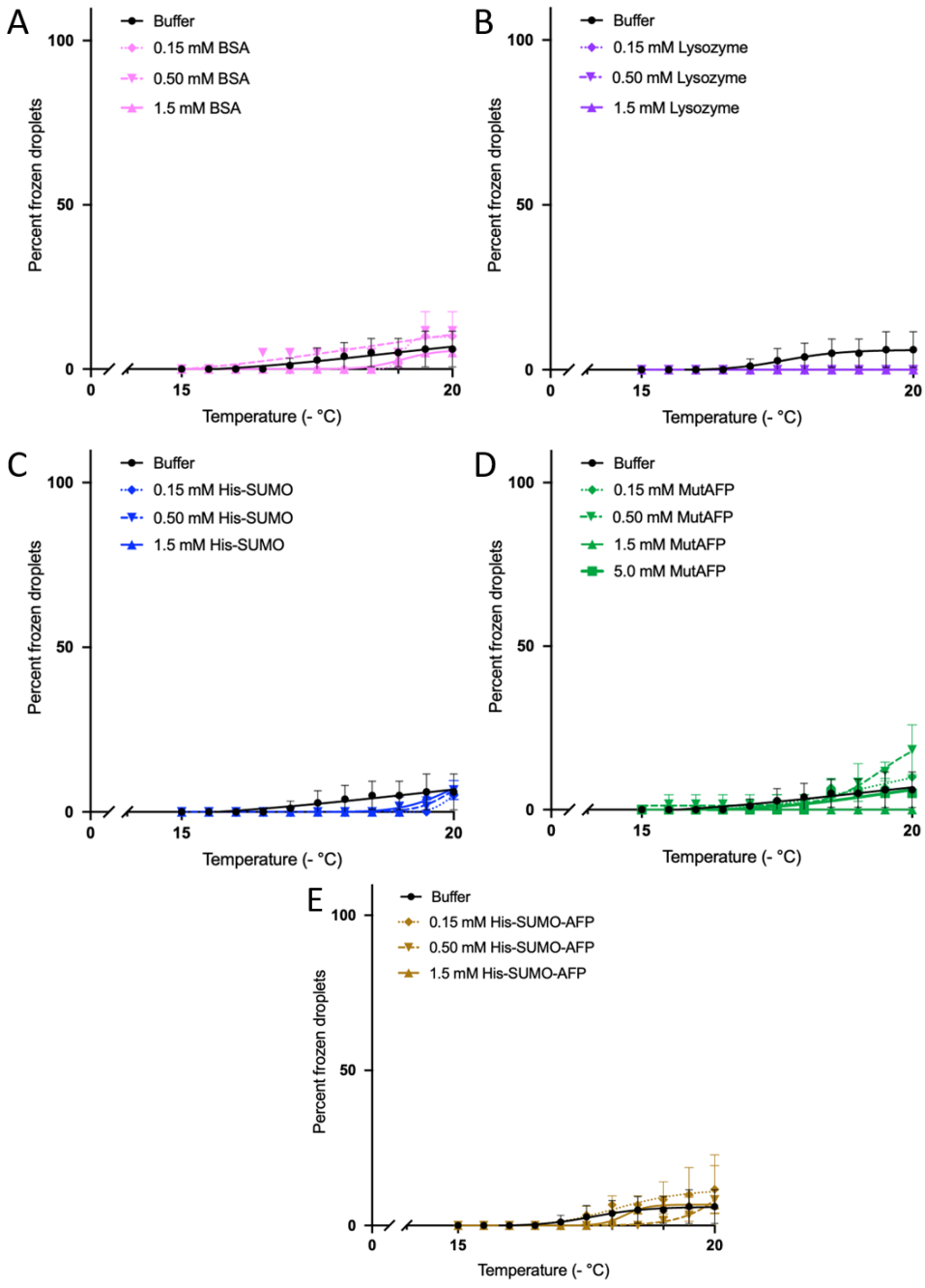


Figure 23. Low sub-zero concentration-dependent freezing profile of AFP-related proteins and control proteins in the absence of exogenous nucleators. Panels A-E are BSA, lysozyme, MutAFP, His-SUMO and His-SUMO-AFP, respectively. Values shown are means ± SD for triplicate sample assays for proteins in 10 mM ammonium bicarbonate buffer and for buffer alone.

3.4 Discussion

The phenomenon in which aqueous solutions remain in the liquid state when cooled below the point of fusion is known as supercooling. Solutions may supercool to varying degrees before ice forms by nucleation, and the temperature at which spontaneous nucleation occurs is the nucleation temperature (Kristiansen, 2001). As noted in the Introduction, homogeneous and heterogeneous nucleation are different from one another (Kanji et al., 2017). In heterogeneous ice nucleation, a particle in the solution or a surface upon which is placed forms a structure compatible with an ice surface. As the temperature decreases, water begins to stably rest on this structure, forming hydrogen bonds, and an embryo crystal forms (Knight, 2005). This happens at a higher temperature than homogeneous nucleation (Knight, 2005).

3.4.1 Effects of Proteins on AgI-Nucleated Ice

The dose-dependent inhibitory effect of AFP on heterogenous ice nucleation induced by AgI at high sub-zero temperatures was consistent with previous findings from studies of AFGP (Parody-Morreale et al., 1988) and a flounder AFP preparation (Schwidetzky et al., 2020). However, by using pure recombinant AFP, the current study reveals that the effect is due to the specified protein and not a secondary protein or salt contaminant. In addition, the use of non-AFP controls allowed the general effects of protein presence to be distinguished from specific ice binding effects of AFPs.

There are few reports in the literature regarding the possible effects of non-INP

proteins on ice nucleation; however, the freezing profile of the two unrelated control proteins, BSA and lysozyme, in AgI-induced nucleation approached the freezing profile of the buffer control, indicating that they had little or no effect on ice nucleation. In addition, the concentration dependence of the nucleation curves for these proteins showed no appreciable variation. Although lysozyme and BSA showed no discernable effect, the His-SUMO protein and MutAFP appeared to lower the nucleation temperature somewhat, with His-SUMO lowering it by close to a degree. The concentration dependence of their effect were also evident in this study, although they were both shown not to have antifreeze activity in Chapter 2. These proteins may each have a surface with a set of polar residues that interact weakly with water to prevent ice formation or that interact with a particular surface of the AgI. This question will require further study.

3.4.2 Inhibition of Nucleation by AFP and Other Proteins

Both ice nucleation suppression and ice crystal absorption inhibition can contribute to freeze avoidance. Type I AFPs are known to inhibit the growth of ice crystal by absorbing to the crystal surface to make the addition of water molecules energetically unfavorable (Salvay et al., 2010). Nonetheless, water molecule aggregates (ice nuclei) critical for ice nucleation may be too small to be allow AFPs to adsorb and block ice growth, and therefore, the absorption inhibition of AFP would have no effect on the initiation of ice nucleation (Wilson & Leader, 1995). AFPs have been found to inhibit ice nucleation to an

extent comparable to polyvinyl-pyrrolidone, which is a material without thermal hysteresis activity, suggesting that the absorption inhibition of AFP on ice crystals does not apply to the suppression of embryonic ice clusters (Franks et al., 1987). A simulation study also indicated that the ice nucleation suppression is achieved by a mechanism different from absorption inhibition of ice crystals, the study suggested that the ice binding site of AFP promotes ice nucleation due to the ice crystals being energetically stable on the ice binding face (Liu et al., 2016). Although the same study also suggested that disordered liquid water is energetically more stable on the non-ice-binding site of AFP, causing the non-ice-binding site of AFP to depress ice nucleation. Other studies also suggested that ice binding site and non-ice binding site of AFP work synergistically with ice binding site binds to ice crystals leaving non-ice binding site exposed to liquid water to depress ice nucleation (Celik et al., 2013; Duman, 2001). In this context, the current finding that AFP enhances ice nucleation at low subzero temperatures in the absence of AgI along with the absence of this activity in proteins including His-SUMO-AFP and MutAFP is informative. These results suggest a clear role for ice binding in the promotion of ice nucleation. In contrast, the modest inhibition of AgI-induced ice nucleation by the active AFPs together with the detectable effect of other non-AFP proteins on that phenomenon is incremental and may not be directly associated with active ice binding. If the ice nucleation inhibition by AFP in this context is due to masking of the ice nucleating surfaces on AgI (Inada et al., 2012; Liu et al., 2016), then it is possible that non-AFPs that interact even very weakly with AgI might lower the nucleation temperature.

3.4.3 Nucleation Inhibition and Promotion

In the winter flounder, AFPs do not appear to have a role in preventing ice crystals from forming. Instead, they are absorbed onto existing ice crystals to inhibit their growth. Yet, as suggested above, there may be some relationship between ice binding and the modulation of nucleation. Since small AFPs absorb to ice crystals to inhibit their growth, while large INPs trigger the formation of new ice crystals at temperatures higher than the homogeneous ice nucleation temperature, it was hypothesized that both AFP and INPs act base on the same principle due to their similarity of interaction with ice (Eickhoff et al., 2019). This is consistent with the report of antifreeze activity in an INP-derived peptide (Kobashigawa et al., 2005). A simulation study also suggested that AFP increases the homogeneous nucleation temperature, and the increase repeats of AFP is associated with further increased homogeneous nucleation temperature, which indicates that AFP might work based on the same principle with INPs but with different sizes (Qiu, Hudait, & Molinero, 2019).

Given that increasing AFP repeats appears to increase nucleation (Qiu et al., 2019) and that extracting a single repeat unit from an INP gives an AFP (Kobashigawa et al., 2005), the role of repeat number (or aggregation) on a conversion in activity from ice nucleation inhibition to ice nucleation promotion could be hypothesized to result from this kind of difference. AFP does have aggregation properties. When frozen and thawed at high concentrations, the AFP form of amyloid fibers (Dubé et al., 2016; Graether et al., 2003). The low sub-zero temperature freezing profile of AFP in this study shows that the higher

concentration of AFP is associated with higher ice nucleation temperature, and the higher AFP concentration are also known to be associated with AFP aggregation, which may suggest that the high concentration of AFP causes aggregation, thereby mimicking the large size of INPs to increase ice nucleation temperature.

3.4.4 The Effect of SUMO Domain and Ice Binding Activity on AFP6 Ice Nucleation

His-SUMO-AFP did not show any low sub-zero temperature ice nucleation promotion. Unlike MutAFP, the AFP portion of the His-SUMO-AFP was identical to AFP, and the thermal hysteresis activity data suggests that its AFP portion has near-identical thermal hysteresis activity to AFP (Chapter 3). The results indicate that the His-SUMO domain can prevent the AFP from inducing ice nucleation at low sub-zero temperature. The molecular weight of His-SUMO (~14 kDa) is larger than AFP (~3.3 kDa), and it is possible that the presence of His-SUMO domain on AFP could prevent AFP from undergoing an aggregation process at high concentration. Although, it is still unclear how the His-SUMO domain prevents AFP from enhancing nucleation, it is unlikely that it is prevented by the His-SUMO domain wrapping around the AFP portion, since the thermal hysteresis activity is maintained on His-SUMO-AFP.

3.4.5 Concluding Remarks

The aim of the study was to observe the effect of AFP and related proteins (His-SUMO-AFP, His-SUNO, and MutAFP) on ice nucleation, and the results revealed several factors that may have roles. AFP is capable of inhibiting ice nucleation induced by ice nucleating agents, suggesting a possible interaction between AFP and the ice nucleator AgI. In contrast, at lower temperatures in the absence of AgI, AFP was also found to be capable of promoting ice nucleation, and this effect was not detected in the inactive mutant, MutAFP, suggesting an involvement of the ice binding site of AFP in ice nucleation. The low sub-zero temperature ice nucleation promotion by AFP was also prevented by adding a His-SUMO tag to its N-terminus, suggesting the AFP ice nucleation promotion could depend on the protein size or structure. This would be consistent with a role for protein assembly, if the SUMO tag interferes with that. Although the observations provide insights into nature of AFP regarding ice nucleation, a number of questions remain to be answered concerning the details mechanisms of AFP in ice nucleation.

CHAPTER 4: CONCLUSIONS

This study was an evaluation of ice nucleation by type I AFP, which is the simplest model. This work differs from many of the previous studies in that a pure recombinant type I AFP was used, suitable controls were used and all AFP forms were evaluated for thermal hysteresis activity in addition to ice nucleation studies. Together, these factors allowed the common effects of proteins to be distinguished from the specific effects of the AFP on ice nucleation. Furthermore, the nucleation effects of the AFP could be interpreted in light of the known thermal hysteresis of the AFPs, allowing insight into the role of ice binding.

The AFP exhibited opposite effects on ice nucleation under different conditions. In the presence of AgI, which triggered ice nucleation at high sub-zero temperatures, the AFP inhibited nucleation, as showed by the diminished nucleation temperature. In contrast, the AFP enhanced the ice nucleation when it was allowed to occur at far lower temperatures without an added exogenous nucleator. The unrelated insect and type III AFP were suggested to have contrasting effects depending upon the concentration or exposure of the ice binding face to the solvent (Eickhoff et al., 2019; Liu et al., 2016), and impure type I AFP was suggested to have a transient ice nucleation inhibition with increasing concentration (Wilson et al., 2010). Nonetheless, no clear results were previously obtained on the type I AFP. Furthermore, the temperature dependence of this effect was previously unknown.

If this change is due to lower temperature rather than the absence of AgI, then it

suggests some interesting possibilities. A temperature-dependent change in AFP behavior from an ice nucleation inhibitor to a nucleator suggests either a solution change or a protein change at a very low sub-zero temperature. Study of the variants in parallel provided insight into this. The inactive form, MutAFP, did not nucleate ice at low sub-zero temperature. This suggests that the promotion of ice nucleation at low sub-zero temperature is associated with ice binding, which is consistent earlier findings that ice binding site of AFP is required for ice nucleation (Liu et al., 2016) and the finding that a fragment of an ice nucleator displays antifreeze activity (Kobashigawa et al., 2005). Yet, the nucleation of ice requires more than ice binding. As demonstrated by the His-SUMO-AFP. Although this larger construct had normal antifreeze activity, it did not nucleate ice under the conditions in which the AFP did. Therefore, it appeared that the larger His-SUMO attachment interfered with another behavior of the AFP required for ice nucleation. Nucleators are proposed to be distinct from AFPs due to the repeated nature and size of their ice-binding sites (Kobashigawa et al., 2005). Therefore, the transition of the AFP in this study to a nucleating form may be a result of protein assembly into larger structures. The ice-induced formation of amyloid by this AFP demonstrates its propensity for assembly, at least under one form (Dubé et al., 2016; Graether et al., 2003). By extension, it could be argued that the bulky His-SUMO attachment impedes an assembly of the AFP that is required for its transition to a nucleating form. Considering the findings above, it is possible that AFP forms amyloid at very low sub-zero temperature and consequently promotes ice nucleation (Dubé et al., 2016; Graether et al., 2003; Gurian-Sherman &

Lindow, 1993; Qiu et al., 2019). If the relatively large size of His-SUMO tag interferes with an aggregation or amyloid formation process, this can be investigated in future work.

Although it is still unconfirmed in this study whether the effect of ice nucleation inhibition at high sub-zero temperature is caused by direct interactions between AFP and the ice nucleating agent, it was suggested by many studies that the AFP can bind to ice nucleating sites of AgI to inhibit its ice nucleating function due to the similarities of ice crystals and the surface of AgI (Inada et al., 2012; Liu et al., 2016). Taken together, these results suggest a testable model in which monomeric AFP binds to ice nucleating agents to inhibit ice nucleating at high sub-zero temperature, but with low sub-zero temperature causing AFP to aggregate and therefore promotes ice nucleation.

Although it was suggested that the aggregates of AFP can induce ice nucleation (Qiu et al., 2019), to test this hypothesis, it would be necessary to determine whether AFP can aggregate at low temperature, whether through amyloid formation or another mechanism. However, the induction of AFP aggregation by repeated freezing and thawing could be inconsistent; therefore, it might be necessary to cross-link AFP monomers or express AFP multimers to confirm that the increased repeats of AFP is associated with increased ability of ice nucleation promotion. Also, it is known from this study that adding an unrelated large domain to the N-terminus of AFP can prevent it from promoting ice nucleation at low sub-zero temperature. Therefore, the effect of this addition on amyloid propensity should be tested in order to determine whether there might be a relationship between these features.

In future, the investigation of different temperature regimens, freezing speeds and freezing volumes could be explored. Binding of the AFP to a solid surface, e.g. through its His tag, might also allow informative experiments. It is unlikely that these properties of the AFP are meaningful in the flounder where they occur naturally; however, they may find applications in freezing technology.

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