

# **Exploring several nonconventional interactions of ice-binding proteins**

by

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A thesis submitted to the Department of Biology

In conformity with the requirements for  
the degree of Doctor of Philosophy

Queen's University

Kingston, Ontario, Canada

(June, 2016)

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## Abstract

Traditionally, ice-binding proteins (IBPs), also known as antifreeze proteins (AFPs), have been defined by two universal activities: ice recrystallization inhibition and thermal hysteresis. However, there remains the possibility IBPs have other complementary functions given the diversity found within this protein group. This thesis explores some of these in both natural and applied settings, in the hopes of furthering our understanding of this remarkable group of proteins.

Plant IBPs could function as part of a defensive strategy against ice nucleators produced by certain pathogens. To assess this hypothesis, recombinant IBPs from perennial ryegrass and purple false brome were combined with the ice nucleation protein (INP) from the plant pathogen, *Pseudomonas syringae*. Strikingly, the plant proteins depressed the freezing point of the bacterial INP, while a fish AFP could not, nor did the INPs have any effect on IBP activity. Thus, the interaction between these two different proteins suggests a role in plant defensive strategies against pathogenic bacteria as another IBP function.

In addition, the potential use of hyperactive insect IBPs in organ preservation was investigated. Current kidney preservation techniques involve storing the organ at 4 °C for a maximum of 24 h prior to transplantation. Extending this “safe” time would have profound effects on renal transplants, however, ischemic injury is prevalent when storage periods are prolonged. Experiments described here allowed subzero preservation for 72 h with the addition of a beetle IBP to CryoStasis® solution. Kidneys stored using the traditional technique for 24 h and the method developed here for 72 h showed similar levels of biomarker enzymes, underscoring the potential utility of insect IBPs for future transplant purposes.

Finally, IBP function in the freeze-tolerant gall fly, *Eurosta solidaginis*, was examined. Larvae representing the mid-autumn stage displayed ice-binding activity, suggesting an IBP is being expressed, possibly as a protective measure against freezing damage when fall temperatures can unpredictably drop. IBP activity was also observed in the larvae’s host plant, *Solidago spp.* Mass spectrometry analysis of ice-

affinity purified plant extracts provided three candidate pathogenesis-related proteins that could be responsible for the detected activity, further demonstrating additional functions of IBPs.

## **Co-Authorship**

### **Chapter 2: Perturbation of bacterial ice nucleation activity by a grass antifreeze protein**

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Biochemical and Biophysical Research Communications (2014) 452: 636-641.

I was responsible for data collection and analysis. I also wrote the manuscript with editorial input from V.K. Walker.

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### **Chapter 4: Kidney preservation at subzero temperatures using a novel storage solution and insect antifreeze proteins**

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In preparation for submission to Cryoletters

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the cell staining analysis presented in Figure 4.1. I wrote the manuscript with editorial input from all other authors.

### **Chapter 5: Goldenrod and a gall fly: two ice-binding proteins**

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In preparation for eventual submission

I was primarily responsible for experimental design, data collection and analysis. H. Hussain was an undergraduate student who performed the preliminary screening of goldenrod for IBP activity. K.B. Storey provided the acclimated *E. solidaginis* larvae. I wrote the manuscript with editorial input from V.K. Walker.

## Acknowledgements

First and foremost, I need to thank my wonderful supervisor, Dr. Virginia Walker. Without your guidance and support, this thesis would not exist. You knew when pushing was required, but also showed incredible kindness and compassion when life threw curve balls. I am forever grateful for the opportunity you provided me and the priceless knowledge I have gained working in your lab.

I want to thank my committee members, Drs. Fredrick Kan, Olga Kukal, and William Plaxton for their insightful assistance during the progression of this thesis. A special thank you is required to Olga Kukal and Tom Allen, for taking a chance and allowing me to work on the CryoStasis project.

I would also like to thank all past and present members of the “Bug Group”, as well as other graduate students I have met along the way. A special thank you needs to be given to Kristin Spong for being a true friend. All the laughter, advice, and sincerity are irreplaceable.

A huge thank you to my family. To Mom and Dad - you have always been my cheerleaders in the background. This PhD would have never been finished without your caring and selfless support. My fiancé Scott, you came into my life at the beginning of this journey and have continuously shown incredible encouragement, even when I expressed doubts. Thank you for all the emotional (and financial!) support you have provided throughout this process. Aaron, thank you for your wondrous smile and amusing “toddler chatter”. Even on the darkest days, coming home to you always puts a smile on my face.

While unconventional, I would like to close this section with a quote that resonated with me during the progression of this thesis. While generally credited to Winston Churchill, to the best of my knowledge, the true author is unknown:

“Success is not final, failure is not fatal: it is the courage to continue that counts” - unknown

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## List of Abbreviations

AFGL	antifreeze glycolipid
AFGP	antifreeze glycoprotein
AFP	antifreeze protein (interchangeable with IBP)
ANOVA	analysis of variance
<i>BdIRI/BdIBP</i>	<i>Brachypodium distachyon</i> IBP
CrS	CryoStasis solution
<i>DcIBP</i>	<i>Dendroides canadensis</i> IBP
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetra acid
EGTA	ethylene glycol tetraacetic acid
GFP	green fluorescent protein
GMO	genetically modified organism
IAP	ice affinity purification
IBP	ice-binding protein (interchangeable with AFP and IRIP)
INA	ice nucleation activity
INA <sup>+</sup>	ice nucleating active
INP	ice nucleation protein
IPTG	isopropyl $\beta$ -D-1-thiogalactopyranoside
IR	ice recrystallization
IRI	ice recrystallization inhibition
IRIP	ice recrystallization inhibition protein (interchangeable with IBP)
IVC	inferior vena cava
kDa	kiloDalton
LB	Luria-Bertani medium
LC MS/MS	liquid chromatography with tandem mass spectrometry
<i>LpAFP/LpIBP</i>	<i>Lolium perenne</i> IBP
LRR	leucine rich repeat
mOsm	milliosmoles
MP	machine perfusion
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
OD	optical density

PR	pathogenesis-related
PTU	phenylthiourea
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RCH	rapid cold hardening
ROS	radical oxygen species
SEM	standard error of the mean
TEV	tobacco etch virus
TH	thermal hysteresis
<i>TmAFP/TmIBP</i>	<i>Tenebrio molitor</i> IBP
Tris	tris(hydroxymethyl) aminomethane
TSB	tryptic soy broth
UW	University of Wisconsin solution

# Chapter 1

## General Introduction and Literature Review

### 1.1 Low temperature stress and survival strategies

Exposure to subzero conditions presents a number of distinctive challenges that organisms must successfully overcome to survive. An array of potentially detrimental effects have been reported including the production of radical oxygen species (ROS), DNA damage, alterations in membrane fluidity, as well as changes in protein stability and function (Suzuki and Mittler, 2005; Margesin *et al.*, 2007). Additionally, subzero temperatures present the unique challenge of ice crystallization. Water sequestration into ice crystals, combined with solute exclusion from the growing ice, results in osmotic gradients that encourage cellular dehydration (Mazur, 1970) resulting in a potentially toxic environment. The formation of intra- and extracellular crystals can also physically damage cellular barriers, increasing susceptibility to disrupted ion gradients and lethal changes in cellular volume (Mazur, 1970).

To successfully adapt to these conditions, some overwintering species have evolved a number of different mechanisms which can be categorized under two different survival strategies. Freeze-avoidance is characterized by the supercooling of cellular fluids to maintain a liquid state below the equilibrium freezing point. The production of ice-binding proteins (IBPs) or the accumulation of compatible solutes are two processes commonly employed to achieve freezing point depression. IBPs permit supercooling by inhibiting the growth of embryonic ice crystals in a non-colligative manner (Raymond and DeVries, 1977), while the synthesis of low-molecular weight compatible solutes, such as soluble sugars and glycerol, provide colligative depression of the freezing point in some species.

Alternatively, freeze-tolerant organisms use a contrasting strategy by permitting the controlled growth and formation of extracellular ice. This can be accomplished in a variety of ways including the use of ice nucleators to initiate ice crystal formation, expression of IBPs to

function as ice recrystallization (IR) inhibitors, as well as compatible solute synthesis to provide protection from intracellular freezing and to stabilize cellular volume (Sidebottom *et al.*, 2000; Mugnano *et al.*, 1996; Storey and Storey, 1983). Additionally, a number of pronounced biochemical and physiological changes within cells are seen in both freeze-avoiding and freeze-tolerating species to cope with subzero temperatures. This includes the reduction of metabolic rates, increased expression of radical oxygen species (ROS) to detoxify substances, the production of cold shock proteins, chaperones and cold-adaptive enzymes (reviewed in Margesin *et al.*, 2007), along with mitochondrial degradation (Kukal *et al.*, 1988) and changes to membrane composition, including increased production of phospholipids and fatty acid unsaturation (Uemura *et al.*, 2006).

## **1.2 Ice-binding proteins: controlling the nucleation and growth of ice**

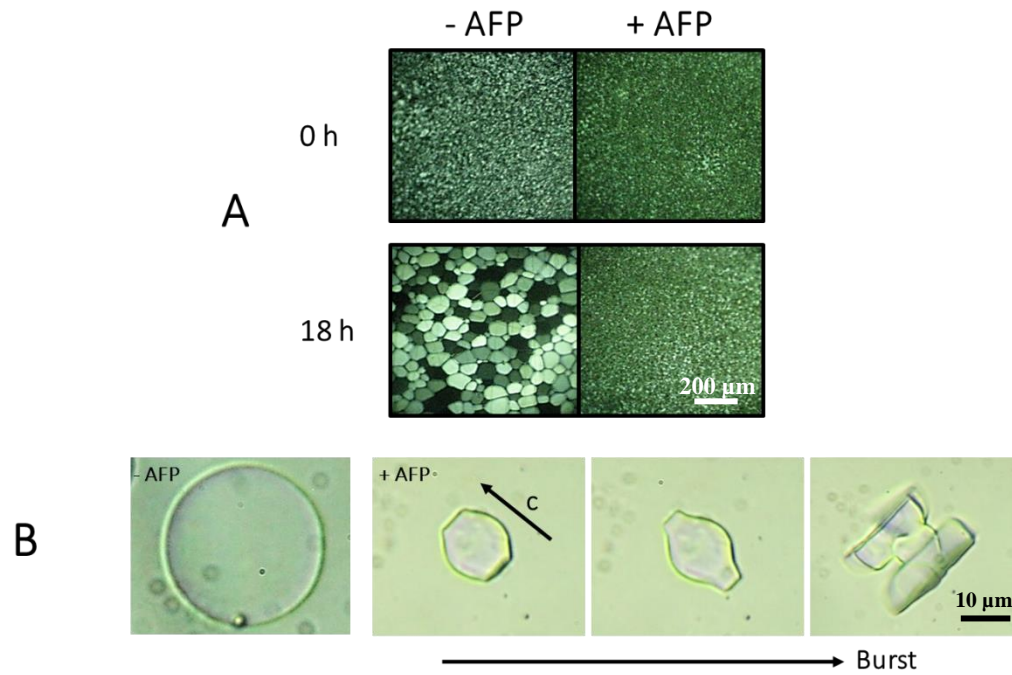
Ice-binding proteins (IBPs) are a noteworthy group of proteins characterized by their ability to interact with ice, directly manipulating its nucleation and growth. Examples of IBPs include the Types I-IV antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs) synthesized by certain polar fish to depress the freezing point of their serum, along with AFPs expressed by some freeze-tolerant plants as protection from the damaging effects of uncontrolled ice crystal growth. Ice nucleation proteins (INPs) are also generally included in the IBP grouping; however, our group believes that this classification is questionable since little evidence exists suggesting these proteins can bind directly to an ice surface (see Chapter 2). Nonetheless, INPs function as heterogeneous ice nucleators, permitting some freeze-tolerant species to actively promote extracellular ice formation, thereby increasing intracellular solute concentration and protecting cellular structures from ice damage.

### ***1.2.1 Antifreeze proteins***

Since the discovery of AFGPs and AFPs in polar fish (Raymond and DeVries, 1977), AFPs, or simply IBPs, have also been reported in a range of cold tolerant species including plants

(Atici and Nalbantoglu, 2003), insects (Graether and Sykes, 2004), fungi (Duman *et al.*, 1993) and bacteria (Kawahara *et al.*, 2004). Curiously, despite this diversity, few sequence and structural similarities exist between them, suggesting most AFPs have likely evolved independently with similarities, a result of convergent evolution. This hypothesis is further strengthened by evidence that many AFPs have evolved from proteins which originally contained binding motifs or nucleic acid sequences for other molecules. This includes carbohydrate binding  $\text{Ca}^{2+}$ -dependent lectins (e.g. Type III AFP), chitinases (e.g. winter rye AFP), polygalacturonase inhibitor proteins (e.g. carrot AFP), as well as trypsinogen-like proteases (e.g. AFGPs) (Ewart *et al.*, 1999). In plants, some of these proteins that share motifs with AFPs are pathogenesis-related (PR) products (Griffith and Yaish, 2004), although the significance of this observation is currently unknown. However, no matter their origin, AFPs remarkably all display the ability to adsorb to growing ice surfaces, thermodynamically restricting the growth of the ice to areas in between adsorbed molecules (Raymond and DeVries, 1977). Informally termed the “mattress-button” model, the growth between adsorbed AFPs creates an increase in ice surface curvature which, once a critical radius is reached, results in the continued growth of the ice lattice to become an energetically-unfavorable event (Barrett, 2001). Consequently, a non-equilibrium depression of the freezing point of the solution occurs without significant alteration to the melting point. This temperature difference, termed thermal hysteresis (TH), is considered one of the ubiquitous functions of AFPs. The interaction with ice surfaces also resulting in AFPs “shaping” the ice crystals, due to adsorption onto different planes of ice (Figure 1.1).

While TH activity can be used as part of a freeze-avoidance strategy, as seen in certain polar fish, freezing may be an inevitable process for other organisms, such as some cold-tolerant



**Figure 1.1** Examples of ice recrystallization (A) and ice crystal morphology (B) in the presence and absence of an AFP (shown using an AFP from perennial ryegrass (A) and gall fly larvae (B), respectively). Magnifications are indicated by the bars; the c-axis is indicated by the arrow (B); “burst” refers to ice crystal growth once the TH limit has been exceeded (B).

plants. In these instances, AFPs function by preventing the formation of large, damaging ice crystals (Knight and Duman, 1986). At temperatures close to 0 °C, water movement in boundaries between ice crystals results in the formation of large crystals at the expense of smaller ones. Referred to as ice recrystallization (IR), absorbed AFPs obstruct this water movement, preserving a small ice crystal size (Figure 1.1; Knight *et al.*, 1995). This second notable function of AFPs, IR inhibition, is crucial for freezing tolerance where the formation of large ice crystals can have injurious consequences on fragile cellular structures. Plant AFPs in particular have been reported to have high IR inhibition but low TH activity (Pudney *et al.*, 2003), and thus their IBPs are often referred to as IR inhibition proteins (IRIPs). Presumably this characteristic is due to the survival strategy of perennials, where controlling the size and growth of ice crystals is more important than the ability to supercool tissue fluids.

The detailed mechanisms behind AFP interactions with an ice surface have been an area of continual development since the initial discovery of IBPs. Over the years, several different hypotheses have been proposed. Originally, it was suggested hydrogen binding was the main influence behind AFP binding (Knight, 1991). Mutagenesis studies of postulated ice-binding faces resulted in the recognition of the hydrophobic composition of these surfaces. This hydrophobic characteristic in turn resulted in the proposal of a second hypothesis stressing the importance of hydrophobic and van der Waals interactions in AFP-ice interactions (Jia and Davies, 2002; Davies *et al.*, 2002). More recent modelling investigations have suggested that the ice-binding faces of AFPs organize water molecules in a clathrate-like formation, allowing absorption onto the water-ice interface of growing ice crystals (Smolin and Daggett, 2008; Nutt and Smith, 2008).

### ***1.2.2 Characteristics of selected AFPs***

#### **AFP from the perennial ryegrass, *Lolium perenne***

The AFP from the perennial ryegrass, *L. perenne* (*LpIBP*), was originally isolated by Sidebottom *et al.* (2000) from the leaves of this freeze-tolerant turf grass. Characterized as a 118-residue protein (~12 kDa) with an irregular repeating ice-binding motif (XNXVX), the protein exhibits low TH activity of only 0.1°C – 0.45°C. However, *LpIBP* does demonstrate high IRI activity (Pudney *et al.*, 2003) compared to AFPs from other species, suggesting the AFP's main function is for IR inhibition, rather than the supercooling of the plant's tissue. The overall structure of this protein is comprised of a parallel  $\beta$ -roll domain with two hydrophobic, flat surfaces on either side (Fig. 1.2). The presence of these two separate flat faces was initially thought to account for the superior IRI activity exhibited by *LpIBP*, creating two ice-binding faces that allow the protein to bind and stabilize adjacent ice crystal boundaries (Kuiper *et al.*, 2001). However, mutagenesis experiments along with crystallography analysis subsequently demonstrated that only one face ('a'-side) was involved in ice-binding (Middleton *et al.*, 2009; Middleton *et al.*, 2012).

#### **AFP from the yellowmeal worm, *Tenebrio molitor***

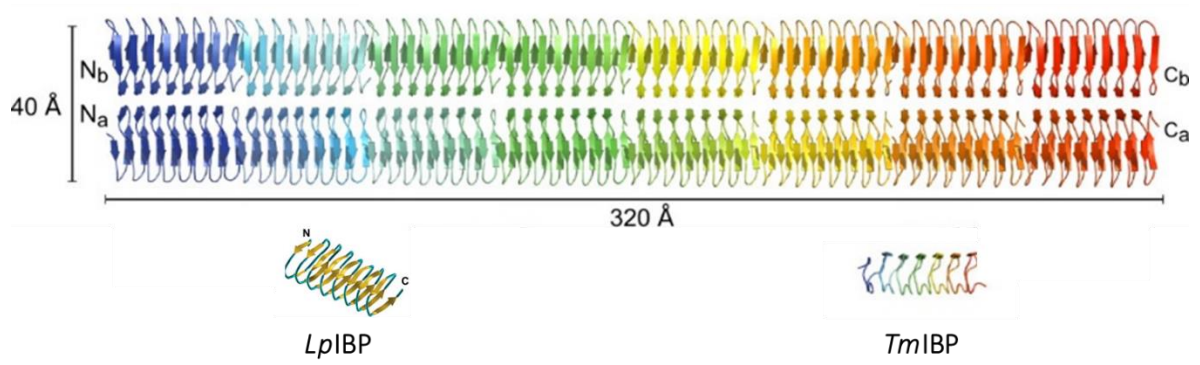
The AFP from the freeze-avoiding yellow mealworm, *T. molitor* (*TmIBP*) was originally isolated from larval hemolymph (Graham *et al.*, 1997). This small protein (~8.4 kDa) folds into  $\beta$ -helical structure (Liou *et al.*, 2000), with a tandemly repeating 12 amino acid sequence (TCTXSXXCXXAX). Strikingly, this AFP exhibits significantly high levels of TH activity, showing 10- to 100-fold greater activity compared to moderately active fish AFPs (Graham *et al.*, 1997; Liou *et al.*, 1999). This increase in activity was postulated to be due to the regularity of the ice-binding face (Fig. 1.2), characterized by a highly conserved TXT ice-binding motif (Liou *et al.*, 2000), providing the larva the ability to supercool their bodily fluids to temperatures as low as -12 °C.

### 1.2.3 Ice nucleation proteins

Ice nucleation activity (INA) has been reported in several different organisms, such as larvae of the gall fly, *Eurosta solidaginis*, where it is reportedly due to  $\text{Ca}(\text{PO}_4)_2$  crystals (Mugnano *et al.*, 1996). However, INA conferred by microorganisms are more active and are the most powerful nucleation agents, aside from ice itself. As such, bacterial INPs are well characterized. At least three different bacterial genera have been identified as INP producers, including isolates from *Pseudomonas*, *Erwinia*, and *Xanthomonas* (Kawahara, 2002). INPs are hypothesized to function as a template for ice nucleation, displayed as highly repetitive aggregates on the outer membrane of these certain Gram negative strains (Wolber and Warren, 1989; Gurian-Sherman and Lindow, 1993; Vanderveer *et al.*, 2014). Most of these species are also frequently viewed as plant pathogens, where INP expression is postulated to be responsible for frost damage seen in plants at temperatures just below 0 °C (Lindow *et al.*, 1983).

Within ice nucleating strains (INA<sup>+</sup>), INPs are categorized based upon differences in INA levels (Yankofsky *et al.*, 1981). Type I nuclei are the most active biological nucleators, promoting ice formation at temperature > -5 °C. Type II and III nucleators promote ice formation at temperatures between -5 °C to -7 °C, and below -7 °C, respectively. While each type of nuclei can be found in cells belonging to a single culture, levels of INA depend on several factors including membrane anchoring, culture conditions, and posttranslational modifications (Kozloff *et al.*, 1991; Turner *et al.*, 1991; Cochet and Widehem, 2000) and perhaps dimerization (Figure 1.2; Garnham *et al.*, 2011).

INA has been best characterized in the widely distributed plant epiphyte, *Pseudomonas syringae*. Encoded by a single gene, *inaZ* (Tegos *et al.*, 2000), the 120 kDa INP produced by these isolates can be divided into non-repetitive N- and C- terminal domains involved in membrane anchoring and possibly protein stability, respectively, as well as a highly repetitive central region.



**Figure 1.2** Size comparison between dimerized bacterial INPs from *P. borealis* (above) and two known AFPs from *L. perenne* (*LpIBP*) and *T. molitor* (*TmIBP*; below). Modified from Garnham *et al.* (2011) and Kuiper *et al.* (2001).

The central domain has been modeled as a  $\beta$ -helical structure, similar to the structure seen in some plant and insect AFP models but are approximately 10-fold longer (Figure 1.2; Graether and Jia, 2001; Garnham *et al.*, 2011). While the exact mechanism behind INPs' nucleation activity is not yet fully understood, the central domain is believed to provide a template for ice nucleation, consisting of ~ 48 repeats of 16 residues (Green *et al.*, 1988). INPs are proposed to assemble into an overlapping structure on the surface of bacterial cells, dimerizing with adjacent INPs to increase the surface area available for water organization and hence, ice propagation (Wu *et al.*, 2009; Garnham *et al.*, 2011).

### **1.3 Biotechnological applications of IBPs**

The intrinsic abilities of IBPs to both prevent and control the growth of ice have been proposed or studied for a range of applications in agriculture, medicine, animal reproduction, frozen food engineering, ice-inhibiting coatings, as well as hydrocarbon exploration and transport (summarized in Table 1.1). While few applications have yet to see wide-scale use, such new developments improve our understanding of how IBPs might assist with the industrial and societal challenges experienced during subzero temperatures.

#### ***1.3.1 Applications of IBPs in agriculture***

Exposure to low temperatures can result in a significant decrease in crop yield (Xin and Browse, 2000). In temperate climates and at high altitudes, these temperatures are a restrictive factor for agricultural output. Subzero temperatures are particularly damaging, especially when they lead to ice crystallization resulting in damage to delicate cellular structures. For these reasons, it has been of interest to increase cold- and freeze-tolerance in economically-important crops (reviewed in Sanghera *et al.*, 2011). Recombinant IBPs have been expressed in several

**Table 1.1** Summary of several biotechnological applications of IBPs

	<b>IBP (Gene origin)</b>	<b>Industry</b>	<b>Application</b>
<b>Fish</b>	AFGP (Antarctic notothenioids)	Animal Reproduction Medicine Food engineering	Cryopreservation of animal oocytes,embryos, and sperm <sup>1</sup> Cryopreservation of cardiomyocytes, hearts, and livers <sup>1</sup> Improving quality of frozen meat <sup>2</sup>
	Type I (Winter flounder)	Agriculture Animal Reproduction Medicine Food engineering Petroleum	Transformation of tomato, potato, and tobacco <sup>3</sup> Cryopreservation of animal sperm <sup>1</sup> Cryopreservation of red blood cells <sup>1</sup> Improving quality of frozen meat <sup>2</sup> Hydrate inhibition <sup>4</sup>
	Type II (Sea raven)	Agriculture Medicine	Transformation of tobacco <sup>3</sup> Cryopreservation of red blood cells <sup>1</sup>
	Type III (Antarctic eel pout)	Animal Reproduction Medicine Biomaterials Food engineering Petroleum	Cryopreservation of animal oocytes,embryos, and sperm <sup>1</sup> Cryopreservation of red blood cells <sup>1</sup> Ice-inhibiting polymers <sup>5</sup> Ice cream manufacturing and sumiri preservation <sup>2</sup> Hydrate inhibition <sup>6</sup>
<b>Insect</b>	<i>Cf</i> BP (Spruce bud worm)	Agriculture Petroleum	Transformation of tobacco <sup>3</sup> Hydrate inhibition <sup>4</sup>
	<i>Decl</i> BP (Fire-coloured beetle)	Agriculture	Transformation of mustard weed <sup>3</sup>
	<i>Tm</i> BP (Yellow mealworm)	Petroleum	Hydrate inhibition <sup>6</sup>
	<i>sf</i> BP (Snow flea)	Biomaterials	Ice-inhibiting polymers <sup>5</sup>
<b>Plant</b>	<i>Dacl</i> BP (Carrot)	Agriculture	Transformation of tobacco and mustard weed <sup>3</sup>
	<i>Lpl</i> BP (Perennial ryegrass)	Agriculture Medicine Petroleum	Transformation of mustard weed <sup>7</sup> Hepatoma tumor cell preservation <sup>8</sup> Hydrate inhibition <sup>6</sup>
	Winter rye IBP	Food engineering	Ice cream manufacturing <sup>2</sup>
<b>Microbes</b>	<i>Cn</i> BP (Antarctic diatom)	Biomaterials	Ice-inhibiting polymers <sup>9</sup>

<sup>1</sup> reviewed in Brockbank *et al.*, 2011; <sup>2</sup> reviewed in Petzold and Aguilera, 2009; <sup>3</sup> reviewed in Griffith and Yaish, 2004; <sup>4</sup> Zeng *et al.*, 2003; <sup>5</sup> Esser-Kahn *et al.*, 2010; <sup>6</sup> Gordienko *et al.*, 2010; <sup>7</sup> M. Bredow, 2016, unpublished; <sup>8</sup> Capicciotti *et al.*, 2015; <sup>9</sup> Gwak *et al.*, 2015

different plants to assess if they can augment the plants' tolerance to subzero temperature exposure.

An assortment of plants, including tobacco, rice, tomatoes, potatoes and mustard weed, have been transformed with several different fish, insect, and plant IBPs. The Type I fish AFP gene sequence from winter flounder (*Pseudopleuronectes americanus*) has been transferred to several plant species, including tomatoes (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*) to assess if the transfer of IR inhibition ability has the potential to increase the quality of frozen foods. In both cases, a fusion protein version of Type I was found to accumulate within the cells and IR inhibition was detected in the transgenic lines (Hightower *et al.*, 1991). It was not reported if the recombinant IBP expression permitted supercooling of the host plants. Several other studies have explored the transformation of plants with the Type I AFP. Kenward *et al.* (1993) determined that low temperatures (4 °C) were required for the AFP to stably accumulate within the plant tissue, while transgenic potatoes (*Solanum tuberosum*) expressing recombinant Type I AFP presented better cold tolerance, tolerating a 1 °C drop in temperature compared to wild-type controls (Wallis *et al.*, 1997). Tobacco has also been transformed with the Type II fish AFP from the sea raven (*Hemirhamphus americanus*) and tested in field trials for resistance to early fall frosts. While IR inhibition and minimal TH was detected in apoplastic extracts, no difference in frost resistance was observed between transformed lines and wild-type plants (Kenward *et al.*, 1999).

Fish AFPs are not likely ideal for conferring freeze resistance to crops due to the morphology of ice crystals that form in their presence. By binding onto the pyramidal planes of ice crystals, fish AFPs result in the formation of a bipyramidal crystals, with sharp projections growing from the c-axis once the TH limit has been reached (Fletcher *et al.*, 2001). This needle-like growth could cause damage to surrounding cells by piercing cellular membranes (Carpenter and Hansen, 1992; Ishiguro and Rubinsky, 1994). However, several insects and plants IBPs have

also been investigated. Tobacco has been transformed with both the spruce budworm (*Choristoneura fumiferana*) and carrot (*Daucus carota*) IBPs which may have a potentially less damaging “burst” morphology (e.g. Figure 1.1B). In each instance, IR inhibition and low levels of TH were detected in the transgenic lines (Holmberg *et al.*, 2001; Worrall *et al.*, 1998). Expression of the carrot IBP in particular permitted supercooling of the plant to -2 °C (Fan *et al.*, 2002). The mustard weed, *Arabidopsis thaliana*, has also been transformed with the carrot IBP, as well as the IBP originally isolated from the fire coloured beetle (*Dendroides canadensis*; *DcIBP*). Plants transformed with the carrot IBP exhibited typical IBP-associated ice shaping (Meyer *et al.*, 1999), while *DcIBP* lowered the freezing point of the whole plants and TH activity was detectable in apoplastic extractions (Huang *et al.*, 2002). In our lab, the transfer of sequences encoding multiple isoforms of *LpIBP* to *A. thaliana* conferred dramatic freeze tolerance to host plants (M. Bredow, 2016, unpublished), underscoring the potential of a gene transfer strategy utilizing the IBP from a freeze-tolerant species.

### ***1.3.2 Applications of IBPs in medicine and animal reproduction***

Cryopreservation involves the storage of biological substances in a frozen state; however, purely freezing most mammalian cells and tissues generally results in nonfunctional material once thawed. Cryoinjury occurs during the preservation process, due to stress along cellular membranes, dehydration, intra- and extracellular ice crystals formation, as well as toxic solute build-up from water sequestration into the ice (reviewed in Gao and Critser, 2000). For instance, sperm cells are known to have an unusual array of lipids and sterols, resulting in challenges for the cell when forced to undergo phase transitions (Holt *et al.*, 1992; Holt, 2000). As such, a loss of membrane integrity is often observed when spermatozoa are frozen and subsequently thawed, resulting in the loss of up to 50% of viable spermatozoa during the freeze-thaw process (Watson, 2000). As previously noted, there are multiple examples of species that can survive subzero temperatures, using a variety of different mechanisms as outlined in Section 1.1 of this thesis. It is

these organisms that have inspired studies investigating the use of naturally-occurring compounds in cryopreservation techniques, including IBPs.

IBPs have been used for experimental cryopreservation of mammalian cells, embryos, and tissues. High concentrations of AFGP (40 mg/mL) added to vitrification solutions improved post-thaw viability of immature pig oocytes, as well as two-cell stage murine and pig embryos (Rubinsky *et al.*, 1992). Interestingly, the addition of an AFP from the Antarctic eel pout did not show any improvements in mammalian post-thaw viability (Rubinsky *et al.*, 1992). The cryopreservation of chimpanzee and bovine sperm in solutions with added Type III fish AFP, as well as ram sperm with Type I and AFGPs, displayed improved motility, osmotic resistance, and post-thaw acrosome integrity (Younis *et al.*, 1998; Prathalingam *et al.*, 2006; Payne *et al.*, 1994a). Micromolar concentrations of fish AFPs were also found to decrease levels of hemolysis in frozen red blood cells, although this protective effect was abolished at millimolar concentrations (Carpenter and Hansen, 1992; Chao *et al.*, 1996). In more complex tissue systems, whole rat livers frozen with a high concentration of glycerol and AFGPs were observed to have better bile production and less hepatocyte damage (Rubinsky *et al.*, 1994).

Contrariwise, several studies have shown opposite effects of IBP addition. Fish AFPs and AFGP appeared to have a cytotoxic effect on sperm motility at temperatures above 0 °C (Younis *et al.*, 1998; Payne *et al.*, 1994), while mouse sperm frozen with Type I, III, and AFGP displayed lower viability post thaw (Koshimoto and Mazur, 2002). Similarly, frozen equine embryos were unsuccessfully preserved with a fish AFP (Lagneaux *et al.*, 1997). Hepatoma tumor cells suffered more damage compared to controls when frozen in the presence of a plant IBP, while cardiomyocytes frozen with AFGP showed intracellular freezing; both cases attributed to AFP-mediated ice crystal morphology (Capicciotti *et al.*, 2015; Mugnano *et al.*, 1995). Whole rat hearts frozen with AFGP also showed poor recovery post-thaw (Wang *et al.*, 1994).

Despite these conflicting results, which could be attributed to differences in cooling and warming rates (Mazur, 1970), there is still interest in the applications of AFPs for cryopreservation and other medical purposes. This mainly is driven by evidence that suggests fish AFPs can interact with and bind to lipid membranes, conceivably protecting the membrane from hypothermic damage when exposed to low temperatures. Fish AFPs and AFGPs appear to interact with and stabilize model lipid membranes during phase transitions (Tomczak *et al.*, 2002; Kun *et al.*, 2008). While the mechanisms behind these interactions are currently unknown, it has been proposed that AFGPs form a monolayer on the membrane to prevent cell leakage, although this interaction appears to be dependent upon the lipid structure and composition of the membrane (Garner *et al.*, 2008). Type I AFPs are hypothesized to insert directly into the membrane, providing stability by improving the orderly packing of acyl chains in the membrane (Kun *et al.*, 2008). Prathalingam *et al.* (2006) observed that GFP-tagged Type I and Type III AFPs bound directly to bovine sperm membranes, while Arav *et al.* (1993) reported that AFGPs, Type I, II and III fish AFPs were able to protect the integrity of pig oocyte oolemma after rapid cooling to -130 °C. The addition of Type III AFPs into cosmetic face creams is another example of the potential for cell preservation by AFPs, even at elevated temperatures (LiftLabs, AF/Protein Inc., E. Entiss, pers. comm.). Clearly, more could be done to further our understanding of IBP applications in medical and animal reproduction technology.

### ***1.3.3 Applications of IBPs in frozen food***

Ice crystal morphology plays an important role in the quality and texture of frozen foods. Large, irregular ice crystals create an unappealing texture resulting in low product quality, due to damaged cell membranes and the subsequent loss of fluid during thawing, or simply from the “crunchy” texture (reviewed in Griffith and Ewart, 1995). While rapid cooling can initiate the formation of small ice crystals, growth does occur at high subzero temperatures from thawing in transit and during defrost cycles in storage.

The importance of controlling freezing is best exemplified in the ice cream industry where ice crystal morphology directly influences quality. A smooth, creamy texture is obtained through small ice crystals, while large crystals result in an inferior product characterized by a grainy consistency. Sugars present in the mixture play a large role in crystallization since their addition can depress the freezing point, leading to a portion of water that remains unfrozen at high, subzero temperatures (reviewed in Petzold and Aguilera, 2009). To combat this, stabilizers such as carrageen and xanthan gum are generally introduced to function as IR inhibitors (Sutton and Wilcox 1998a, 1998b). Novel inhibitors from other natural sources have also been explored, including the use of several different IBPs. IBPs from winter rye (*Secale cereal*; Regand and Goff, 2006), as well as the Type III fish AFP (Warren *et al.*, 1992 – USA patent) have both been studied for use in ice cream manufacturing. Termed “ice structuring proteins” by the industry, these IBPs have shown the potential to preserve quality texture during ice cream storage by inhibiting IR. Indeed, Unilever includes a plant IBP in its more expensive ice cream formulations (Breyer’s). This IBP was selected due to its high IR inhibition activity and its stability even after pasteurization temperatures.

IBP treatment has also been explored in the freezing of meat products. Soaking bovine and ovine muscle in Type I fish AFP and the AFGP from Antarctic cod (*Dissostichus mawsoni*), and subsequent storage at -20 °C showed smaller ice crystal formation compared to non-AFP controls (Payne *et al.*, 1994b). Similarly, lambs intravenously injected with AFGPs prior to slaughter displayed reduced ice crystal size during freezing, in addition to reduced drip loss after thawing (Payne and Young, 1995). Likewise, fresh pork and dough pretreated with a crude IBP extract from the recombinant bacteria, *Lactococcus lactis*, displayed both lower drip loss and better fermentation following freezing, respectively (Yeh *et al.*, 2009). Type III fish AFP was also shown to better protect Ca<sup>2+</sup> ATPase activity in surimi muscle following freezing compared to conventional cryoprotectants (Boonsupthip and Lee, 2003). It has also been suggested that IBPs

could improve the preservation of fruit, such as strawberries, that generally result in changes to texture and flavor from cellular damage during the freezing process. The introduction of IBPs could promote small ice crystal size to prevent damage to cellular membranes, as well as inhibit solute effects leading to dehydration of cells and a loss of cellular integrity (Griffith and Ewart, 1995).

#### ***1.3.4 Applications of IBPs as ice-inhibiting polymers***

Protein-polymer biomaterials are created through the conjugation of proteins or peptides onto synthetic surfaces, permitting enhanced control over biological function for a range of industrial applications (Krishan and Kiick, 2010). In particular, efforts have been made to adhere IBPs to different substrates, including plastic, where they can function as an “anti-icing” coating to prevent ice accumulation on machinery components. Recombinant Type III fish AFP and the hyperactive insect IBP from the snow flea have been attached onto synthetic surfaces using oxime formation. In both cases, the IBP-polymer showed enhanced TH activity, as well as inhibited the formation of frost on a glass surface (Esser-Kahn *et al.*, 2010). Likewise, a recombinant fusion IBP from the Antarctic marine diatom, *Chaetoceros neogracile*, has been successfully attached onto an aluminum surface, resulting in a significant decrease in the supercooling point (Gwak *et al.*, 2015).

#### ***1.3.5 Applications of IBPs as hydrate inhibitors***

Hydrate formation is a major hurdle for the petroleum industry. Forming under high pressure and low temperature conditions within gas and oil pipes, hydrates can lead to serious, and even fatal, consequences (Mehta *et al.*, 2006). One of the more tragic incidents involving hydrate plugs is the 1988 Piper Alpha blowout in the North Sea, resulting in the death of 167 oil workers. Structurally different from a typical ice lattice, clathrate hydrates consist of an ice-like cage surrounding guest molecules of hydrocarbon gases. Currently, gas hydrates are mitigated by the use of kinetic inhibitors, such as methanol, poured into the pipe, disrupting their formation

(Koh *et al.*, 2002). However, while effective, these inhibitors are highly toxic to the environment. As such, environmental-friendly, or “green”, inhibitors are sought.

IBPs have been explored to fulfill this “green” inhibitor requirement. Studies have demonstrated their ability to delay the formation and growth of hydrates (Walker *et al.*, 2015). *Lp*IBP and Type III AFP from the ocean pout (Gordienko *et al.*, 2010), as well as the insect AFPs from spruce budworm and the fish Type I from the winter flounder (Zeng *et al.*, 2003) have all been shown to adsorb onto a model hydrate surface (made with tetrahydrofurane) or different types of gas hydrates. Together, these experiments showed that AFPs could inhibit growth better than the chemical inhibitor, polyvinylpyrrolidone (PVP). Type I AFP from the winter flounder was also found to significantly inhibit both the nucleation and growth of propane and methane hydrates, as well as abolish the “memory effect” associated with hydrate nucleation rates (Zeng *et al.*, 2006). *Lp*IBP, Type III fish AFP, and an insect IBP from the yellow mealworm, *T. molitor*, were all found to show better inhibition compared to conventional industry inhibitors, although not as well as next generation inhibitors (Ohno *et al.*, 2010). Despite the distinct morphology of gas hydrates compared to ice, it appears that IBPs adsorb to both crystal types in a similar way (Walker *et al.*, 2015; Sun *et al.*, 2015).

## **1.4 Biotechnological applications of INPs**

### ***1.4.1 Applications of INPs in agriculture and food***

Similar to AFPs but to a lesser extent, INPs have been studied for use in a range of commercial purposes, with emphasis on the INP produced by *P. syringae*. An INP-depleted mutant strain of *P. syringae* was tested to protect crops from frost damage (Lindow and Panopoulos, 1988), which interestingly was the first genetically modified organism (GMO) to be released to the environment, only after many years in U.S. courts. Bacterial and fungal INPs have also been suggested for use as biological pest controls, inhibiting the supercooling capabilities of freeze-tolerant insects (reviewed in Lee *et al.*, 1993).

INPs have been explored for use in several food processing applications. This includes refining freeze-concentration techniques through the initiation of a large ice crystal size (Widehem and Cochet, 2003), and as additives to frozen foods, with the aim of improving food quality by decreasing the degree of supercooling (Li and Lee, 1995), as well as the energy savings accrued by freezing at higher subzero temperatures.

#### **1.4.2 Other applications of INPs**

Artificial snow production at numerous ski resorts is currently the most widely used application, enabling snow production at temperatures as high as 1.1 °C (SnowMax<sup>®</sup>; Wolber, 1993; Cochet and Widehem, 2000). Likewise, seeding clouds with *P. syringae* has been shown to be an effective inducer of precipitation (Ward and Demott, 1989). INPs have also been proposed as an integral part of freezers and air conditioners since lower energy costs can be achieved by freezing at higher temperatures (Tsuchiya *et al.*, 2004).

### **1.5 Thesis objectives and hypotheses**

Despite all that is currently known about IBPs and their functions, there still remains unresolved questions. In particular, while IBPs are most well known for their “antifreeze” properties (TH) and the ability to inhibit IR, it is plausible that they also hold additional functions given the extensive diversity seen within this protein class. For example, recent experiments have identified an alternative function of IBPs for surface adhesion in Antarctic bacteria (Guo *et al.*, 2012). This thesis will explore some non-conventional interactions and applications of IBPs in the hopes of enhancing our understanding of the various roles these proteins play. Thus, my hypothesis is that IBPs have alternative functions or biotechnological applications that could be harnessed to the betterment of society. This thesis explores three such possibilities, posed as questions below.

### **1.5.1 Can plant IBPs function as part of a pathogen defense mechanism against ice-nucleating epiphytes? (Chapters 2 and 3)**

As previously noted, it is recognized that some epiphytic bacteria produce INPs as a way of damaging the leaves and stems of plants, providing access to a rich pool of nutrients (Lindow *et al.*, 1978). Examinations of leaves from the perennial rye grass, *L. perenne*, showed 40% of the bacterial community were known INP producers (Hirano and Upper, 2000). While plant IBPs provide protection from the damaging effects of IR once freezing has occurred (Sidebottom *et al.*, 2000), it is possible that bacterial INPs and plant AFPs could also interact. Could plant IBPs function as a defense mechanism, offering protection not only from IR, as shown by others, but also against the INA of colonizing pathogenic bacteria? It has been previously suggested that insect INPs and IBPs could interact (Olsen and Duman, 1997a, 1997b), but the characterization of any possible interactions between these two different proteins has not been hitherto explored, or examined as a protective mechanism.

### **1.5.2 Can hyperactive insect IBPs be used to extend the preservation time of rat kidneys at low, but non-freezing temperatures? (Chapter 4)**

Current kidney preservation techniques allow storage for approximately 24 h at 4 °C, before cold-induced ischemic injury starts to accumulate (reviewed in Guibert *et al.*, 2011). While it has been shown that decreasing the storage temperature below 0 °C can extend storage times, generally toxic substances are used as freezing point depressors (Scotte *et al.*, 1996). The addition of IBPs to organ preservation solution is not a unique concept, but previously other researchers have solely focused on fish AFPs which only offer moderate TH activity (Amir *et al.*, 2004). Thus, this chapter investigates the use of a proprietary storage solution with and without amendment using a recombinant insect IBP from the yellow mealworm, *T. molitor*.

### ***1.5.3 Does a freeze-tolerant animal produce IBPs? (Chapter 5)***

IBP function has been reasonably established in freeze-intolerant animals, with TH activity permitting the ability to supercool bodily fluids in temperature below 0 °C. Yet, freeze-tolerant animals permit the formation of extracellular ice while maintaining an intracellular liquid state, similar to a strategy known in plants. In such organisms, IBP function is considerably less understood. The larvae of the goldenrod gall fly, *E. solidaginis*, is a well-characterized freeze-tolerant insect (McMullen and Storey, 2008; Pfister and Storey, 2006; Joannis and Storey, 1997). With the ability to endure freezing of ~65% of their total body water, these larvae have been shown to accumulate high concentrations of cryoprotectants (glycerol and sorbitol) and add ice nucleators to their body fluids while overwintering. These mechanisms permit survival in winter temperatures as low as -30 °C (Duman, 2001). Currently, it is unknown if *E. solidaginis* larvae produce ice-binding proteins (IBPs). This chapter explores the possibility that they do.

## 1.6 References

- Amir, G., Rubinsky, B., Horowitz, L., Miller, L., *et al.* (2004). Prolonged 24-hour subzero preservation of heterotopically transplanted rat hearts using antifreeze proteins derived from arctic fish. *The Annals of Thoracic Surgery*, 77: 1648-1655.
- Arav, A., Rubinsky, B., Fletcher, G., & Seren, E. (1993). Cryogenic protection of oocytes with antifreeze proteins. *Molecular Reproduction and Development*, 36: 488-493.
- Atici, O., & Nalbantoglu, B. (2003). Antifreeze proteins in higher plants. *Phytochemistry*, 64: 1187-1196.
- Barrett, J. (2001). Thermal hysteresis proteins. *International Journal of Biochemistry & Cell Biology*, 33: 105-117.
- Boonsupthip, W., & Lee, T. C. (2003). Application of Antifreeze Protein for Food Preservation: Effect of Type III Antifreeze Protein for Preservation of Gel - forming of Frozen and Chilled Actomyosin. *Journal of Food Science*, 68: 1804-1809.
- Brockbank, K. G., Campbell, L. H., Greene, E. D., *et al.* (2011). Lessons from nature for preservation of mammalian cells, tissues, and organs. *In Vitro Cellular & Developmental Biology-Animal*, 47: 210-217.
- Capicciotti, C. J., Poisson, J. S., Boddy, C. N., & Ben, R. N. (2015). Modulation of antifreeze activity and the effect upon post-thaw HepG2 cell viability after cryopreservation. *Cryobiology*, 70: 79-89.
- Carpenter, J. F., & Hansen, T. N. (1992). Antifreeze protein modulates cell survival during cryopreservation: mediation through influence on ice crystal growth. *PNAS*, 89: 8953-8957.
- Chao, H., Davies, P. L., & Carpenter, J. F. (1996). Effects of antifreeze proteins on red blood cell survival during cryopreservation. *Journal of Experimental Biology*, 199: 2071-2076.
- Cochet, N., & Widehem, P. (2000). Ice crystallization by *Pseudomonas syringae*. *Applied Microbiology and Biotechnology*, 54: 153-161.
- Davies, P. L., Baardsnes, J., Kuiper, M. J., & Walker, V. K. (2002). Structure and function of antifreeze proteins. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 357: 927-933.
- Duman, J. G., & Olsen, T. M. (1993). Thermal hysteresis protein activity in bacteria, fungi, and phylogenetically diverse plants. *Cryobiology*, 30:322-328.
- Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annual Review of Physiology*, 63: 327-357.
- Esser-Kahn, A. P., Trang, V., & Francis, M. B. (2010). Incorporation of antifreeze proteins into polymer coatings using site-selective bioconjugation. *Journal of the American Chemical Society*, 132: 13264-13269.

- Ewart, K. V., Lin, Q., & Hew, C. L. (1999). Structure, function and evolution of antifreeze proteins. *Cellular and Molecular Life Sciences CMLS*, 55: 271-283.
- Fan, Y., Liu, B., Wang, H., Wang, S., & Wang, J. (2002). Cloning of an antifreeze protein gene from carrot and its influence on cold tolerance in transgenic tobacco plants. *Plant Cell Reports*, 21: 296-301.
- Fletcher, G. L., Hew, C. L., & Davies, P. L. (2001). Antifreeze proteins of teleost fishes. *Annual Review of Physiology*, 63: 359-390.
- Gao, D., & Critser, J. K. (2000). Mechanisms of cryoinjury in living cells. *ILAR Journal*, 41: 187-196.
- Garnham, C. P., Campbell, R. L., Walker, V. K., & Davies, P. L. (2011). Novel dimeric  $\beta$ -helical model of an ice nucleation protein with bridged active sites. *BMC Structural Biology*, 11: 1.
- Garner, J., Inglis, S. R., Hook, J., Separovic, F., *et al.* (2008) A solid-state NMR study of the interaction of a fish antifreeze protein with phospholipid membranes. *European Journal of Biochemistry*, 271:3285-3296.
- Gordienko, R., Ohno, H., Singh, V. K., Jia, Z., Ripmeester, J. A., & Walker, V. K. (2010). Towards a green hydrate inhibitor: imaging antifreeze proteins on clathrates. *PLoS One*, 5: e8953.
- Graether, S.P., and Sykes, B.D. (2004) Cold survival in freeze-intolerant insects: The structure and function of  $\beta$ -helical antifreeze proteins. *European Journal of Biochemistry*, 271: 3285-3296.
- Graether, S. P., & Jia, Z. C. (2001). Modeling *Pseudomonas syringae* ice-nucleation protein as a beta-helical protein. *Biophysical Journal*, 80: 1169-1173.
- Graham, L. A., Liou, Y. C., Walker, V. K., & Davies, P. L. (1997). Hyperactive antifreeze protein from beetles. *Nature*, 388: 727-728.
- Green, R. L., Corotto, L. V., & Warren, G. J. (1988). Deletion mutagenesis of the ice nucleation gene from *Pseudomonas syringae* S203. *Molecular & General Genetics*, 215: 165-172.
- Griffith, M., & Ewart, K. V. (1995). Antifreeze proteins and their potential use in frozen foods. *Biotechnology Advances*, 13: 375-402.
- Griffith, M., & Yaish, M. W. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. *Trends in Plant Science*, 9: 399-405.
- Guibert, E. E., Petrenko, A. Y., Balaban, C. L., Somov, A. Y., Rodriguez, J. V., & Fuller, B. J. (2011). Organ preservation: current concepts and new strategies for the next decade. *Transfusion Medicine and Hemotherapy*, 38: 125-142.
- Guo, S., Garnham, C. P., Whitney, J. C., Graham, L. A., & Davies, P. L. (2012). Re-evaluation of a bacterial antifreeze protein as an adhesin with ice-binding activity. *PloS One*, 7: e48805.
- Gurian-Sherman, D., & Lindow, S.E. (1993) Bacterial ice nucleation: significance and molecular basis. *FASEB Journal* 7: 1338-1342.

- Gwak, Y., Park, J. I., Kim, M., *et al.* (2015). Creating Anti-icing Surfaces via the Direct Immobilization of Antifreeze Proteins on Aluminum. *Scientific reports*, 5: 12019.
- Hightower, R., Baden, C., Penzes, E., Lund, P., & Dunsmuir, P. (1991). Expression of antifreeze proteins in transgenic plants. *Plant Molecular Biology*, 17: 1013-1021.
- Hirano, S. S., & Upper, C. D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - a pathogen, ice nucleus, and epiphyte. *Microbiology and Molecular Biology Reviews*, 64: 624.
- Holmberg, N., Farrés, J., Bailey, J. E., & Kallio, P. T. (2001). Targeted expression of a synthetic codon optimized gene, encoding the spruce budworm antifreeze protein, leads to accumulation of antifreeze activity in the apoplasts of transgenic tobacco. *Gene*, 275:115-124.
- Holt, W. V., Head, M. F., & North, R. D. (1992). Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: observations with experimental cryomicroscopy. *Biology of Reproduction*, 46: 1086-1094.
- Holt, W. V. (2000). Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology*, 53: 47-58.
- Huang, T., Nicodemus, J., Zarka, D. G., Thomashow, M. F., Wisniewski, M., & Duman, J. G. (2002). Expression of an insect (*Dendroides canadensis*) antifreeze protein in *Arabidopsis thaliana* results in a decrease in plant freezing temperature. *Plant Molecular Biology*, 50: 333-344.
- Ishiguro, H., & Rubinsky, B. (1994). Mechanical interactions between ice crystals and red blood cells during directional solidification. *Cryobiology*, 31: 483-500.
- Jia, Z. C., & Davies, P. L. (2002). Antifreeze proteins: An unusual receptor-ligand interaction. *Trends in Biochemical Sciences*, 27: 101-106.
- Joanisse, D. R., & Storey, K. B. (1998). Oxidative stress and antioxidants in stress and recovery of cold-hardy insects. *Insect Biochemistry and Molecular Biology*, 28: 23-30.
- Kawahara, H. (2002). The structures and functions of ice crystal-controlling proteins from bacteria. *Journal of Bioscience and Bioengineering*, 94: 492-496.
- Kenward, K. D., Altschuler, M., Hildebrand, D., & Davies, P. L. (1993). Accumulation of type I fish antifreeze protein in transgenic tobacco is cold-specific. *Plant Molecular Biology*, 23: 377-385.
- Kenward, K. D., Brandle, J., McPherson, J., & Davies, P. L. (1999). Type II fish antifreeze protein accumulation in transgenic tobacco does not confer frost resistance. *Transgenic Research*, 8: 105-117.
- Knight, C. A., & Duman, J. G. (1986). Inhibition of recrystallization of ice by insect thermal hysteresis proteins - a possible cryoprotective role. *Cryobiology*, 23: 256-262.

- Knight, C.A., Cheng, C.C., & DeVries, A.L. (1991) Adsorption of  $\alpha$ -helical antifreeze peptides on specific ice crystal surface planes. *Biophysical Journal*, 59: 409-418.
- Knight, C. A., Wen, D. Y., & Laursen, R. A. (1995). Nonequilibrium antifreeze peptides and the recrystallization of ice. *Cryobiology*, 32: 23-34.
- Koh, C. A., Westacott, R. E., Zhang, W., Hirachand, K., Creek, J. L., & Soper, A. K. (2002). Mechanisms of gas hydrate formation and inhibition. *Fluid Phase Equilibria*, 194: 143-151.
- Koshimoto, C., & Mazur, P. (2002). Effects of warming rate, temperature, and antifreeze proteins on the survival of mouse spermatozoa frozen at an optimal rate. *Cryobiology*, 45: 49-59.
- Kozloff, L. M., Turner, M. A., & Arellano, F. (1991). Formation of bacterial-membrane ice-nucleating lipoglycoprotein complexes. *Journal of Bacteriology*, 173: 6528-6536.
- Krishna, O. D., & Kiick, K. L. (2010). Protein - and peptide - modified synthetic polymeric biomaterials. *Peptide Science*, 94: 32-48.
- Kuiper, M. J., Davies, P. L., & Walker, V. K. (2001). A theoretical model of a plant antifreeze protein from *lolium perenne*. *Biophysical Journal*, 81: 3560-3565.
- Kukal, O., Duman, J. G., & Serianni, A. S. (1989). Cold-induced mitochondrial degradation and cryoprotectant synthesis in freeze-tolerant arctic caterpillars. *Journal of Comparative Physiology B*, 158: 661-671.
- Kun, H., Minnes, R., and Mastai, Y. (2008) Effects on antifreeze peptides on the thermotropic properties of a model membrane. *Journal of Bioenergetics and Biomembranes*, 40: 389-396.
- Lagneaux, D., Huhtinen, M., Koskinen, E., & Palmer, E. (1997). Effect of anti - freeze protein (AFP) on the cooling and freezing of equine embryos as measured by DAPI - staining. *Equine Veterinary Journal*, 29: 85-87.
- Lee, R. E., Lee, M. R., & Strong-Gunderson, J. M. (1993). Insect cold-hardiness and ice nucleating active microorganisms including their potential use for biological control. *Journal of Insect Physiology*, 39: 1-12.
- Li, J., & Lee, T. C. (1995). Bacterial ice nucleation and its potential application in the food industry. *Trends in Food Science & Technology*, 6: 259-265.
- Lindow, S. E., Army, D. C., & Upper, C. D. (1978). Distribution of ice nucleation-active bacteria on plants in nature. *Applied and Environmental Microbiology*, 36: 831-838.
- Lindow, S. E. (1983). The role of bacterial ice nucleation in frost injury to plants. *Annual Review of Phytopathology*, 21: 363-384.
- Lindow, S. E., & Panopoulos, N. J. (1988). Field tests of recombinant ice--*Pseudomonas syringae* for biological frost control in potato. In: Sussman, M., Collins, C. H., & Skinner, F. A. (eds) *Proceedings of the 1<sup>st</sup> international conference on the release of genetically engineered microorganisms*. Academic Press, London, pp 121-138.

- Liou, Y. C., Thibault, P., Walker, V. K., Davies, P. L., & Graham, L. A. (1999). A complex family of highly heterogeneous and internally repetitive hyperactive antifreeze proteins from the beetle *Tenebrio molitor*. *Biochemistry*, *38*:11415-11424.
- Liou, Y. C., Tocilj, A., Davies, P. L., & Jia, Z. (2000). Mimicry of ice structure by surface hydroxyls and water of a  $\beta$ -helix antifreeze protein. *Nature*, *406*: 322-324.
- Margesin, R., Neuner, G., & Storey, K. B. (2007). Cold-loving microbes, plants, and animals-fundamental and applied aspects. *Naturwissenschaften*, *94*: 77-99.
- McMullen, D. C., & Storey, K. B. (2008). Suppression of Na<sup>+</sup> K<sup>+</sup>-ATPase activity by reversible phosphorylation over the winter in a freeze-tolerant insect. *Journal of Insect Physiology*, *54*: 1023-1027.
- Mehta, A., Walsh, J., & Lorimer, S. (2000). Hydrate challenges in deep water production and operation. *Annals of the New York Academy of Sciences*, *912*: 366-373.
- Meyer, K., Keil, M., & Naldrett, M. J. (1999). A leucine-rich repeat protein of carrot that exhibits antifreeze activity. *FEBS letters*, *447*:171-178.
- Middleton, A. J., Brown, A. M., Davies, P. L., & Walker, V. K. (2009). Identification of the ice-binding face of a plant antifreeze protein. *FEBS letters*, *583*: 815-819.
- Middleton, A. J., Marshall, C. B., Faucher, F., *et al.* (2012). Antifreeze protein from freeze-tolerant grass has a beta-roll fold with an irregularly structured ice-binding site. *Journal of Molecular Biology*, *416*: 713-724.
- Mugnano, J. A., Wang, T., Layne, J. R., DeVries, A. L., & Lee, R. E. (1995). Antifreeze glycoproteins promote intracellular freezing of rat cardiomyocytes at high subzero temperatures. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *269*: R474-R479.
- Mugnano, J., Lee, R., & Taylor, R. (1996). Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *The Journal of Experimental Biology*, *199*: 465-471.
- Mazur, P. (1970). Cryobiology - freezing of biological systems. *Science*, *168*: 939.
- Mazur, P. (1984). Freezing of living cells: mechanisms and implications. *American Journal of Physiology*, *247*: 125-42.
- Nutt, D. R., & Smith, J. C. (2008). Dual function of the hydration layer around an antifreeze protein revealed by atomistic molecular dynamics simulations. *Journal of the American Chemical Society*, *130*: 13066-13073.
- Ohno, H., Susilo, R., Gordienko, R., *et al.* (2010). Interaction of antifreeze proteins with hydrocarbon hydrates. *Chemistry—A European Journal*, *16*: 10409-10417.

- Olsen, T. M., & Duman, J. G. (1997a). Maintenance of the supercooled state in the gut fluid of overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of ice nucleators and antifreeze proteins. *Journal of Comparative Physiology B*, *167*: 114-122.
- Olsen, T. M., & Duman, J. G. (1997b). Maintenance of the supercooled state in overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of hemolymph ice nucleators and antifreeze proteins. *Journal of Comparative Physiology B*, *167*: 105-113.
- Payne, S. R., Oliver, J. E., & Upreti, G. C. (1994a). Effect of antifreeze proteins on the motility of ram spermatozoa. *Cryobiology*, *31*: 180-184.
- Payne, S. R., Sandford, D., Harris, A., & Young, O. A. (1994b). The effects of antifreeze proteins on chilled and frozen meat. *Meat Science*, *37*: 429-438.
- Payne, S. R., & Young, O. A. (1995). Effects of pre-slaughter administration of antifreeze proteins on frozen meat quality. *Meat Science*, *41*: 147-155.
- Petzold, G., & Aguilera, J. M. (2009). Ice morphology: fundamentals and technological applications in foods. *Food Biophysics*, *4*: 378-396.
- Pfister, T. D., & Storey, K. B. (2006). Insect freeze tolerance: Roles of protein phosphatases and protein kinase A. *Insect Biochemistry and Molecular Biology*, *36*: 18-24.
- Prathalingam, N. S., Holt, W. V., Revell, S. G., *et al.* (2006). Impact of antifreeze proteins and antifreeze glycoproteins on bovine sperm during freeze-thaw. *Theriogenology*, *66*: 1894-1900.
- Pudney, P. D. A., Buckley, S. L., Sidebottom, C. M., *et al.* (2003). The physico-chemical characterization of a boiling stable antifreeze protein from a perennial grass (*Lolium perenne*). *Archives of Biochemistry and Biophysics*, *410*: 238-245.
- Raymond, J. A., & Devries, A. L. (1977). Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *PNAS*, *74*: 2589-2593.
- Regand, A., & Goff, H. D. (2006). Ice recrystallization inhibition in ice cream as affected by ice structuring proteins from winter wheat grass. *Journal of Dairy Science*, *89*: 49-57.
- Rubinsky, B., Arav, A., & Devries, A. L. (1992). The cryoprotective effect of antifreeze glycopeptides from antarctic fishes. *Cryobiology*, *29*: 69-79.
- Rubinsky, B., Arav, A., Hong, J. S., & Lee, C. Y. (1994). Freezing of mammalian livers with glycerol and antifreeze proteins. *Biochemical and Biophysical Research Communications*, *200*: 732-741.
- Sanghera, G. S., Wani, S. H., Hussain, W., & Singh, N. (2011). Engineering cold stress tolerance in crop plants. *Current genomics*, *12*: 30.
- Scotte, M., Eschwege, P., Cherruau, C., *et al.* (1996). Liver preservation below 0 C with UW solution and 2, 3-butanediol. *Cryobiology*, *33*: 54-61.

- Sidebottom, C., Buckley, S., Pudney, P., *et al.* (2000). Phytochemistry - heat-stable antifreeze protein from grass. *Nature*, 406: 256-256.
- Smolin, N., & Daggett, V. (2008). Formation of ice-like water structure on the surface of an antifreeze protein. *The Journal of Physical Chemistry B*, 112: 6193-6202.
- Storey, J. M., & Storey, K. B. (1983). Regulation of cryoprotectant metabolism in the overwintering gall fly larva, *Eurosta solidaginis*: temperature control of glycerol and sorbitol levels. *Journal of Comparative Physiology*, 149: 495-502.
- Sun, T., Davies, P. L., & Walker, V. K. (2015). Structural Basis for the Inhibition of Gas Hydrates by  $\alpha$ -Helical Antifreeze Proteins. *Biophysical Journal*, 109:1698-1705.
- Sutton, R. L., & Wilcox, J. (1998a). Recrystallization in ice cream as affected by stabilizers. *Journal of Food Science*, 63: 104-107.
- Sutton, R. L., & Wilcox, J. (1998b). Recrystallization in model ice cream solutions as affected by stabilizer concentration. *Journal of Food Science*, 63: 9-11.
- Suzuki, N., & Mittler, R. (2006). Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiologia Plantarum*, 126: 45-51.
- Tegos, G., Vargas, C., Perysinakis, A., *et al.* (2000). Release of cell-free ice nuclei from *Halomonas elongate* expressing the ice nucleation gene *inaZ* of *Pseudomonas syringae*. *Journal of Applied Microbiology* 89: 785-792.
- Tomczak, M. M., Hinch, D. K., Estrada, S. D., *et al.* (2002) A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. *Biophysical Journal*, 82: 874-881.
- Tsuchiya, Y., Sasaki, K., & Hasegawa, H. (2004). Effect of freeze-thaw repetitions upon the supercooling release ability of ice-nucleating bacteria. *Journal of Bioscience and Bioengineering*, 97: 71-74.
- Turner, M. A., Arellano, F., & Kozloff, L. M. (1991). Components of ice nucleation structures of bacteria. *Journal of Bacteriology*, 173: 6515-6527.
- Uemura, M., Tominaga, Y., Nakagawara, C., Shigematsu, S., *et al.* (2006). Responses of the plasma membrane to low temperatures. *Physiologia Plantarum*, 126: 81-89.
- Vanderveer, T. L., Choi, J., Miao, D., & Walker, V. K. (2014). Expression and localization of an ice nucleating protein from a soil bacterium, *Pseudomonas borealis*. *Cryobiology*, 69: 110-118.
- Walker, V. K., Zeng, H., Ohno, H., Daraboina, N., *et al.* (2015). Antifreeze proteins as gas hydrate inhibitors. *Canadian Journal of Chemistry*, 93: 839-849.
- Wallis, J. G., Wang, H., & Guerra, D. J. (1997). Expression of a synthetic antifreeze protein in potato reduces electrolyte release at freezing temperatures. *Plant Molecular Biology*, 35: 323-330.

- Wang, T., Zhu, Q., Yang, X., *et al.* (1994). Antifreeze glycoproteins from antarctic notothenioid fishes fail to protect the rat cardiac explant during hypothermic and freezing preservation. *Cryobiology*, *31*: 185-192.
- Ward, P. J., & DeMott, P. J. (1989). Preliminary Experimental Evaluation of Snomax (TM) Snow Inducer, Nucleus *Pseudomonas syringae*, as an Artificial Ice for Weather Modification. *The Journal of Weather Modification*, *21*: 9-13.
- Warren, G. J., Mueller, G.M., & McKown, R.L. (1992) Ice crystal growth suppression polypeptides and method of making. US Patent 5,118,792.
- Watson, P. F. (2000). The causes of reduced fertility with cryopreserved semen. *Animal reproduction science*, *60*, 481-492.
- Widehem, P., & Cochet, N. (2003). *Pseudomonas syringae* as an ice nucleator - application to freeze-concentration. *Process Biochemistry*, *39*: 405-410.
- Wolber, P., & Warren, G. (1989) Bacterial ice-nucleation proteins. *Trends in Biochemical Sciences*, *14*: 179-182.
- Wolber, P. K. (1993). Bacterial ice nucleation. *Advances in Microbial Physiology*, *34*: 203-203.
- Worrall, D., Elias, L., Ashford, D., Smallwood, M., *et al.* (1998). A carrot leucine-rich-repeat protein that inhibits ice recrystallization. *Science*, *282*: 115-117.
- Wu, Z., Qin, L., & Walker, V.K. (2009) Characterization and recombinant expression of a divergent ice nucleation protein from '*Pseudomonas borealis*'. *Microbiology* *155*: 1164-1169.
- Xin, Z., & Browse, J. (2000). Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant, Cell & Environment*, *23*: 893-902.
- Yankofsky, S. A., Levin, Z., Bertold, T., & Sandlerman, N. (1981). Some basic characteristics of bacterial freezing nuclei. *Journal of Applied Meteorology*, *20*: 1013-1019.
- Yeh, C. M., Kao, B. Y., & Peng, H. J. (2009). Production of a recombinant type 1 antifreeze protein analogue by *L. lactis* and its applications on frozen meat and frozen dough. *Journal of agricultural and food chemistry*, *57*: 6216-6223.
- Younis, A. I., Rooks, B., Khan, S., & Gould, K. G. (1998). The effects of antifreeze peptide III (AFP) and insulin transferrin selenium (ITS) on cryopreservation of chimpanzee (*Pan troglodytes*) spermatozoa. *Journal of andrology*, *19*: 207-214.
- Zeng, H., Wilson, L. D., Walker, V. K., & Ripmeester, J. A. (2003). The inhibition of tetrahydrofuran clathrate-hydrate formation with antifreeze protein. *Canadian Journal of Physics*, *81*:17-24.
- Zeng, H., Moudrakovski, I. L., Ripmeester, J. A., & Walker, V. K. (2006). Effect of antifreeze protein on nucleation, growth and memory of gas hydrates. *AIChE journal*, *52*: 3304-3309.

## Chapter 2

# Perturbation of bacterial ice nucleation activity by a grass antifreeze protein

### 2.1 Abstract

Certain plant-associated bacteria produce ice nucleation proteins (INPs) which allow the crystallization of water at high subzero temperatures. Many of these microbes are considered plant pathogens since the formed ice can damage tissues, allowing access to nutrients. Intriguingly, certain plants that host these bacteria synthesize antifreeze proteins (AFPs). Once freezing has occurred, plant AFPs likely function to inhibit the growth of large damaging ice crystals. However, we postulated that such AFPs might also serve as defensive mechanisms against bacterial-mediated ice nucleation. Recombinant AFP derived from the perennial ryegrass *Lolium perenne* (*LpAFP*) was combined with INP preparations originating from the grass epiphyte, *Pseudomonas syringae*. The presence of INPs had no effect on AFP activity, including thermal hysteresis and ice recrystallization inhibition. Strikingly, the ice nucleation point of the INP was depressed up to 1.9 °C in the presence of *LpAFP*, but a recombinant fish AFP did not lower the INP-imposed freezing point. Assays with mutant *LpAFPs* and the visualization of bacterially-displayed fluorescent plant AFP suggest that INP and *LpAFP* can interact. Thus, we postulate that in addition to controlling ice growth, plant AFPs may also function as a defensive strategy against the damaging effects of ice-nucleating bacteria.

### 2.2 Introduction

Ice-binding proteins function as part of a survival strategy for some organisms that cannot avoid exposure to subzero temperatures. These proteins include antifreeze proteins (AFPs) and ice nucleation proteins (INPs), which manipulate the growth of ice or the crystallization temperature. First discovered in insects (Ramsay, 1964), then in polar fish (DeVries and

Wohlschl, 1969), AFPs adsorb to embryonic ice crystals resulting in a depression of the freezing point relative to the melting point (Raymond and DeVries, 1977). The difference between the melting and freezing temperature is measured as the thermal hysteresis (TH) activity. In plants, AFPs have been isolated from several species including the perennial ryegrass, *Lolium perenne* (Moffatt *et al.*, 2006; Sidebottom *et al.*, 2000). Generally, plant AFPs are characterized by lower TH activity compared to the AFPs found in some insects and polar fish. Since certain plants cannot avoid freezing, the primary function of a plant AFP is to inhibit the growth of large, damaging ice crystals, with little impact on freezing point depression (Sidebottom *et al.*, 2000). Because of this characteristic, plant AFPs are also referred to as ice recrystallization (IR) inhibitors.

INPs operate in a seemingly opposite manner compared to AFPs, functioning as heterogeneous nucleators that catalyze ice crystallization at high subzero temperatures. Although several different organisms have been reported to have ice nucleation activity (INA), only those INPs produced by bacteria have been well characterized. INPs have been isolated from approximately ten different bacterial species belonging to at least three different genera: *Pseudomonas*, *Erwinia*, and *Xanthomonas* (Kawahara, 2002), with the encoding DNA sequences almost certainly exchanged by horizontal transfer between species (Warren and Wolber, 1991). INPs form aggregates on the outer membrane where they function as a template for ice formation (Gurian-Sherman and Lindow, 1993).

INP producing plant-associating bacteria are frequently viewed as plant pathogens, but this is not always the case. Certainly it is recognized that several epiphytic bacteria produce INPs as a way of initiating wounding to leaves and stems, permitting access to a rich pool of nutrients (Lindow, 1983). Some of these bacteria are also known to invade the plant during favorable conditions, gaining access to the apoplast through openings on the plant's surface (Melotto *et al.*, 2006). In surveys of *L. perenne* leaves, 40% of the bacterial community was represented by

*Pseudomonas fluorescens*, *Pseudomonas* spp., *Erwinia herbicola*, and *Xanthomonas campestris*, all associated with INP production (Hirano and Upper, 2000).

As indicated, *L. perenne*'s AFP (*LpAFP*) is postulated to offer host protection by inhibiting IR once freezing has occurred (Sidebottom *et al.*, 2000). Although this is undoubtedly true, we wondered if there was also an interaction between the bacterial INPs and the AFPs in the plant extracellular fluids. In this regard, the evolutionary origin of *LpAFP* is unknown; some of these plant AFP sequences appear to be related to defensive agents such as pathogenesis-related proteins (Griffith and Yaish, 2004; van Loon and Pieterse, 2006). Could *LpAFP* also play a defensive role, offering protection not only from IR, but also against the INA of potentially pathogenic bacteria? Previously, it has been suggested that insect AFPs and INPs could interact (Olsen and Duman, 1997a, 1997b), but to our knowledge quantified studies and detailed characterization of any interactions have not been done. It is also important to test the interaction of INPs and AFPs derived from other species to determine if any such interaction is specific to plant AFPs. Such analysis, we hope, will also contribute to our structural and functional understanding of these two distinct ice-associating proteins.

## **2.3 Materials and Methods**

### **2.3.1 Protein and sample preparation**

Recombinant AFPs including *LpAFP* (GenBank: AJ277399), two mutated versions of *LpAFP* (N72Y and T43Y), *LpAFP* tagged with green fluorescent protein (*LpAFP*-GFP), and fish type III AFP derived from the ocean pout, *Macrozoarces americanus*, were purified as previously described (Lauersen *et al.*, 2011; Middleton *et al.*, 2009; Davies and Hew, 1990; Gordienko *et al.*, 2010).

*P. syringae* INP preparations were purchased from Ward's Natural Science (USA) and used at concentrations ranging from 50 µg/mL to 5 µg/mL. *P. syringae* B728a (Wilson and Lindow, 1994) and *P. borealis* DL7 (Wu *et al.*, 2009) were cultured for 24-48 h at 22°C in 10%

tryptic soy broth (TSB) and subsequently cold conditioned for two days at 4 °C before used as an additional source of INPs (Vanderveer *et al.*, 2014). Cytochrome c was used as a control to distinguish any protein-mediated concentration effects.

### **2.3.2 Ice nucleation assays**

Ice nucleation activity (INA) was assayed using a procedure modified from a standard technique (Vali, 1971). Briefly, freezing points were obtained by pipetting 20 replicate samples (2 µl) on a polarized film, which was subsequently placed over an insulated chamber containing 50% ethylene glycol. While lowering the chamber temperature (-1 to -12°C at 0.2°C/min), images of the polarized film as well as the thermistor output were automatically recorded every 60 s. The temperature at which 90% of the samples froze ( $T_{90}$ ) was considered the nucleation point, while samples with freezing points below -9 °C were not considered to have significant INA. Using Vali's (1971) equation, the cumulative number of ice nuclei per mL in each sample ( $K(T)$ ) was calculated as:

$$K(T) = -\ln(N(T)/N_0) * V^{-1}$$

with  $N(T)$  representing the number of unfrozen drops at temperature  $T$ ,  $N_0$  representing the total drop number, and  $V$  representing the drop volume. INA (20 replicate samples) was determined at least three times with different protein preparations at all reported concentrations.

### **2.3.3 Antifreeze activity assays**

IR inhibition was assayed using capillary assays (Tomczak *et al.*, 2003) and a modified version of the splat assay (Knight *et al.*, 1988), exactly as described (Middleton *et al.*, 2014). A Clifton nanolitre osmometer was used to determine the TH of the AFPs (Chakrabarty and Hew, 1991), as well as to visualize ice crystal morphology (Middleton *et al.*, 2014). All IR inhibition and TH assays were performed three or more times.

### 2.3.4 Fluorescence microscopy

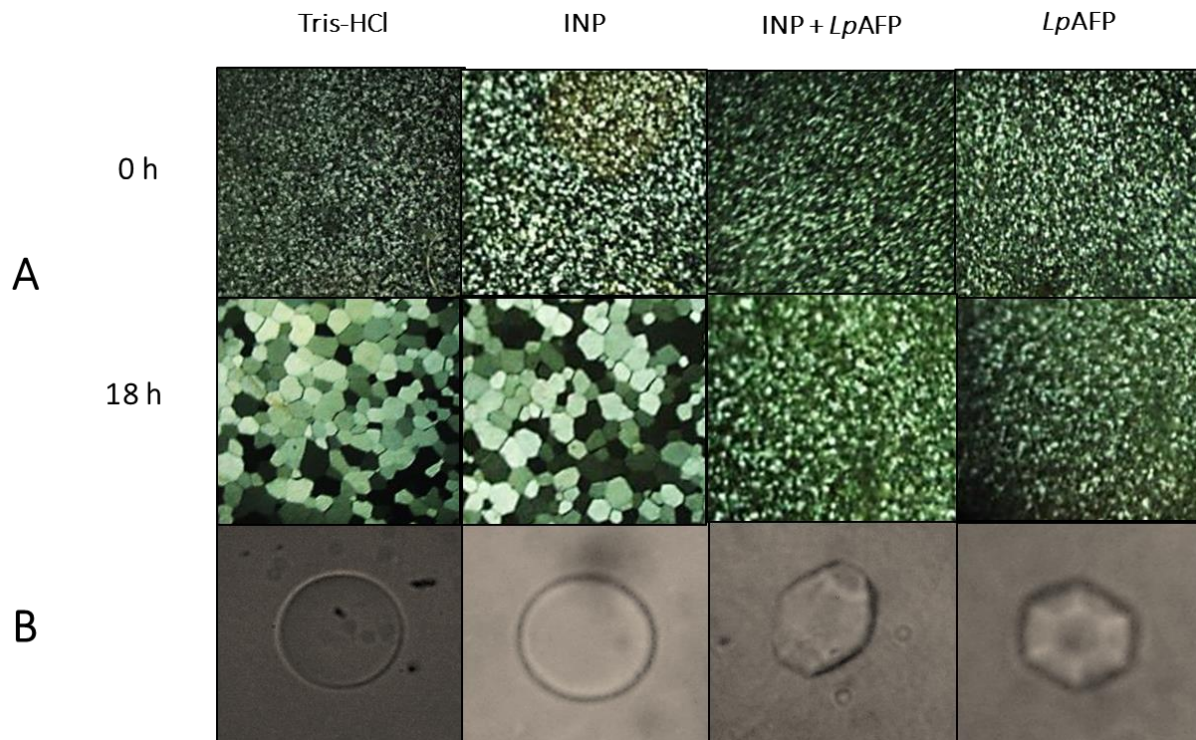
Purified *LpAFP*-GFP (to a final concentration of 0.5 mg/mL) was added to 1 mL aliquots of the cold-acclimated *P. syringae* or *P. borealis* cultures and allowed to incubate for 30 min at 4 °C. Samples (5 µL) were placed on clean microscope slides and visualized using a cold stage (Physiotemp Inc.) set at 4 °C on an inverted Zeiss Axiovert 200M microscope under fluorescent light conditions (543 nm).

## 2.4 Results

### 2.4.1 Impact of INPs on AFP activity

Samples of recombinant AFPs were mixed with INP preparations and assayed for IR inhibition activity (Figure 2.1A) and for changes in the morphology of individual ice crystals at their equilibrium temperature (Figure 2.1B). Ice crystals formed in the presence of INPs alone at any of the tested concentrations were disk-like, reflecting uninhibited ice growth on all six sides of the embryonic crystal (Figure 2.1B). Ice crystals grown in solutions containing any of the tested AFPs (type III AFP, wild type *LpAFP*, or *LpAFP*s bearing mutations on the non-ice binding face) resulted in morphologies that were clearly distinct. Ice crystals were either bipyramidal (Type III AFP) or hexagonal (*LpAFP*s) in shape. The addition of INPs to any of these AFPs at any of the tested concentrations resulted in no visible disruption to the AFP-dependent appearance of the ice crystals (Figure 2.1B).

In the absence of INPs, the mean TH values of the type III and *LpAFP* preparations (all at 1 mg/mL) were  $0.62 \pm 0.1$  °C and  $0.20 \pm 0.02$  °C, respectively. After the addition of the INP preparations, the TH values did not change significantly (two-tailed, unpaired t-test,  $P > 0.05$ ) with mean values of  $0.59 \pm 0.01$  °C and  $0.21 \pm 0.02$  °C for the fish and plant proteins, respectively. Predictably, both AFPs inhibited IR in either the capillary or splat assays, with ice crystals remaining small at annealing temperatures of -4 °C (Figure 2.1A). In the absence of AFPs (buffer alone or control protein), these conditions resulted in large ice crystals. Similar to the TH assays,



**Figure 2.1** Representative ice crystals from an IR inhibition splat assay (A) and typical ice crystal morphologies (B) in the presence of *P. syringae* ice nucleation protein (INP; 0.05 mg/mL) and *L. perenne* AFP (*LpAFP*; 1 mg/mL). Both assays were performed in triplicate.

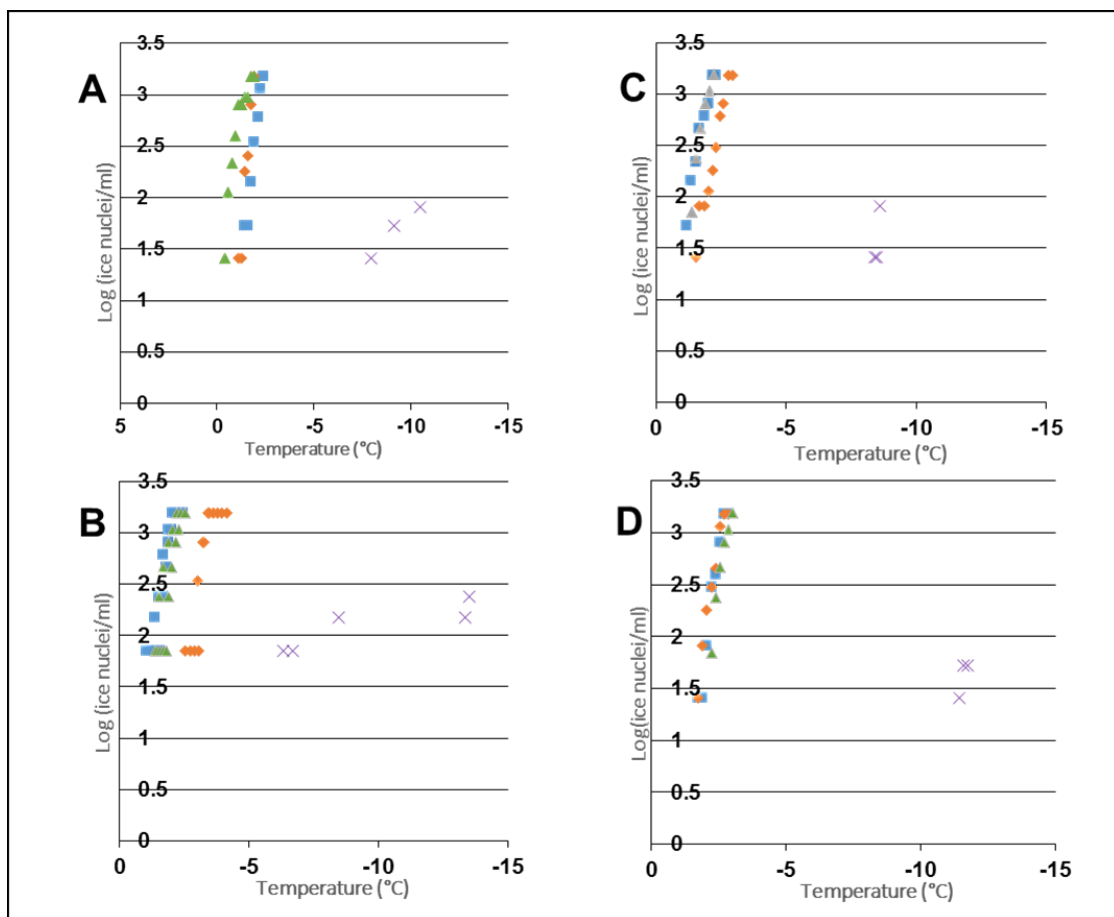
IR assays in the presence of INPs and any of the AFP samples showed that effective IR inhibition was maintained.

#### **2.4.2 Impact of AFPs on INP activity**

Initially, the impact of AFPs on INP activity was assessed using a concentration matrix with *P. syringae* preparations ranging from 50 µg/mL to 0.5 µg/mL and AFP concentrations ranging from 50 µg/mL to 3 mg/mL. Following that survey, a more thorough investigation was carried out using two different concentrations of type III AFP and *Lp*AFP (1 and 2 mg/mL) and two INP levels (50 µg/mL and 5 µg/mL).

In the absence of AFPs, the mean INP-mediated freezing point was -3.08 °C and -3.82 °C (at 50 µg/mL and 5 µg/mL, respectively). When type III AFP was added to the INP preparation, there was no significant depression of the freezing temperature at any of the tested concentrations compared to the non-AFP controls (two tailed, unpaired t-test;  $P > 0.05$ ; Table 2.1). Figure 2.2A represents typical results, showing the effect of type III AFP on the cumulative number of ice nuclei per ml of an INP preparation (at 50 µg/mL). At one concentration combination (2 mg/mL type III AFP and 5 µg/mL INP), however, there appeared to be a minor increase ( $P < 0.05$ ) in the freezing temperature by an average of 0.33 °C.

In contrast to the results for type III AFP, the addition of *Lp*AFP (either 1.0 or 2.0 mg/mL) significantly depressed the freezing point of the INP preparations compared to non-AFP controls (two tailed, unpaired t-test;  $P < 0.05$ ); *Lp*AFP depressed the freezing point of the INP up to 1.9 °C (Table 2.1). Graphically, the effects of *Lp*AFP on the cumulative number of ice nuclei are typified by Figure 2.2B, which demonstrates the depression of the freezing point with the addition of *Lp*AFP (1 mg/mL) when added to the INP (50 µg/mL). The addition of the *Lp*AFP mutant T43Y (1 mg/mL) also significantly depressed the freezing point of tested INPs (Figure 2.2C; two tailed, unpaired t-test,  $P < 0.05$ ), but the *Lp*AFP mutant N72Y did not (Figure 2.2D). The addition of *Lp*AFP –GFP also resulted in a significant depression of the INP freezing point,



**Figure 2.2** Representative graphs for ice nucleation activity shown as the logarithm of the cumulative number of ice nuclei per mL with (A) Type III (1 mg/mL), (B) *LpAFP* (1 mg/mL), (C) *LpAFP* T43Y (1 mg/mL) and (D) *LpAFP* N72Y (1 mg/mL). Samples include an INP preparation from *P. syringae* (blue squares), INPs combined with either Type III AFP (A) or *LpAFP* (B,C,D) (orange diamonds), INPs combined with cytochrome c (green triangles), and either Type III AFP or *LpAFP* alone (purple X). All ice nucleation assays were performed in triplicate.

but the decrease was more modest than with *LpAFP*, showing an average depression of 0.63 °C (Table 2.1).

To ensure the changes in the freezing temperatures were not due to protein colligative effects, cytochrome C was used as a control in all experiments. Cytochrome C did not alter the freezing temperature of INP preparations except at very high concentrations (>3 mg/mL). At these high concentrations, cytochrome C significantly altered the freezing point, consistent with the expected concentration dependent effect.

#### *Visualization of the interaction between INPs and LpAFP*

After cold conditioning *P. syringae* and *borealis* cultures for two days at 4 °C, ice nucleation assays, averaging -2.8°C and -2.9°C respectively, ensured that ice nucleating activity was present. Aliquots of these bacteria incubated with *LpAFP*-GFP resulted in the appearance of concentrated green fluorescence at the pole of some cells in several samples of both *Pseudomonas* species (Figure 2.3); however, it must be noted that these results were not always consistent. In this regard, it must be recalled that not all cells in *Pseudomonas sp.* cultures synthesize INPs, possibly explaining why fluorescence was not observed on every cell and in every slide [8].

## **2.5 Discussion**

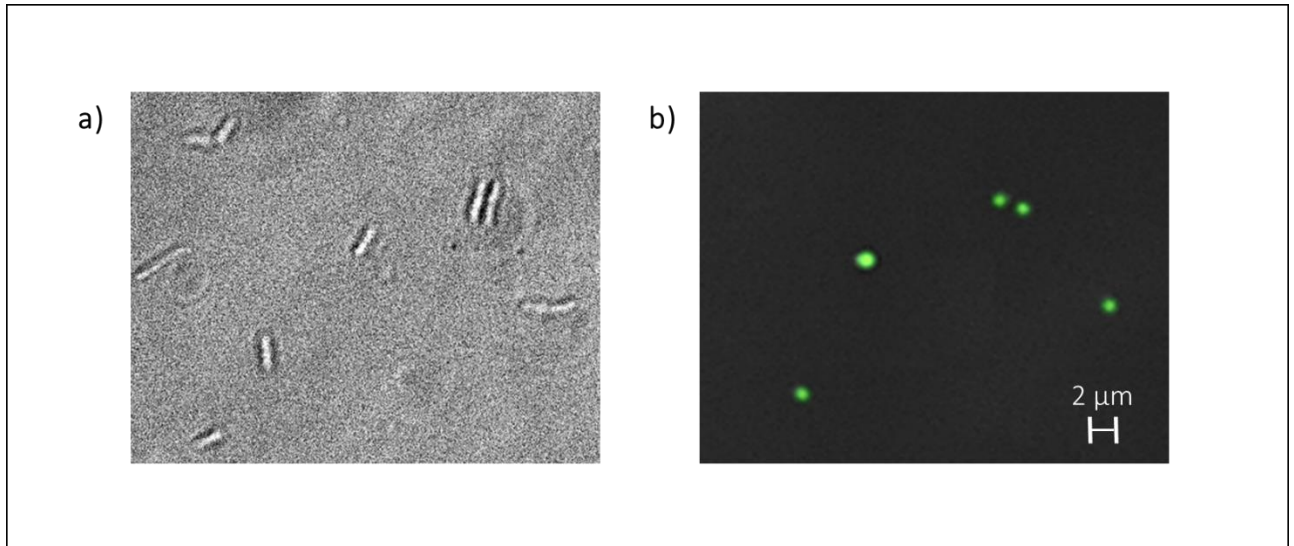
AFPs are thought to facilitate the freezing survival of perennials by inhibiting the growth of large ice crystals in the apoplast. However, we wondered if they also might serve to counter the damaging effects of INP-producing bacteria by depressing the freezing temperature. Such freezing point depression would restrict the initial ice crystal size and thus would presumably lead to reduced cell damage. Our results strongly support this hypothesis.

As far as we are aware, this hypothesis has not been previously investigated. However, others have examined the interaction of animal AFPs and ice nucleators, with sometimes conflicting results. For example, fish antifreeze glycoproteins (AFGPs) as well as insect AFPs

**Table 2.1** Mean differences in freezing temperatures for *P. syringae* INP preparations in the presence or absence of recombinant AFPs.

Protein Addition <sup>2</sup>		[INP] <sup>1</sup>	
		0.05 mg/mL	0.005 mg/mL
Type III AFP	2 mg/mL	+0.4°C ± 0.2	+0.33°C ± 0.3 *
	1 mg/mL	+0.13°C ± 0.4	+0.33°C ± 0.3
<i>Lp</i> AFP	2 mg/mL	-1.9°C ± 0.42 *	-1.31°C ± 0.29 *
	1 mg/mL	-1.02°C ± 0.31 *	-0.9°C ± 0.29 *
<i>Lp</i> AFP-GFP	2 mg/mL	-0.39°C ± 0.39	-0.86°C ± 0.29 *
	1 mg/mL	-0.63°C ± 0.41 *	-0.82°C ± 0.17 *
Cytochrome c	2 mg/mL	+0.21°C ± 0.43	-0.08°C ± 0.8
	1 mg/mL	+0.43°C ± 0.34	-0.04°C ± 0.27

1. The standard deviation for each value is also shown. Each experiment was repeated three or more times. Values displayed with an asterisks (\*) denotes samples that were significantly different from cytochrome C controls.
2. The mean freezing point in the presence of INPs alone was -3.08°C ± 0.78 (0.05 mg/mL) and -3.82°C ± 0.78 (0.005 mg/mL). These values were subtracted from the observed values to give the relative differences reported above.



**Figure 2.3** Visualization of cold-acclimated *Pseudomonas borealis* DL7 cells incubated with GFP labelled *LpAFP*. Cells viewed under normal/bright light conditions are shown in (a) and compared to those viewed under fluorescence (543 nm) in (b). Magnifications are shown by the bars. Note that not all bacteria show fluorescence.

were reported to inhibit bacterial INA (Parody-Morreale *et al.*, 1988; Duman, 2001) and fish type I and III AFPs depressed the nucleation temperature of AgI, an effective abiotic ice nucleator (Inada *et al.*, 2012). In contrast, type I and III AFP have been reported to actually enhance ice nucleation (Wilson *et al.*, 2010; Holt, 2003). While the concept of an AFP, which lowers the freezing temperature, interacting with INPs to essentially promote ice nucleation in these studies would be seemingly contradictory, Holt (2003) suggested that type III AFP could increase the freezing point of *P. syringae* by binding adjacent nucleators to form a larger surface area for ice nucleation. In this regard, it should be noted that we observed no AFP-associated enhancement of INA, with the single marginal exception involving the highest type III AFP concentration paired with the lowest INP concentration (Table 1). Other studies serve to further complicate the literature. For example, a bacterial AFP from *P. putida* was shown to display a moderate level of ice nucleation activity (Xu *et al.*, 1998). Similarly, under certain circumstances including at high concentrations ( $\geq 8$  mg/mL), type I AFP acted as a nucleator (Wilson *et al.*, 2010). It should be noted that fish are freeze intolerant organisms; if they freeze, they will die. Thus a fish AFP-mediated enhancement of ice nucleation as found by some researchers is unlikely to be adaptive. Our results show that enhancement of INP activity was not found at all INP and type III AFP concentrations in any case.

In contrast to the results with the fish AFP, when *LpAFP* was added to INP preparations, the nucleation temperature was reliably decreased (Table 2.1; Figure 2.2B). Remarkably, when *LpAFP* (2 mg/mL) was added to INP (50  $\mu$ g/mL), there was a mean freezing point depression of  $1.9^{\circ}\text{C} \pm 0.34^{\circ}\text{C}$ . This temperature is substantially greater than the recorded maximum TH activity of  $0.45^{\circ}\text{C}$  for this protein (Pudney *et al.*, 2003) indicating that its “anti-nucleator” activity is greater than its ability to depress the freezing point. As well, the extent of the freezing point depression indicates that *LpAFP* was not just adsorbing to embryonic ice crystals.

The observation that a consistent decrease in INA was achieved with an AFP found naturally in the same environment as the bacterial INP is noteworthy. Certain perennials have evolved several different cold-adaptive mechanisms, including cold-induced cell wall modification proteins, pathogenesis-related (PR) proteins to combat psychrophilic pathogens, and AFPs to inhibit IR (Hiilovaara-Teijo *et al.*, 1999). Some of the PR proteins have themselves been shown to possess antifreeze activity, thought to be due to cross-adaptation [Moffatt *et al.*, 2006, Hiilovaara-Teijo *et al.*, 1999]. Reports of similar motifs between AFPs and PR proteins, however, do not extend to the ice-binding regions of *LpAFP* (Griffith and Yaish, 2004). Therefore, we suggest that *LpAFP* not only functions to prevent IR once freezing takes place, but may also serve to lower the high freezing temperature and corresponding large ice crystal sizes dictated by the near ubiquitous INP-producing bacteria. These microbes are not only found on the surface of the leaves (Hirano and Upper, 2000), but also in the apoplast, the site of *L. perenne*'s AFP (Lauersen *et al.*, 2011).

More difficult to understand is how INPs and *LpAFP*s would interact. It was proposed that type III AFP inhibited ice nucleation via a complex interaction with ice crystals and foreign particles, such as dust (Du *et al.*, 2003), but given that we saw no freezing point depression with type III AFPs, coupled with our observation that *LpAFP* decreases the INP-*LpAFP* solution freezing point more than its TH value, this model is not satisfactory. Rather, we speculate that some *LpAFP* molecules can associate with the flat, repetitive ice nucleation sites on the INPs. These large proteins have been suggested to assemble in an overlapping, 'stair-like' fashion on the surface of bacterial cells since ice may preferentially form on steps (Wu *et al.*, 2009). If *LpAFP* associated with even some of these flat surfaces, it could disrupt the aggregation of INP clusters at high subzero temperatures, thus preventing the formation of water clusters with a critical radius that would facilitate the further propagation of ice (Wilson and Leader, 1995).

A physical interaction between *LpAFP* and bacterial INPs has not previously been suggested, but the striking structural similarities including two comparable flat, relatively hydrophobic faces on opposite sides of both their respective  $\beta$ -roll structures (Middleton *et al.*, 2009; Garnham *et al.*, 2011) could facilitate this. The parallels between the two distinct ice-associating proteins was convincingly demonstrated by the appearance of an ice crystal exhibiting a AFP-like inhibition morphology in experiments using a recombinant peptide fragment representing  $\leq 8\%$  of the *P. syringae* INP (Kobashigawa *et al.*, 2005). Our observations (Figure 2.3) showing GFP-labelled *LpAFP* localized to the poles of *P. syringae* and *P. borealis* where INPs appear to cluster (Vanderveer *et al.*, 2014) helps support a physical interaction hypothesis. Interestingly, X-ray crystallographic analysis of purified *LpAFP* shows intermolecular interaction between *LpAFPs* themselves along the  $\beta$ -roll surface (Middleton *et al.*, 2012) and thus it is possible that *LpAFP* and INP could also interact. In contrast to the two-flat sided  $\beta$ -roll structure of *LpAFP*, type III is globular (Jia *et al.*, 1996); presumably then, there would be no structural theoretical basis for a direct interaction of the non-plant AFP with INPs. Although the appearance of fluorescence on *Pseudomonas* was repeatable with the two species of ice nucleating bacteria, it was not always seen. These experiments were challenging not only because not all bacteria within a given strain produce INPs, but also likely because *LpAFP*-GFP itself did not reduce INA activity as much as the wild-type protein (Table 2.1). We speculate that the presence of the GFP-tag reduced the affinity of the protein to the INP, as seen previously with another ice-like substrate (Gordienko *et al.*, 2010).

Although the INP's INA was decreased in the presence of *LpAFP*, there was no impact on any of the ice-binding activities of any of the tested AFPs. The absence of notable INP-mediated changes to IR inhibition, TH levels, or ice morphologies characteristic of each AFP indicates that INPs do not compete with AFPs for ice adsorption. Our hypothesis of a physical interaction between *LpAFP* and INPs is consistent with these observations due to the large

discrepancy in the size of the two proteins. INP monomers are more than 12-fold larger than *LpAFP*. Perhaps only a single molecule of *LpAFP* is enough to ‘spoil’ the ice nucleation surface of the INP. In turn, if these ‘spoiled’ INPs were then less likely to form ordered INP-INP aggregates, this could result in a significant depression of the INP-mediated freezing point as demonstrated in our experiments. Is it by chance or evolutionary design that only one of the two flat sides of *LpAFP* binds ice (Middleton *et al.*, 2009), hypothetically allowing *LpAFP* to ‘spoil’ INA without interfering with the active ice-binding site?

Regrettably, “a-side” (ice adsorption side) mutations could not be tested since they cannot be purified by ice-affinity. However, our results involving non-ice binding “b-side” *LpAFP* mutants support our proposed model of INP inhibition. The freezing point depression clearly seen with wild-type *LpAFP* was abolished when a single steric mutation (N72Y) was introduced on the b-side of *LpAFP*’s  $\beta$ -roll structure. A secondary steric mutation, T43Y, on the b-side modestly depressed the freezing point of *LpAFP*, but this particular residue is located near the N-terminus at a bulge that interrupts the flat face, suggesting that the interaction between *LpAFP* and INPs involves the flat surface (Middleton *et al.*, 2012). Taken together, our results suggest that a freeze-tolerant perennial grass with *LpAFP* should suffer less freeze damage, both by inhibition of IR as well as the lowering of the freezing point and initiating small ice crystal size, and together these activities will help ensure its winter survival.

## 2.6 Acknowledgments

We acknowledge Dr. P. Davies for use of his nanolitre osmometer, Dr. D. Guttman for the *P. syringae* B728a strain and Mr. T. DeFalco for preliminary observations. We also thank T. Papanicolaou and M. Bredow for their assistance with the fluorescence microscopy images. This work was funded by an NSERC (Canada) grant to VKW.

## 2.7 References

- Chakrabarty, A., & Hew, C.L. (1991). The effect of enhanced  $\alpha$ -helicity on the activity of a winter flounder antifreeze polypeptide. *Eur. J. Biochem.* 202: 1057-1063.
- Davies, P.L., & Hew, C.L. (1990). Biochemistry of fish antifreeze proteins, *FASEB J.* 4: 2460-2468.
- DeVries, A.L., & Wohlschl, D. (1969). Freezing resistance in some Antarctic fishes, *Science* 163: (1969) 1073.
- Du, N., Liu, X.Y. , & Hew, C.L. (2003). Ice nucleation inhibition. *J. Biol. Chem.* 278: 36000-36004.
- Duman, J.G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annu. Rev. Physiol.* 63: 327-357.
- Garnham, C.P., Campbell, R.L., Walker, V.K., & Davies, P.L. (2011). Novel dimeric  $\beta$ -helical model of an ice nucleation protein with bridged active sites. *BMC Struct. Biol.* 11:36-47.
- Gordienko, R., Ohno, H., Singh V.K., *et al.* (2010). Towards a Green Hydrate Inhibitor: Imaging Antifreeze Proteins on Clathrates. *PLoS One* 5:e8953.
- Griffith, M., & Yaish, M.W.F. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. *Trends Plant Sci.*, 9: 399-405.
- Gurian-Sherman, D., & Lindow, S.E. (1993). Bacterial ice nucleation: significance and molecular basis. *FASEB J.*, 7:1338-1342.
- Hiilovaara-Teijo, M., & Plava, E.T. (1999). Molecular responses in cold adapted plants. In: R. Margesin, F. Schinner (Eds.), *Cold-Adapted Organisms—Ecology, Physiology, Enzymology, Molecular Biology*. Springer, Heidelberg, pp 349–384.
- Hirano, S.S., & Upper, C.D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. R.*, 64: 624-653.
- Holt, C.B. (2003). The effect of antifreeze proteins and poly(vinyl alcohol) on the nucleation of ice: A preliminary study. *CryoLetters*, 24: 323-330.
- Inada, T., Koyama, T., Goto, F., & Seto, F. (2012). Inactivation of ice nucleating activity of silver iodide by antifreeze proteins and synthetic polymers. *J. Phys. Chem.*, 116: 5364-5371.
- Jia, Z., Deluca, C.I., Chao, H., & Davies, P.L. (1996). Structural basis for the binding of a globular antifreeze protein to ice. *Nature*, 384: 285-288.
- Kawahara, H. (2002). The structures and functions of ice crystal-controlling proteins from bacteria. *J. Biosci. Bioeng.*, 94: 492-496.

- Knight, C.A., Hallett, J., & Devries, A.L. (1988). Solute effects on ice recrystallization: an assessment technique. *Cryobiology*, 25: 55-60.
- Kobashigawa, Y., Nishimiya, Y., Miura, K., *et al.* (2005). A part of ice nucleation protein exhibits the ice-binding ability. *FEBS Letters*, 579: 1493-1497.
- Lauersen, K.J., Brown, A., Middleton, A., *et al.* (2011). Expression and characterization of an antifreeze proteins from the perennial rye grass, *Lolium perenne*. *Cryobiology*, 62: 194-201.
- Lindow, S.E. (1983). The role of bacterial ice nucleation in frost injury to plants. *Annu. Rev. Phytopathol.*, 21: 363-384.
- Melotto, M., Underwood, W., Koczan, J., *et al.* (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell*, 126: 969-980.
- Middleton, A., Brown, A.M., Davies, P.L., & Walker, V.K. (2009). Identification of the ice-binding face of a plant antifreeze protein. *FEBS Letters*, 583: 815-819.
- Middleton, A., Marshall, C.B., Faucher, F., *et al.* (2012) Antifreeze protein from freeze-tolerant grass has a beta-roll fold with an irregularly structured ice-binding site. *J. Mol. Biol.*, 416: 713-724.
- Middleton, A.J., Vanderbeld, B., Bredow, M., *et al.* (2014). Isolation and characterization of ice-binding proteins from higher plants. In: D.K. Hinch, E. Zuther (Eds.), *Plant Cold Acclimation: Methods and Protocols*. Springer, Heidelberg, pp 255-277.
- Moffatt, B., Ewart, V., & Eastman, A. (2006). Cold comfort: plant antifreeze proteins. *Physiol. Plantarum.*, 126: 5-16.
- Olsen, T.M. & Duman, J.G. (1997a). Maintenance of the supercooled state in overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of hemolymph ice nucleators and antifreeze proteins. *J. Comp. Physiol. B*, 167: 105-113.
- Olsen, T.M. & Duman, J.G. (1997b). Maintenance of the supercooled state in the gut fluid of overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of ice nucleators and antifreeze proteins. *J. Comp. Physiol. B*, 167: 114-122.
- Parody-Morreale, A., Murphy, K.P., Cera, E.D., *et al.* (1988). Inhibition of bacterial ice nucleators by fish antifreeze glycoproteins. *Nature*, 333: 782-783.
- Pudney, P.D.A., Buckley, S.L., Sidebottom, C.M., *et al.* (2003). The physico-chemical characterization of a boiling stable antifreeze protein from a perennial grass (*Lolium perenne*). *Arch. Biochem. Biophys.*, 410: 238-245.
- Ramsay, R.A. (1964). The rectal complex of the mealworm, *Tenebrio molitor*. *Phil. Trans. R. Soc. London B*, 248: 279-314.
- Raymond, J.A. & Devries, A.L. (1977). Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *PNAS*, 74: 2589-2593.

- Sidebottom, C., S. Buckley, S., Pudney, P. *et al.* (2000). Phytochemistry - heat-stable antifreeze protein from grass. *Nature*, 406: 256-256.
- Tomczak, M.M., Marshall, C.B., Gilbert, J.A., & Davies, P.L. (2003). A facile method for determining ice recrystallization inhibition by antifreeze proteins. *Biochem. Bioph. Res. Co.*, 311: 1041-1046.
- Vali, G. (1971). Quantitative evaluation of experimental results on heterogeneous freezing nucleation of supercooled liquids. *J. Atmos. Sci.*, 28: 402.
- Vanderveer, T.L., Choi, J., Miao, D., & Walker, V.K. (2014). Expression and localization of an ice nucleating protein from a soil bacterium, *Pseudomonas borealis*. *Cryobiology*, 69: 110-118.
- van Loon, L.C., Rep, M., & Pieterse, C.M.J. (2006). Defense-related proteins in infected plants. *Annu. R. Phytopathol.*, 44: 135-162.
- Warren, G., & Wolber, P. (1991). Molecular aspects of microbial ice nucleation. *Mol. Microbiol.*, 5: 239-243.
- Wilson, M., & Lindow, S.E. (1994). Ecological similarity and coexistence of epiphytic ice-nucleating (Ice<sup>+</sup>) *Pseudomonas syringae* and a non-ice-nucleating (Ice<sup>-</sup>) biological control agent. *Appl. Environ. Microb.*, 60: 3128-3137.
- Wilson, P.W., & Leader, J.P. (1995). Stabilization of supercooled fluids by thermal hysteresis proteins. *Biophys. J.*, 68: 2098-2107.
- Wilson, P.W., Osterday, K.E., Heneghan, A.F., & Haymet, A.D.J. (2010) Type I antifreeze proteins enhance ice nucleation above certain concentrations. *J. Biol. Chem.*, 285: 34741-34745.
- Wu, Z., Qin, L., & Walker, V.K. (2009). Characterization and recombinant expression of a divergent ice nucleation protein from '*Pseudomonas borealis*'. *Microbiology*, 155:1164-1169.
- Xu, H., Griffith, M., Patten, C.L., & Glick, B.R. (1998) Isolation and characterization of an antifreeze protein with ice nucleation activity from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Can. J. Microbiol.*, 44:64-73.

## Chapter 3

# Further evidence that a grass ice-binding protein suppresses bacterial ice nucleation

### 3.1 Abstract

An ice-binding protein (IBP) with ice recrystallization (IR) inhibition activity from the model cereal, *Brachypodium distachyon* (*BdIRI*), depressed the freezing point of an ice nucleation protein (INP) preparation from the plant pathogen, *Pseudomonas syringae*. Although *BdIRI* is distinct, the data collected are consistent with the phenomena reported for *P. syringae* INP preparations when combined with an IBP from *Lolium perenne* (*LpAFP*) (Tomalty and Walker, 2014). Here we show a graphical representation of the freezing point depression, as well as representative images of IR inhibition activity and ice crystal morphologies. These data augment evidence in support of our hypothesis that plant IBPs could function as a defense mechanism against pathogenic bacterial ice nucleation.

#### 3.1.1 Specification table

Subject area	<i>Biology</i>
More specific subject area	<i>Depression of bacterial ice nucleation activity by a plant ice-binding protein</i>
Type of data	<i>Graph, Table, Figure</i>
How data were acquired	<i>Ice nucleation assay, Splat assay, Nanolitre osmometer</i>
Data format	<i>Analyzed</i>
Experimental factors	<i>Presence or absence of a recombinant plant IBP</i>
Experimental features	<i>Recombinant plant IBP was combined with INP from a plant pathogen. Protein combinations were subsequently assessed from ice nucleation, ice recrystallization inhibition, and ice crystal morphology.</i>
Data source location	<i>Kingston ON, Canada</i>
Data accessibility	<i>Within this article</i>

### 3.1.2 Value of the data

- Provides further insight into the role of plant ice-binding proteins
- Evidence that plant ice-binding proteins can act as “anti-nucleators”
- Provides additional support of the hypothesis that plant IBPs also function as a pathogen defense mechanism within the plant

## 3.2 Data

This paper presents data supporting our original research article “Perturbation of bacterial ice nucleation by a grass antifreeze protein” (Tomalty and Walker, 2014). Using the same methods outlined in the original article, a recombinant IBP from purple false brome, *B. distachyon* (*BdIRI*), was combined with *P. syringae* INP preparations. Although this protein is distinct from the IBP from the perennial rye grass, *L. perenne* (*LpAFP*), *BdIRI* was able to significantly depress the freezing point of the INP preparation. The presence of INPs did not affect ice-binding activity as assessed by IR inhibition, thermal hysteresis and ice crystal shaping abilities of *BdIRI*.

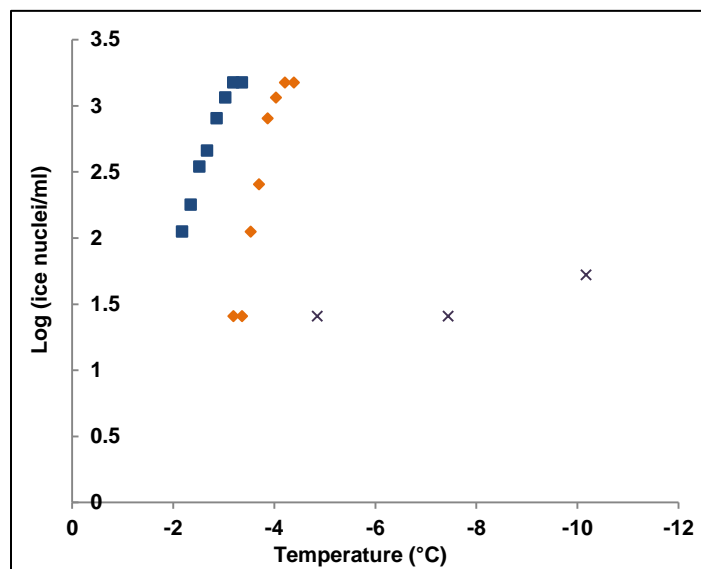
## 3.3 Experimental Design, Materials and Methods

### 3.3.1 Protein preparation

Recombinant *BdIRI* (Bradi5g27350.1) was purified using a modified version of a well-established protocol (Middleton *et al.*, 2009), while *P. syringae* INP preparations were purchased from Ward’s Natural Science (USA). Concentrations of the INP preparation tested ranged from 50 µg/mL to 5 µg/mL, while the concentration of *BdIRI* was 1 mg/mL.

### 3.3.2 Ice nucleation assays

Ice nucleation activity was assessed using a previously described technique (Tomalty and Walker, 2014; Vali, 1971). For each sample, the cumulative number of ice nuclei per ml was calculated using Vali’s equation (1971) and subsequently graphed (Fig 3.1). The addition of recombinant *BdIRI* resulted in the significant depression (two-tailed, unpaired t-test,  $P < 0.05$ ) of



**Figure 3.1** Representative graphs for ice nucleation activity shown as the logarithm of the cumulative number of ice nuclei per mL. Samples include a *P. syringae* INP preparation (blue squares), INPs combined with *BdIRI* (orange diamonds), and *BdIRI* alone (purple Xs).

the freezing point of INP preparations at all concentrations tested when compared to cytochrome c controls (Table 3.1). Mean freezing differences seen with *LpAFP* (Tomalty and Walker, 2014) are also shown in Table 1 for comparison purposes. All ice nucleation assays were performed in triplicate.

### **3.3.3 *Ice recrystallization inhibition and ice morphology assessments***

A modified version of the splat assay was used to assess IR inhibition, while ice morphology and thermal hysteresis were assayed with a Clifton nanolitre osmometer (Middleton *et al.*, 2014). The presence of the INP preparation did not affect the IR inhibition and ice crystal morphology of *BdIRI* (Fig. 3.2). The INP preparation from *P. syringae* also did not significantly affect the TH of *BdIRI*. In the presence of INPs, *BdIRI* displayed a mean TH value of 0.047 °C, while in the absence of INPs, a TH value of 0.052 °C was seen. All IR inhibition were performed in triplicate and TH/ice morphology assays were performed in duplicate.

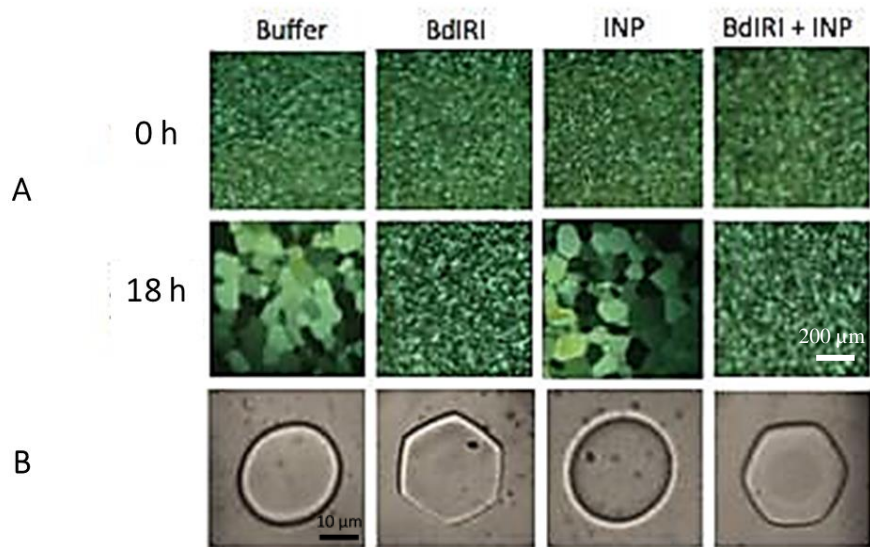
## **3.4 Acknowledgements**

Thank you to Dr. P. Davies for use of his nanolitre osmometer. This work was funded by an NSERC grant to VKW.

**Table 3.1** Mean differences in nucleation temperatures for INP preparations from *P. syringae* combined with recombinant *BdIRI*.

Protein Addition	[INP] <sup>1</sup>	
	0.05 mg/mL	0.005 mg/mL
<i>BdIRI</i> (1 mg/mL)	-1.26 °C ± 0.26 °C*	-2.41 °C ± 0.49°C*
<i>LpAFP</i> (1 mg/mL) <small>(Tomalty and Walker, 2014)</small>	-1.02 °C ± 0.31 °C*	-0.9 °C ± 0.29 °C*

1. Values notated with asterisks (\*) indicate samples that were significantly different from cytochrome c controls.



**Figure 3.2** Representative ice crystals from an IR inhibition splat assay (A) and typical ice crystal morphologies (B) in the presence of *P. syringae* ice nucleation protein (INP; 0.05 mg/mL) and *B. distachyon* AFP (BdIRI; 0.5 mg/mL). Both assays were performed in triplicate; magnifications are shown by the scale bars.

### 3.5 References

Middleton, A., Brown, A.M., Davies, P.L., and Walker, V.K. (2009). Identification of the ice-binding face of a plant antifreeze protein. *FEBS Letters*, 583: 815-819.

Middleton, A.J., Vanderbeld, B., Bredow, M. *et al.* (2014). Isolation and characterization of ice-binding proteins from higher plants. In: D.K. Hinch, E. Zuther (Eds.), *Plant Cold Acclimation: Methods and Protocols*. Springer, Heidelberg, pp. 255-277.

Tomalty, H.E. and Walker, V.K. (2014). Perturbation of bacterial ice nucleation by a grass antifreeze protein. *BBRC*, 452: 636-641.

Vali, G. (1971). Quantitative evaluation of experimental results on heterogeneous freezing nucleation of supercooled liquids. *J. Atmos. Sci.*, 28: 402.

## Chapter 4

### **Kidney preservation at subzero temperatures using a novel storage solution and insect ice-binding proteins**

#### **4.1 Abstract**

**BACKGROUND:** Contemporary kidney preservation methods involve storing at 4 °C up to 24 h prior to transplantation. By decreasing the storage temperature to below 0 °C, we hypothesized that the “safe” storage time could be significantly lengthened. **OBJECTIVE:** The efficacy of a proprietary CryoStasis (CrS) storage solution for the subzero preservation of kidneys was tested, with or without addition of a hyperactive insect antifreeze protein (*TmAFP*).

**MATERIALS AND METHODS:** Rat kidneys were stored in either University of Wisconsin (UW) solution (4 °C, 24 h), CrS (-2 °C, 48 h), or CrS with 61.5 μM *TmAFP* (-4.4 °C, 72 h). Following storage, viability was assessed with MTT reduction assays and live *vs.* dead cell (FDA/PI) staining. Markers of ischemic damage were analyzed using fluorometric substrates for caspase-3 and calpain activity. **RESULTS:** Kidneys stored in CrS for 48 h and CrS with *TmAFP* for 72 h displayed similar levels of enzymatic activity compared to 24 h UW controls. **CONCLUSION:** This methodology shows promise to prolong the safe storage time of kidneys and offers the potential of increased organ availability for renal transplants.

#### **4.2 Introduction**

Over the past 30 years, there has been little modification to donor kidney preservation using cold static storage. Conventional techniques involve organ perfusion with a cold preservation solution (most commonly University of Wisconsin solution, UW) and storage on ice at ~ 4 °C in a cooler (Belzer and Southard, 1988). Using this method, kidneys reliably withstand ischemic conditions for 24 h. Prolongation of storage times would have a profound effect on renal transplantations logistics. The geographical range between donor and recipient would be

increased, allowing for more optimal tissue matching. Organs might also sustain less ischemic damage, reducing the rate of early graft dysfunction following reimplantation.

Extension of safe storage time might be achieved through the supercooling of organs and preservation at high subzero temperatures in a non-frozen state (al-Abdulla *et al.*, 1995; Scotte *et al.*, 1996; Amir *et al.*, 2004a, 2004b; Okamoto *et al.*, 2008; Berendsen *et al.*, 2014). However, exposure to these low temperatures presents a unique set of challenges, including the probability of ice crystallization. To overcome this obstacle, additives such as DMSO and ethylene glycol are generally included to function as colligative freezing point depressors (al-Abdulla *et al.*, 1995, Scotte *et al.*, 1996; Okamoto *et al.*, 2008; Berendsen *et al.*, 2014). Such additives allow tissue storage below 0 °C, but they can also exert toxic effects, even at low concentrations (Yang *et al.*, 1993; Best, 2015). We have investigated a proprietary CryoStasis solution (CrS) which can colligatively depress the freezing point to -2 °C without the toxicity associated with other antifreeze agents. The freezing point can be further depressed by amending CrS with recombinant ice-binding or antifreeze proteins (IBPs/AFP), allowing storage up to ~ -5 °C.

IBPs have been identified in a wide array of cold-tolerant fish, insects, plants, and microorganisms (Ramsay, 1964; DeVries and Wohlschl, 1969; Sidebottom *et al.*, 2000; Raymond, 2011). Despite this diversity, all IBPs possess several distinctive properties that contribute to their protection conferred during exposure to subzero conditions. These include noncolligative freezing point depression of aqueous solutions, also known as thermal hysteresis (TH), the inhibition of ice recrystallization once freezing has occurred, and the “poisoning” of ice nucleation activity (Raymond and DeVries, 1977; Knight *et al.*, 1984; Tomalty and Walker, 2014). The concept of utilizing IBPs for organ preservation is not a new endeavor and has been investigated with varied success in the past (Wang *et al.*, 1994; al-Abdulla *et al.*, 1995; Soltys *et al.*, 2001; Amir *et al.*, 2004a, 2004b). However, previous studies have focused for the most part on the addition of fish AFPs, which only offer moderate levels of TH activity (~ 1 - 1.5 °C).

Hyperactive IBPs are characterized by high levels of TH activity, displaying close to 10-fold more activity compared to the moderately active fish AFPs (Scotter *et al.*, 2006). These types of IBPs have been described in insects such as the mealworm beetle, *Tenebrio molitor* (*TmAFP*), which was cloned and sequenced approximately 20 years ago. This IBP is expressed during development, allowing larvae to overwinter in a nonfrozen state (Graham *et al.*, 1997). With TH values of  $\sim -2.5$  °C at relatively low concentrations (Liou *et al.*, 2000), the use of this hyperactive insect IBP along with CrS could allow further decrease of the organ storage temperature, possibly extending the safe time for kidneys to be exposed to subzero ischemic conditions.

### **1.3 Materials and Methods**

#### ***4.3.1 Preservation solutions***

Kidneys were perfused with and stored in either University of Wisconsin Belzer cold storage solution (UW; Bridge to Life, USA), CryoStasis storage solution (CrS; Westport, ON), or CrS supplemented with 61.5  $\mu$ M recombinant AFP. Filter sterilization of the solutions ensured sterility, when appropriate.

#### ***4.3.2 Antifreeze protein preparation***

Recombinant AFP from *T. molitor* (*TmAFP*; isoform 4-9) was initially purified as a MBP-His<sub>6</sub>-TEV-*TmAFP* fusion protein. Briefly, the *TmAFP* fusion vector was transformed into *E. coli* Origami B (DE3) plysS cells (Novagen, USA). Transformed bacteria were grown overnight in  $\sim$ 200 mL Luria-Bertani (LB) medium with 25  $\mu$ g/mL chloramphenicol and 100  $\mu$ g/mL ampicillin. Overnight cultures were used to inoculate 3 L of LB medium in a New Brunswick BioFlo 110 fermentor (Enfield, CT) (Gordienko *et al.*, 2010). LB medium was supplemented with 5 g/L yeast, 6 g/L glucose, 12 g/L Na<sub>2</sub>HPO<sub>4</sub>, 6 g/L K<sub>2</sub>HPO<sub>4</sub>, 2 g/L NH<sub>4</sub>Cl, 0.022 g/L CaCl<sub>2</sub>, and 0.482 g/L MgSO<sub>4</sub>. The culture was grown at 37 °C, 500 RPM agitation, and 4 L/min air flow

until dissolved oxygen levels reached ~ 20%. The temperature was decreased to 15 °C and recombinant protein expression induced using 0.5 mM IPTG for 40 h.

After recovery of the cells by centrifugation (6,000 x g, 4 °C, 20 min), the fusion protein was purified as previously described by Bar *et al.* (2006) with modifications. Briefly, cells were lysed via sonication in 50 mM Tris, 300 mM NaCl, 5 mM imidazole, pH 8.0 and centrifuged at 16,000 x g, 4 °C for 40 min. The supernatant was incubated with Ni-NTA agarose beads (Qiagen) for 1-2 h and loaded into a column. The beads were washed with 2x bed volume of lysis buffer and the fusion protein eluted using a gradient of increasing imidazole concentrations (5 – 250 mM). Fractions were analyzed on SDS-PAGE gels, with the purest fractions pooled, concentrated to ~50% initial volume and dialyzed overnight at 4 °C in 50 mM Tris, 300 mM NaCl, pH 8.0.

To remove the carrier protein, MBP-His<sub>6</sub>-TEV-*TmAFP* was digested with TEV protease (1:100 molar ratio of enzyme: substrate) at 4 °C for 4 h in 50 mM Tris, 300 mM NaCl, 3 mM reduced glutathione, and 0.3 mM oxidized glutathione, pH 8.0. After cleavage, TEV protease and MBP-His<sub>6</sub> was removed by incubating the mixture with Ni-NTA beads for 1-2 h at 4 °C. Beads were subsequently loaded into a column and the flow through collected. This solution contained cleaved *TmAFP*.

Following TEV cleavage, *TmAFP* preparations were verified for purity on a SDS-PAGE gel and lyophilized. Protein concentrations were determined using a Pierce BCA assay (Thermo Scientific, USA) and TH activity assessed using a Clifton nanolitre osmometer (Chakrabartty and Hew, 1991). Lyophilized *TmAFP* was dissolved in CrS to a final concentration of 61.5 µM and stored at -20 °C.

#### ***4.3.3 Surgical procedure***

Male Sprague-Dawley rats (275-300 g body weight) were obtained from Charles River Laboratories (Montreal, Que.). Under isoflurane anesthesia, the abdominal aorta and inferior vena cava (IVC) were isolated above and below the renal vessels through a midline incision. Heparin

was administered via the IVC (70 U/kg) and allowed to circulate for two min. Subsequently, the aorta was clamped at the iliac bifurcation, as well as above the renal arteries. A catheter was inserted into the aorta inferior to the left renal artery and secured in place.

The left kidney was perfused with cold preservation solution (~10-20 mL; 2 °C for UW, -2 °C for CrS and -4 °C for CrS + *TmAFP*) through the catheter, where the solution was then drained directly into the peritoneal cavity through the left renal vein, which was divided distally. Once adequately perfused, as indicated by the appearance of pale colouring, the kidney was removed and placed in a sterile container filled with 10 mL of the appropriate preservation solution cooled to the applicable temperature (UW: 4 °C; CrS: -2 °C; CrS + *TmAFP*: -4.4 °C). Kidneys perfused with CrS containing *TmAFP* were initially perfused with 10-15 mL of CrS, followed by 5 mL of CrS + *TmAFP*. This study was conducted in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Queen's University Animal Care Committee.

#### **4.3.4 Organ storage**

To determine optimal storage time for each preservation solution, initially the kidneys were held over three different time points (24, 48, and 72 h) in either UW or CrS solution at 4 °C and -2 °C, respectively. Following each storage point, tissue viability was assessed using live *vs.* dead cell staining and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium salt reduction assays as outlined below.

In the latter half of the study, kidneys were stored for times determined by analysis of the initial results. UW perfused kidneys were stored for 24 h at 4 °C, while CrS kidneys were held at -2 °C for 48 h. Finally, CrS with 61.5 µM *TmAFP* was held for 72 h at -4.4 °C. Dehydrogenase activity was again assessed following the storage periods using the MTT assay. Markers of ischemic damage (caspase-3 and calpain activity) were also assayed, as detailed below.

#### ***4.3.5 Fluorescein diacetate and propidium iodide cell staining (FDA/PI)***

Kidney cortical regions were thinly sectioned, stained with 2  $\mu$ L propidium iodide (PI; stock 2 mg PI/1 mL PBS) and fluorescein diacetate (FDA; stock 5 mg FDA/ 1 mL acetone), and gently compressed under a coverslip. Sections were examined under fluorescent light conditions (FITC filter) at 750 x magnification on an epifluorescent Olympus BH-2 microscope mounted with a 12 megapixel Canon camera. Dead cells, indicated by the emittance of red fluorescent light, were manually counted using ImageJ software (version 2.0.0-rc-43/1.50) after being photographed. Cell viability (%) was calculated by subtracting the number of PI stained cells from mean cell number estimates and dividing by total cell numbers.

#### ***4.3.6 MTT reduction assay***

Kidneys were decapsulated and the cortices sampled with sterile razor blades. Tissue samples ranged from approximately 25 – 55 mg, with three samples collected per kidney. These samples were then placed in 1 mL of sterile MTT solution (0.2 mg/mL MTT in PBS) and incubated for 2 h at 37 °C. Following the incubation period, samples were centrifuged at 16,060 x g for 2 min, the MTT solution was decanted and 1 mL of 100% dimethyl sulfoxide (DMSO) added. Tissue samples were incubated again for 2 h at 37 °C. Absorbance of each sample was determined at 570 nm, followed by a background reading at 690 nm using a SpectraMax Plus plate reader (Molecular Devices, USA). Background readings were subtracted from the 570 nm readings and results were graphed as mean absorbance/tissue mass.

#### ***4.3.7 Caspase-3 and calpain assays***

The remaining cortex of each kidney was finely minced with a sterile razor blade and homogenized in lysis buffer (25 mM HEPES- HCl, pH 7.2, 2 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 1 mM PMSF, 1  $\mu$ M pepstatin A) using ten strokes in a Dounce homogenizer. Homogenates were centrifuged at 100,000 x g for 1 h at 4 °C, flash frozen and

stored at -80 °C. Total protein concentration of each homogenate was determined using  $A_{280}$  readings from a Nanodrop 2000 (Thermo Scientific, USA)

Caspase-3 activity was assessed using an EnzChek kit (Molecular Probes, USA) with modification to the protocol. Briefly, 200  $\mu\text{g}$  of lysate protein was mixed with 50  $\mu\text{L}$  of 10 mM Z-DEVD-AMC substrate. The reaction was made to 100  $\mu\text{L}$  with lysis buffer. Substrate cleavage was monitored for 1 h at 37 °C using excitation and emission wavelengths of 360 and 465 nm respectively on a SpectraMax Gemini plate reader (Molecular Devices, USA). Using AMC standard curves, caspase-3 like activity was assayed as  $\mu\text{M}$  AMC released over 60 min, as well as pmol released/min/mg of lysate protein.

Calpain activity was assessed as described by Jani *et al.* (2004). Briefly, 100  $\mu\text{g}$  of lysate protein was mixed with reaction buffer (62.5 mM Imidazole-HCl, pH 7.4, 10 mM mecarphoethanol) either with or without 5 mM  $\text{CaCl}_2$  to a final volume of 190  $\mu\text{L}$ . Reaction buffer without calcium was supplemented with 1 mM EDTA and 10 mM EGTA. After a 10 min incubation period at 37 °C, the cleavage reactions were initiated by adding 10  $\mu\text{L}$  of 10 mM N-succinyl-Leu-Tyr-7-Amino-4-methylcoumarin (Sigma-Aldrich, Canada). Cleavage was spectrophotometrically monitored over 1 h at 37 °C using wavelengths of 380 and 460 nm. Calpain-like activity was defined as the difference between dependent and independent calcium fluorescence and presented as  $\mu\text{M}$  AMC released over 60 min and AMC released (pmol) per minute per mg of lysate protein. All enzyme assays were performed in triplicate and data were pooled for statistical analysis.

#### ***4.3.8 Statistical analysis***

Statistical analysis was performed using SigmaPlot software (Systat Software Inc., USA). To determine significance differences between multiple treatment groups, normally distributed data were analyzed with one- and two-way ANOVAs, while nonparametric data were analyzed with ANOVA on ranks. *Post hoc* comparisons were performed using the Holm-Sidak method.

Differences between two groups were determined using t-tests. Statistical significance was concluded when  $p < 0.05$ . All results are reported as means  $\pm$  SEM.

## **4.4 Results**

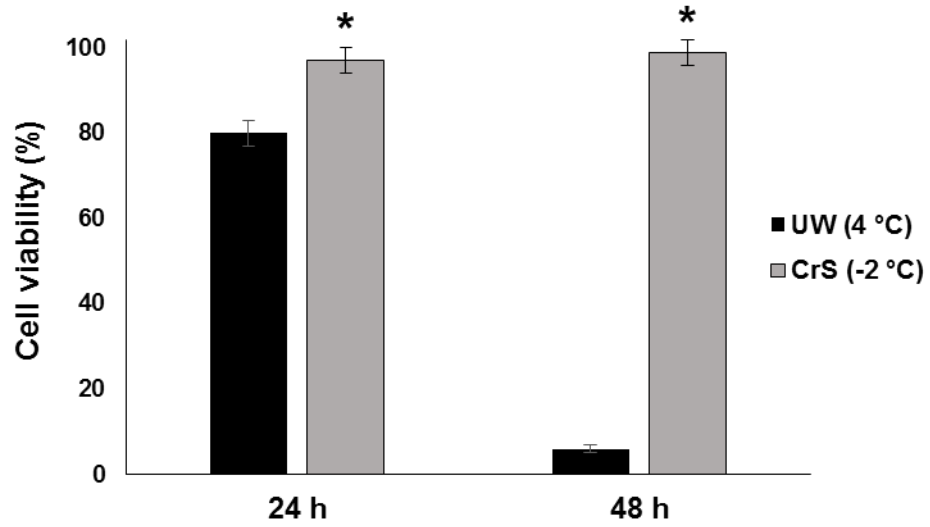
### ***4.4.1 Approximate optimal storage times***

Kidneys preserved in UW can be typically stored for 24 h prior to transplantation (Guibert *et al.*, 2011). Using FDA/PI live vs. dead cell staining along with MTT assays to measure tissue viability, we confirmed this previous result (Figure 4.1). To determine if kidneys could be preserved for a longer period, the CrS preservation solution was used to store kidneys at temperatures below 0 °C. Analysis of FDA/PI stained sections of cortical samples showed CrS-perfused kidneys had significantly higher mean live cell counts compared to those stored in UW. Live cell counts were  $97 \pm 3\%$  and  $99 \pm 1\%$  for 24 and 48 h, respectively. In comparison, UW controls displayed live cell counts of  $80 \pm 3\%$  at 24 h and  $6 \pm 1\%$  at 48 h (Figure 4.1; unpaired t-tests,  $p < 0.05$ ). Complementary results were observed using MTT assays. Kidneys stored in CrS at -2 °C for 24 and 48 h had similar levels of tissue viability as did kidneys held at 4 °C for 24 h in UW. After 72 h storage, viability was significantly decreased in both preservation solutions (Figure 4.2; two-way ANOVA,  $p < 0.05$ ).

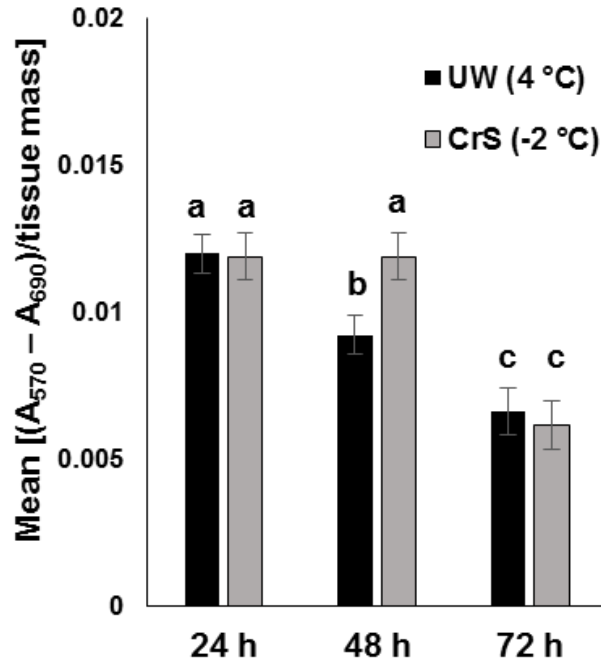
### ***4.4.2 Addition of TmAFP to CrS***

To assess whether the amendment of CrS with a recombinant insect AFP could extend the preservation time further by allowing storage at lower temperatures, kidneys were perfused with either UW, CrS, or CrS + TmAFP and stored at 4 °C for 24 h, -2 °C for 48 h, and -4.4 °C for 72 h, respectively. Assessment of overall viability using MTT assays indicated no significant differences between the three groups despite 2-3 fold longer storage times at the subzero temperatures (Figure 4.3; one-way ANOVA,  $p > 0.05$ ).

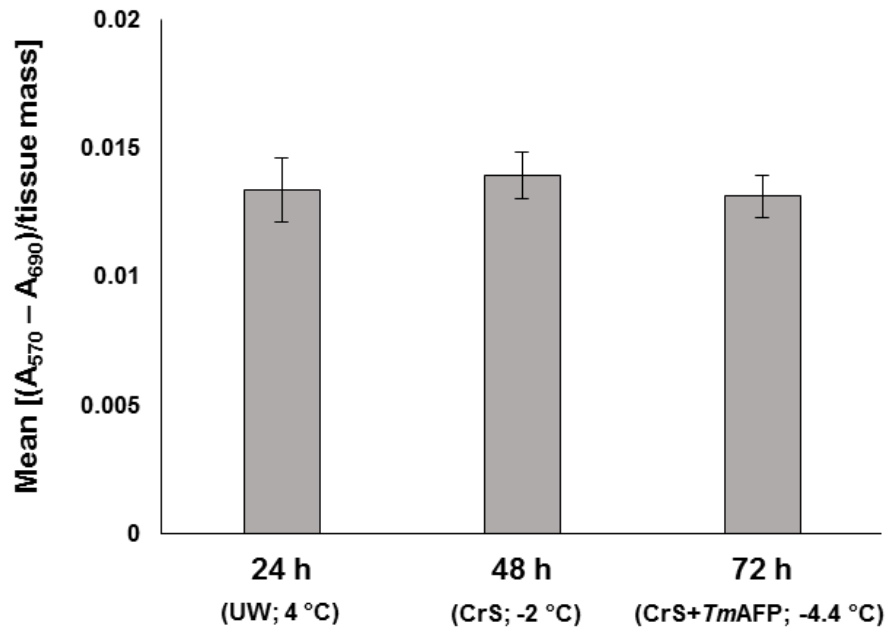
Two enzymes associated with the accumulation of ischemic damage, caspase-3 and calpain, were also assayed. Caspase-3 activity increased in the 24 h UW and 48 h CrS samples,



**Figure 4.1** Comparison of live vs. dead cells, as determined by FDA/PI staining, in kidneys stored in UW or CrS for 24 and 48 h. Asterisks indicate significantly higher live cell counts.



**Figure 4.2** Tissue viability of UW and CrS rat kidneys as assessed by MTT reduction assays at 4 °C (dark bars) and -2 °C (light bars) for 24, 48, and 72 h. Error bars represent  $\pm$  standard error of the mean (SEM) and 2-3 kidneys were used per treatment. Three tissue samples were collected per kidney for the assays; differing letters above the bars indicate significant differences.



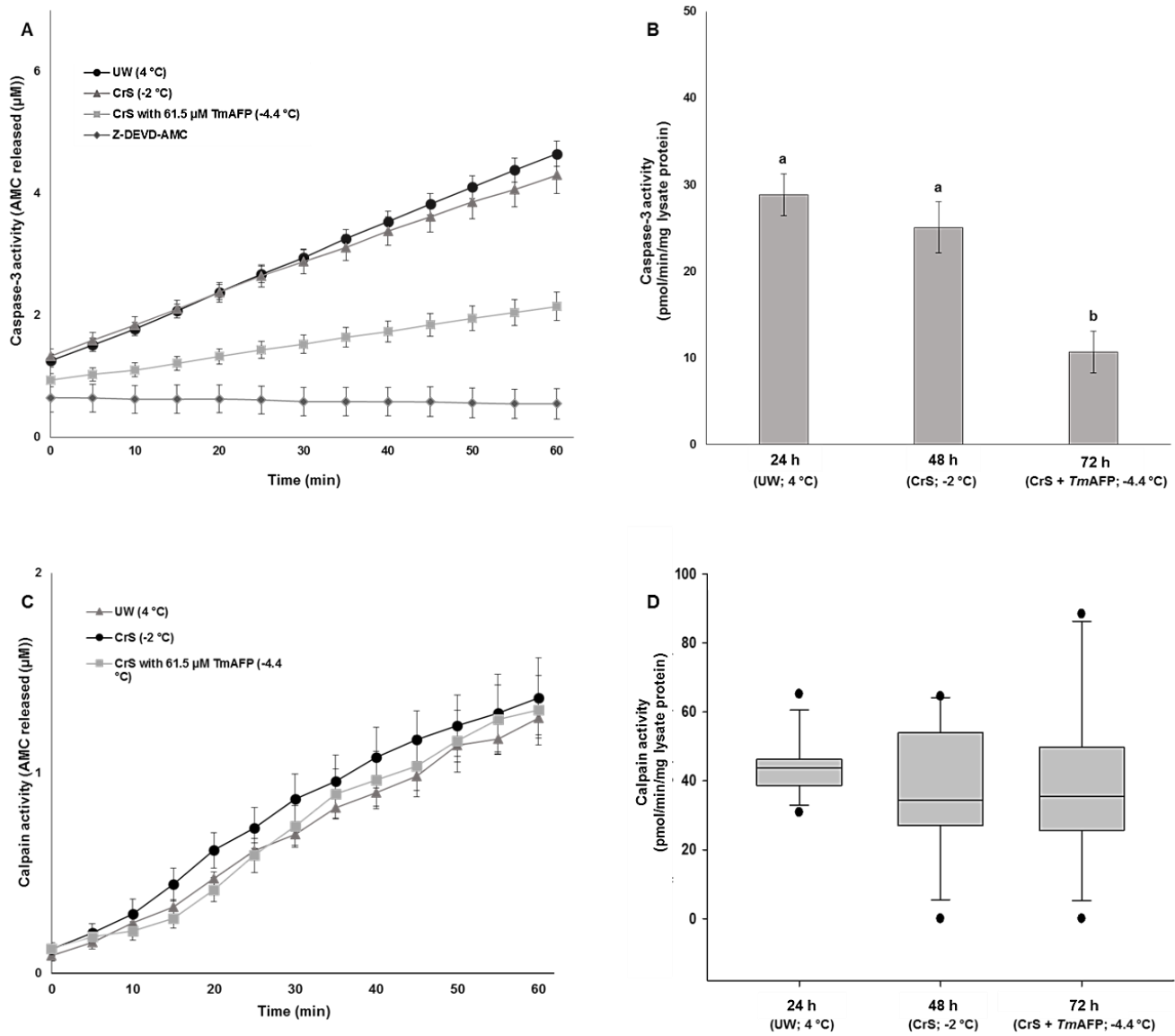
**Figure 4.3** Tissue viability of rat kidneys, as assessed by MTT assays, perfused and held in either UW, CrS, or CrS + *TmAFP* solutions. There was no significant difference in kidneys stored in CrS + *TmAFP* for 72 h at -4.4 °C compared to those stored in either UW (4 °C, 24 h) or CrS (-2 °C, 48 h). Error bars represent  $\pm$ SEM and 4 kidneys were used per treatment. Three tissue samples were collected per kidney for the MTT assays.

with a mean  $28.8 \pm 2.3$  pmol/min/mg and  $25.1 \pm 2.9$  pmol/min/mg of released AMC, respectively. 72 h CrS + *TmAFP* kidneys had significantly lower levels of enzyme activity at  $10.7 \pm 2.4$  pmol/min/mg (Figure 4.4A & 4B; one-way ANOVA,  $p < 0.05$ ). Calpain-like activity was present in all three cold ischemic groups. However, no significant differences in activity were detected between the UW (43.7 pmol of AMC/min/mg), CrS (34.3 pmol of AMC/min/mg), and CrS + *TmAFP* (35.4 pmol of AMC/min/mg) treatments (Figure 4.4C & 4D; ANOVA on ranks,  $p > 0.05$ ).

#### 4.5 Discussion

It has been well established that current cold static preservation methods allow the storage of kidneys for a maximum of 24 h, after which markers of ischemic damage start to rapidly accumulate (Kosieradzki and Rowiński, 2008; Guibert *et al.*, 2011). Storing organs under hypothermic conditions (0 – 4 °C) decreases cellular metabolism, thereby lessening the demand on energy reserves and reducing the accumulation of toxic byproducts. Despite this reduction, detrimental effects continue to accumulate. Lethal cell swelling, cellular acidosis, and production of radical oxygen species upon reperfusion are common drawbacks to this method (Maathuis *et al.*, 2008). We reasoned that these drawbacks could be significantly suppressed by storing at subzero temperatures, thereby further reducing metabolic rate. We have investigated the use of a novel preservation solution, CrS, which might extend the safe cold static storage time of kidneys for transplant purposes.

CrS-perfused kidneys displayed significantly increased counts of live cells in relation to UW controls when analyzed with FDA/PI staining (Figure 4.1). As a membrane-impermeable dye, PI only enters the nuclei upon cellular membrane injury where it intercalates with DNA, emitting red fluorescence. In comparison, FDA is a cell-permeable esterase that elucidates the enzyme activity of living cells by emitting green light when excited by a fluorescent light source. As



**Figure 4.4** Enzyme assessments showing (A) caspase-3 initiated cleavage of the Z-DEVD-AMC substrate over 60 min, (B) the concentration of AMC released (pmol) per min per mg of lysate protein, (C) calpain substrate cleavage over 60 min and (D) the amount of AMC released (pmol) per min per mg of lysate protein. Assays were performed in triplicate and the data were pooled;  $\pm$ SEM is represented by the error bars (A, B, and C); box plots represent the 25<sup>th</sup> and 75<sup>th</sup> percentile with whiskers extended to the 10<sup>th</sup> and 90<sup>th</sup> percentile (D); 4 kidneys were used per treatment.

indicated, higher live cell counts were observed in CrS samples following both 24 and 48 h storage compared to UW controls.

Comparable results were also observed during the assessment of overall tissue viability. The MTT assay measures cell viability through the reduction of yellow water soluble tetrazolium salt to purple insoluble formazan predominantly by intracellular dehydrogenases and oxidoreductases (Mosmann, 1983; Berridge *et al.*, 2005). Generally used for viability assessments of cultured cells, the technique has also been shown to be effective for assessing tissue viability (Tagboto and Griffiths, 2007; Tagboto, 2008; Torre *et al.*, 2012). Initial experiments measuring the reduction of MTT suggest that CrS could safely extend the organ's storage time by 24 h when held at -2 °C. As shown in Figure 4.2, CrS samples exhibited levels of reducing activity after 48 h, similar to those seen in 24 h UW control samples. Furthermore, when CrS was supplemented with *TmAFP* and the storage temperature lowered to -4.4 °C for 72 h, the substrate was reduced to the same level as was observed in UW samples after 24 h and CrS samples after 48 h (Figure 4.3). Taken together, these results suggested that CrS was able to extend the preservation time, with the addition of *TmAFP* further prolonging the storage time. However, one disadvantage of MTT assays is the underestimation of non-viable cells (Wang *et al.*, 2010; Torre *et al.*, 2012). For this reason, it was important to compare markers of ischemic damage between the different preservation solutions.

Prolonged exposure to cold ischemic conditions has been reported to lead to increased kidney injury upon reperfusion, directly impacting the success of graft function following transplantation (Salahudeen *et al.*, 2004). While the causes of delayed graft function are complex and multi-factorial, one source appears to be elevated levels of apoptosis, particularly in tubular cells (Oberbauer *et al.*, 1999; Castaneda *et al.*, 2003). Further investigations into the underlying molecular mechanisms have revealed a variety of pathways involved with cysteine proteases

suggested to be central mediators, including members of the enzyme families caspase-3 and calpain (Kohli *et al.*, 1997; Edelstein *et al.*, 1999; Shi *et al.*, 2000; Jani *et al.*, 2004).

Caspase-3 is a well-studied executioner protease which plays a dominant role in the final stages of apoptosis (Liu *et al.*, 1997). During cold static storage, caspase-3 levels in UW-perfused mouse kidneys were found to increase over 100-fold after 48 h compared to 0 h controls (Jani *et al.*, 2004). As seen here, after 48 h in CrS at -2 °C, rat kidneys showed similar levels of caspase-3 activity as observed in 24 h UW controls, suggesting that the subzero storage slowed the progression of apoptotic pathways. Additionally, CrS with 61.5 µM *TmAFP* displayed significantly lower levels of caspase-3 activity compared to UW and CrS alone (Figure 4.4). The introduction of caspase-3 inhibitors to UW solution reportedly lowered levels of apoptotic cells in both murine kidneys and rat livers (Jani *et al.*, 2004; Natori *et al.*, 2003), as well as improved survival following liver transplantation in rat models (Mueller *et al.*, 2004), further demonstrating the role this enzyme plays in limiting the duration of successful organ storage. Our results suggest that decreasing the storage temperature might produce a similar effect as these inhibitors by suppressing caspase-3 activity.

Calcium-dependent calpain is a cysteine protease implicated in the accumulation of apoptotic cells during ischemic exposure (Sindram *et al.*, 1999). While the detailed mechanisms initiated by calpain are not fully understood, the enzyme has been associated with the occurrence of ischemia/reperfusion injury, with activity observed to significantly increase during cold ischemic conditions in kidneys, livers, and hearts (Sindram *et al.*, 1999, Shi *et al.*, 2000; Chen *et al.*, 2002). We observed that after 48 h in CrS and 72 h in CrS + *TmAFP*, calpain activity was similar to the levels seen in UW after only 24 h. Similar to the results seen with caspase-3 activity, decreasing the storage temperatures appears to suppress the activation of enzymes associated with the progression of cold-induced kidney injury.

With longer wait-lists and more organs being harvested from marginal and non-heart beating donors, quality organ preservation is crucial for successful transplant outcomes. While current cold static preservation methods are effective for short term storage, there is room for improvement. Overall, our results support the hypothesis that preservation using CrS solution at subzero temperatures have the potential to extend safe storage times. We have also shown that some of the underlying molecular mechanisms related to ischemic injury are further suppressed by this method, which could lead to lower rates of early graft dysfunction, as well as better long-term function following transplantation.

#### **4.6 Acknowledgements**

We thank Drs. Z. Jia and P. Davies for use of their Bio Flo 110, nanolitre osmometer and nanodrop, as well as Dr. D. Fass for providing the *TmAFP* construct. We also thank Drs. M. Adams and R. Holden for their helpful insight. This project, originally conceived and funded by CryoStasis Ltd., was partially funded by IRAP, NSERC, and a Mitacs Accelerate grant.

## 4.7 References

- Al-Abdulla, N. A., Cole Jr, G., Braxton, J. H., *et al.* (1995). The effects of supercooling chemicals on myocardial ultrastructure: a transmission electron microscopy case study. *Connecticut medicine*, 59: 387-399.
- Amir, G., Rubinsky, B., Horowitz, L., *et al.* (2004a). Prolonged 24-hour subzero preservation of heterotopically transplanted rat hearts using antifreeze proteins derived from arctic fish. *The Annals of thoracic surgery*, 77: 1648-1655.
- Amir, G., Horowitz, L., Rubinsky, B., *et al.* (2004b). Subzero nonfreezing cryopreservation of rat hearts using antifreeze protein I and antifreeze protein III. *Cryobiology*, 48: 273-282.
- Bar, M., Bar-Ziv, R., Scherf, T., & Fass, D. (2006). Efficient production of a folded and functional, highly disulfide-bonded  $\beta$ -helix antifreeze protein in bacteria. *Protein expression and purification*, 48: 243-252.
- Belzer, F. O., & Southard, J. H. (1988). Principles of solid-organ preservation by cold storage. *Transplantation*, 45: 673-676.
- Berendsen, T. A., Bruinsma, B. G., Puts, C. F., *et al.* (2014). Supercooling enables long-term transplantation survival following 4 days of liver preservation. *Nature medicine*, 20: 790-793.
- Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology annual review*, 11: 127-152.
- Best, B. P. (2015). Cryoprotectant toxicity: facts, issues, and questions. *Rejuvenation research*, 18: 422-436.
- Castaneda, M. P., Swiatecka-Urban, A., Mitsnefes, *et al.* (2003). Activation of mitochondrial apoptotic pathways in human renal allografts after ischemia-reperfusion injury. *Transplantation*, 76: 50-54.
- Chakrabarty, A., & Hew, C. L. (1991). The effect of enhanced  $\alpha$  -helicity on the activity of a winter flounder antifreeze polypeptide. *European Journal of Biochemistry*, 202: 1057-1063.
- Chen, M., Won, D. J., Krajewski, S., & Gottlieb, R. A. (2002). Calpain and mitochondria in ischemia/reperfusion injury. *Journal of Biological Chemistry*, 277: 29181-29186.
- DeVries, A. L., & Wohlschlag, D. E. (1969). Freezing resistance in some Antarctic fishes. *Science*, 163: 1073-1075.
- Edelstein, C. L., Shi, Y., & Schrier, R. W. (1999). Role of caspases in hypoxia-induced necrosis of rat renal proximal tubules. *Journal of the American Society of Nephrology*, 10: 1940-1949.
- Graham, L. A., Liou, Y. C., Walker, V. K., & Davies, P. L. (1997). Hyperactive antifreeze protein from beetles. *Nature*, 388: 727-728.
- Gordienko, R., Ohno, H., Singh, V. K., *et al.* (2010). Towards a green hydrate inhibitor: imaging antifreeze proteins on clathrates. *PLoS One*, 5: e8953.

- Guibert, E. E., Petrenko, A. Y., Balaban, C. L., *et al.* (2011). Organ preservation: current concepts and new strategies for the next decade. *Transfusion Medicine and Hemotherapy*, 38: 125-142.
- Jani, A., Ljubanovic, D., Faubel, S., *et al.* (2004). Caspase inhibition prevents the increase in caspase -3, -2, -8 and -9 activity and apoptosis in the cold ischemic mouse kidney. *American Journal of Transplantation*, 4: 1246-1254.
- Knight, C. A., De Vries, A. L., & Oolman, L. D. (1984). Fish antifreeze protein and the freezing and recrystallization of ice. *Nature*, 208: 295-296.
- Kohli, V., Gao, W., Camargo, C. A., & Clavien, P. A. (1997). Calpain is a mediator of preservation-reperfusion injury in rat liver transplantation. *PNAS*, 94: 9354-9359.
- Kosieradzki, M., & Rowiński, W. (2008). Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplantation proceedings*, 40: 3279-3288.
- Liou, Y. C., Daley, M. E., Graham, L. A., *et al.* (2000). Folding and structural characterization of highly disulfide-bonded beetle antifreeze protein produced in bacteria. *Protein expression and purification*, 19: 148-157.
- Liu, X., Zou, H., Slaughter, C., & Wang, X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, 89: 175-184.
- Maathuis, M. H. J., Leuvenink, H. G., & Ploeg, R. J. (2007). Perspectives in organ preservation. *Transplantation*, 83: 1289-1298.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, 65: 55-63.
- Mueller, T. H., Kienle, K., Beham, A., *et al.* (2004). Caspase 3 inhibition improves survival and reduces early graft injury after ischemia and reperfusion in rat liver transplantation. *Transplantation*, 78: 1267-1273.
- Natori, S., Higuchi, H., Contreras, P., & Gores, G. J. (2003). The caspase inhibitor IDN -6556 prevents caspase activation and apoptosis in sinusoidal endothelial cells during liver preservation injury. *Liver transplantation*, 9: 278-284.
- Oberbauer, R., Rohrmoser, M., Regele, H., *et al.* (1999). Apoptosis of tubular epithelial cells in donor kidney biopsies predicts early renal allograft function. *Journal of the American Society of Nephrology*, 10: 2006-2013.
- Okamoto, T., Nakamura, T., Zhang, J., *et al.* (2008). Successful sub-zero non-freezing preservation of rat lungs at -2 C utilizing a new supercooling technology. *The Journal of heart and lung transplantation*, 27: 1150-1157.
- Ramsay, R.A. (1964). The rectal complex of the mealworm *Tenebrio molitor*, L. (Coleoptera, Tenebrionidae). *Phil. Trans. R. Soc. Lond. B*, 248: 279-314.
- Raymond, J. A. (2011). Algal ice-binding proteins change the structure of sea ice. *PNAS*, 108: E198-E198.

- Raymond, J. A., & DeVries, A. L. (1977). Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *PNAS*, *74*: 2589-2593.
- Salahudeen, A. K., Haider, N., & May, W. (2004). Cold ischemia and the reduced long-term survival of cadaveric renal allografts. *Kidney international*, *65*: 713-718.
- Scotte, M., Eschwege, P., Cherruau, C., *et al.* (1996). Liver preservation below 0 C with UW solution and 2, 3-butanediol. *Cryobiology*, *33*: 54-61.
- Scotter, A. J., Marshall, C. B., Graham, L. A., *et al.* (2006). The basis for hyperactivity of antifreeze proteins. *Cryobiology*, *53*: 229-239.
- Shi, Y., Melnikov, V. Y., Schrier, R. W., & Edelstein, C. L. (2000). Downregulation of the calpain inhibitor protein calpastatin by caspases during renal ischemia-reperfusion. *American Journal of Physiology-Renal Physiology*, *279*: F509-F517.
- Sidebottom, C., Buckley, S., Pudney, P., *et al.* (2000). Phytochemistry: heat-stable antifreeze protein from grass. *Nature*, *406*: 256-256.
- Sindram, D., Kohli, V., Madden, J. F., & Clavien, P. A. (1999). Calpain inhibition prevents sinusoidal endothelial cell apoptosis in the cold ischemic rat liver. *Transplantation*, *68*: 136-140.
- Soltys, K. A., Batta, A. K., & Koneru, B. (2001). Successful nonfreezing, subzero preservation of rat liver with 2, 3-butanediol and type I antifreeze protein. *Journal of Surgical Research*, *96*: 30-34.
- Tagboto, S. K. (2008). The development of a novel in vitro model using kidney biopsy specimens to study the effects of warm and cold ischaemia on the kidney. *Cell & Tissue Transplantation & Therapy*, *1*: 1.
- Tagboto, S., & Griffiths, A. P. (2007). The evaluation of renal ischaemic damage: the value of CD10 monoclonal antibody staining and of biochemical assessments of tissue viability. *BMC clinical pathology*, *7*: 1.
- Tomalty, H. E., & Walker, V. K. (2014). Perturbation of bacterial ice nucleation activity by a grass antifreeze protein. *Biochemical and biophysical research communications*, *452*: 636-641.
- Torre, A., Momier, M., Mazoyer, C., Selva, J., Salle, B., & Lornage, J. (2012). Validation of a new metabolic marker to assess the vascular viability of vitrified whole sheep ovaries. *Human reproduction*, *27*: 1811-1821.
- Wang, P., Henning, S. M., & Heber, D. (2010). Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *PLoS one*, *5*: e10202.
- Wang, T., Zhu, Q., Yang, X., Layne, J. R., & DeVries, A. L. (1994). Antifreeze glycoproteins from antarctic notothenioid fishes fail to protect the rat cardiac explant during hypothermic and freezing preservation. *Cryobiology*, *31*: 185-192.
- Yang, X., Zhu, Q., Layne, J. R., Claydon, M., Hicks, G. L., & Wang, T. (1993). Subzero nonfreezing storage of the mammalian cardiac explant: I. Methanol, ethanol, ethylene glycol, and propylene glycol as colligative cryoprotectants. *Cryobiology*, *30*: 366-375.

## Chapter 5

### Goldenrod and a gall fly: two ice-binding proteins

#### 5.1 Abstract

Ice-binding protein (IBP) expression is seen in a diverse range of species across many phyla. While IBPs are relatively well established in freeze-avoidance overwintering mechanisms in some species, they are considerably less understood in freeze-tolerant organisms. Winter survival and polyol production in the freeze-tolerant gall fly, *Eurosta solidaginis*, has been well documented. However, it is unknown whether the larvae produce an ice-binding protein as part of their low temperature response. We assayed ice-binding activity in collected larvae acclimated to three different temperatures (15 °C, 4 °C, -15 °C), representing three periods in the mid-autumn to winter transition. Larva representing mid-autumn animals (15 °C) displayed ice recrystallization inhibition and ice shaping/thermal hysteresis activity. Hemolymph samples from October-collected larva also showed ice-binding activity. Thus, *E. solidaginis* may produce IBPs as a protective measure against freezing damage when autumn temperatures can unexpectedly drop below 0 °C. Assessment of the larva's host plant, *Solidago canadensis*, indicated that October collections also displayed characteristics associated with the presence of an IBP. Liquid chromatography tandem mass spectrometry of ice-affinity purified plant extracts provided three candidate pathogenesis-related proteins that may be responsible for the detected activity, and indicated that the putative *Eurosta* IBP was not derived from its host plant.

#### 5.2 Introduction

Third instar larvae of the goldenrod gall fly, *Eurosta solidaginis*, are well-known for their overwintering abilities. Residing in galls located on the stem of goldenrod (*Solidago* spp.), these larvae survive winter temperatures as low as -30 °C by allowing the freezing of ~ 65% of their total body water (Duman, 2001). During September/early October in the northern hemisphere,

key biochemical and physiological changes start to occur, including the accumulation of high concentrations of cryoprotectants, adjustments in fatty acid composition, increased expression of membrane-bound channel proteins, and the introduction of ice nucleators to their tissues (Storey and Storey, 1983; Philip and Lee, 2010; Bennett *et al.*, 1997; Mugnano *et al.*, 1996).

The cryoprotectant, glycerol, is synthesized at relatively high temperatures ( $> 10\text{ }^{\circ}\text{C}$ ), and is possibly associated with the second to third instar transition during the late summer (Storey and Storey, 1983). Hormones, along with environmental cues from their plant host, are likely to function as initiators (Hamilton *et al.*, 1986; Rojas *et al.*, 1986). In comparison, the production of other cryoprotectants, sorbitol and trehalose, is a direct response to low temperature exposure (Baust and Lee, 1982; Storey and Storey, 1983). Upregulation of membrane-bound aquaporins and aquaglyceroporins to facilitate the rapid redistribution of water and cryoprotectants can be also readily observed in the larvae during overwintering preparations (Philip and Lee, 2010), as well as increases in unsaturated phospholipids and triacylglycerols during the onset of freezing tolerance (Bennett *et al.*, 1997). These larvae are also notable for the ability to allow some intracellular freezing, through the use of calcium phosphate crystals which act as ice nucleators within fat body cells (Mugnano *et al.*, 1996). However, it is unknown whether *E. solidaginis* synthesize any ice-binding proteins (IBPs) as part of their overwintering strategy.

IBPs control the growth of ice. Best known for their ability to depress the freezing point of aqueous solutions, as observed in certain polar fish and freeze-avoiding insects (reviewed in Davies, 2014), IBP expression is less understood in freeze-tolerant animals. To date, the best characterized IBPs in freeze-tolerant species are found in plants (Atici and Nalbantoglu, 2003). Plant IBPs function by controlling the growth of ice crystals at temperatures close to  $0\text{ }^{\circ}\text{C}$  (Knight and Duman, 1996; Marentes *et al.*, 1993), and may also depress the ice nucleation activity (INA) of pathogenic bacteria (Tomalty and Walker, 2014), because uncontrolled ice growth could have devastating effects on cellular structures. Since *E. solidaginis* larvae exposed to low temperatures

would similarly encounter situations of large ice crystal growth and bacterial ice nucleators, it is plausible that they would also synthesize IBPs. However, it is also possible that the larvae could acquire IBPs passively by ingesting their host plant, goldenrod (*Solidago spp.*). IBPs have not been reported in this species. Plants with IBPs are normally perennial species with vegetation that survives low temperatures. In contrast, the vegetative portion of the perennial, *Solidago spp.*, senesces after flowering each year; thus, there may be no requirement for an IBP in this species.

## 5.3 Materials and Methods

### 5.3.1 *E. solidaginis* and *S. canadensis* sample preparations

Initial collections of *E. solidaginis* larvae were made in mid-October 2009 (Ottawa, ON). Galls were removed from green plants, and the larvae acclimated to +15 and -15 °C for three weeks to represent early autumn and mid-winter temperatures, respectively. Following incubation, larvae were flash frozen and stored at -80 °C. Larvae representing late autumn temperatures were left in their galls, acclimated and stored at 4 °C. To test for ice-binding activity, homogenates were made (n = 3 larvae for each temperature group) in 1 mL PBS buffer, pH = 7.5, 1 mM phenylthiourea (PTU), supplemented with a Roche cOmplete protease inhibitor tablet (Sigma Aldrich, Canada). Crude preparations were then centrifuged at 16,000 x g for 5 min, the supernatant collected and the extracts stored at -20 °C. Trial assays were performed to determine the appropriate dilutions for IBP assays. 1:5 and 1:10 dilutions were used for ice nucleation assays, while 1:15 was used for the IR inhibition assays. For the thermal hysteresis (TH) and ice morphology assay, a 1:5 dilution was deemed appropriate. Homogenates used for ice nucleation assays were not centrifuged prior to storage.

Larvae harvested for hemolymph sampling were collected in October 2014, ~150 km SE of Ottawa, ON (Sydenham, ON). Hemolymph samples were collected by cutting the cuticle with dissection scissors and centrifuging the larvae at 380 x g for 2 min, allowing the hemolymph to drain into the bottom of a microcentrifuge tube. Hemolymph from 3 larvae were pooled, flash

frozen and stored at -20 °C. Hemolymph extracts were used directly for TH measurements, while a 1:15 dilution was used for IR inhibition assays.

Goldenrod (likely *S. canadensis*, but to be confirmed) vegetation was also harvested in October 2014 and 2015 at the same site used for the hemolymph samples. Leaves were collected, flash frozen, and stored at -20 °C. Plant samples were prepared for IBP assays by crushing leaves into a fine powder using liquid nitrogen with a mortar and pestle. The powder was suspended in native protein extraction buffer (10 mM Tris-HCl, pH = 7.5, 25 mM NaCl, Roche cOmplete protease inhibitor tablets, 1.5% w/v PVP, and 1.5% w/v PVPP) and left oscillating overnight at 4 °C. Subsequently, the supernatant was strained through cheese cloth and centrifuged at 16,000 x g for 30 min at 4 °C. Supernatants were again collected and subject to three rounds of ice-affinity purification (IAP) as outlined below.

### **5.3.2 *Ice affinity purification***

A chilled (~ -1 °C) brass ice finger was submerged into a 150 mL beaker containing diluted plant supernatant. The ice finger was then cooled at a rate of 0.04 °C/h until approximately 50% of the liquid was incorporated into the growing ice hemisphere. The ice fraction was melted at 4 °C, with 1.5 M Tris-HCl (pH = 7.5) added slowly to a final concentration of 50 mM, replacing the excluded solutes. IAP was sequentially performed for three rounds of purification. Following the final round of IAP, the melted ice fraction was concentrated 100-fold using Millipore centrifugal devices (3 KDa MWCO). Protein concentration was determined spectrophotometrically (Thermo 2000 nanodrop). Concentrated samples were assessed for IR inhibition, as well as TH/ice shaping activity. IAP was also carried out on *Eurosta* larval homogenates (data not shown).

### **5.3.3 *Splat assays***

IR inhibition was assayed using a modified version of the splat assay (Knight *et al.*, 1988). Briefly, samples (10 µL) were released ~1.5 m above a round glass cover slip (Fisher

Scientific, Canada) placed on a cold (-80 °C) metal platform. Cover slips were subsequently submerged in chilled hexane (-4 °C) and photographed. The samples were then allowed to anneal for 18 h, after which a second image was captured.

#### **5.3.4 Thermal hysteresis and ice morphology**

A Clifton nanolitre osmometer was used to measure TH activity as well as visualize ice crystal morphology (Chakrabarty and Hew, 1991). Samples were loaded into oil-covered wells and snap frozen at -40 °C. Following freezing, the temperature was slowly increased until a single ice crystal remained, with the highest temperature at which the crystal remained stable for 1 min recorded as the sample's melting point. An image of the ice crystal's morphology was also captured. The ice crystal was subsequently cooled (40 mOsm/min) until rapid growth was observed, or the "burst point". The difference between the temperature at which the crystal grew and the melting temperature was recorded as the sample's TH value.

#### **5.3.5 Ice nucleation assays**

INA was assayed using a modified version of a previously described protocol (Vali, 1971; Maki *et al.*, 1974; Kozloff *et al.*, 1983). Freezing points were determined by pipetting 2 µL drops (10 drops/sample) onto polarized film and placement in an insulated chamber containing 50% ethylene glycol. The temperature of the chamber was lowered from -1 to -12 °C at 0.2 °C/min while automatically recording the temperature and an image of the polarized film every 60 s. The temperature at which 90% of the samples froze ( $T_{90}$ ) was considered the freezing point. No significant INA was concluded for samples with  $T_{90} > -9$  °C. The cumulative number of ice nuclei per mL for each sample ( $K(T)$ ) was calculated using Vali's (1971) equation:

$$K(T) = -\ln(N(T)/N_0) * V^{-1}$$

In the above equation,  $N(T)$  represents the number of unfrozen drops at temperature  $T$ ,  $N_0$  signifies the total drop number, and  $V$  is the drop volume.

### **5.3.6 *Liquid chromatography with tandem mass spectrometry (LC MS/MS)***

Concentrated samples from IAP of October-collected goldenrod leaves were analyzed by mass spectrometry (Sick Kids Proteomics, Analytics, Robotics & Chemical Biology Centre; SPARC Biocentre; Toronto, ON). Briefly, samples were trypsin digested in solution and analyzed using Q-Exactive instrumentation. Sequenced peptide fragments were screened through NCIB green plants database for protein identification. Similarly, *Eurosta* samples were analyzed using LC MS/MS and screened through publically-available databases.

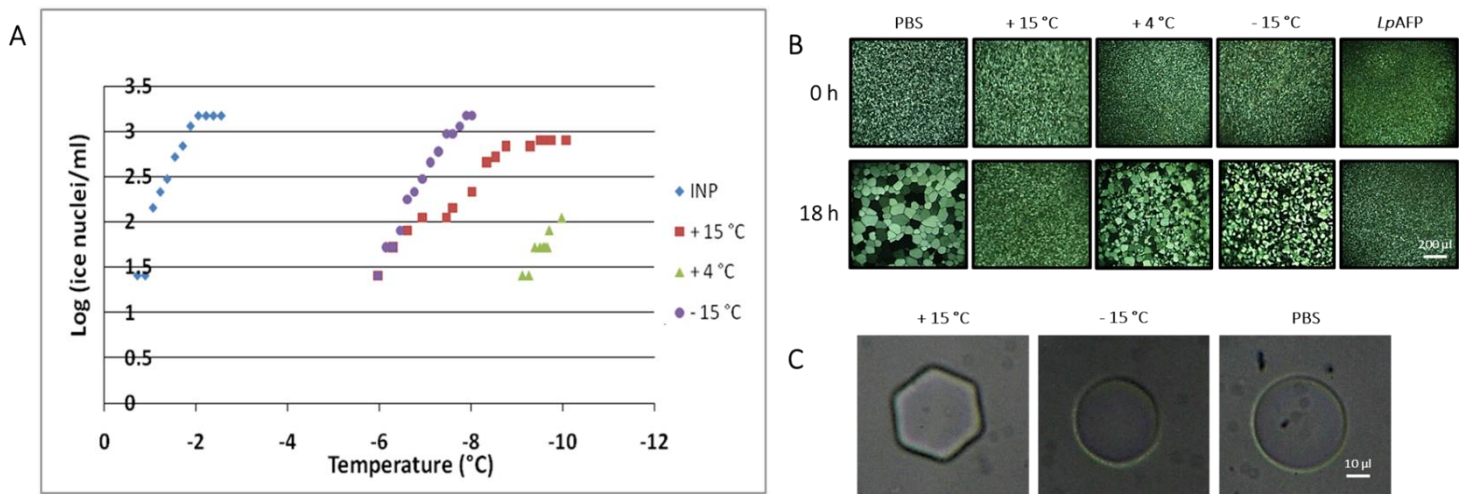
## **5.4 Results**

### **5.4.1 *Ice-binding activity of larvae representing the autumn to winter transition***

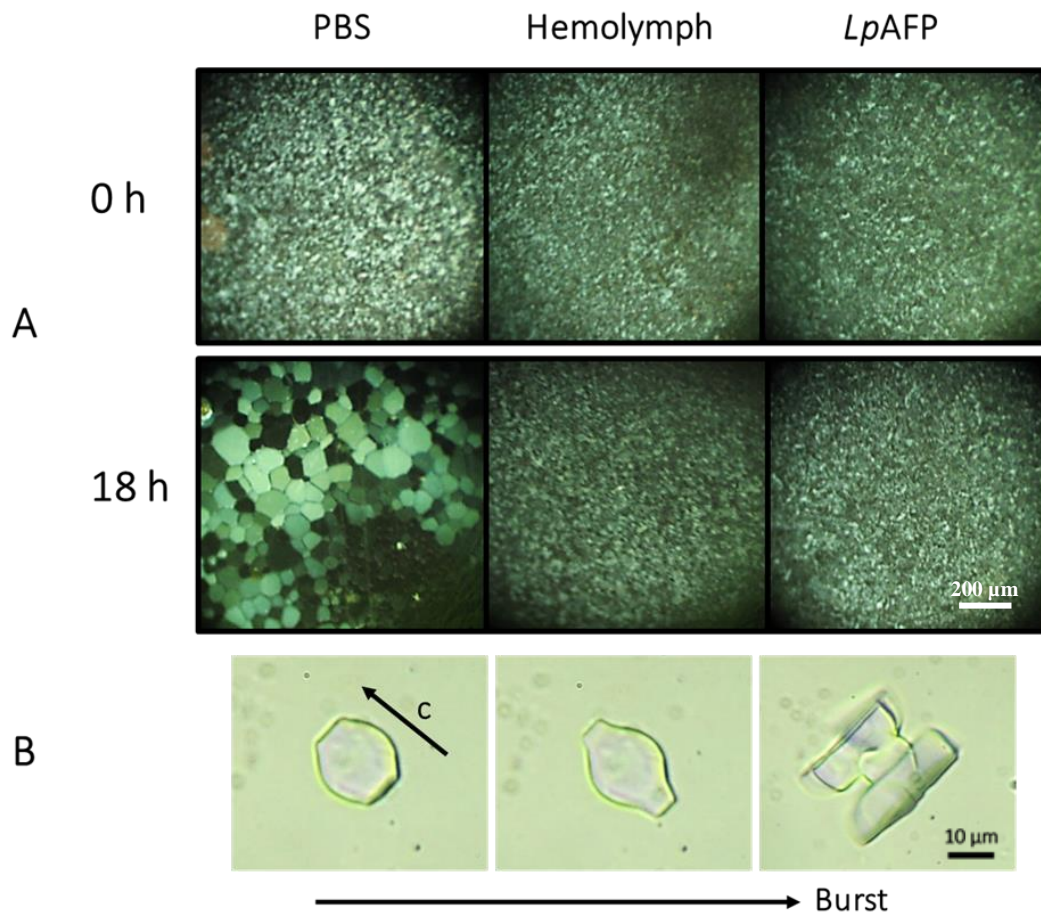
After acclimating larvae to three different conditions representing the broad temperature transitions of early autumn to winter, larval homogenates were assessed for INA, IR inhibition, as well as TH activity and ice crystal morphology. Type III INA was detected in larvae kept at -15 °C, in agreement with previous reports of calcium phosphate crystal nucleators (Mugano *et al.*, 1996). There was no evidence of INA in any of the other larval samples (Fig. 5.1A). Larvae kept at 15 °C displayed IR inhibition, maintaining a small ice crystal size after an 18 h incubation period at -4 °C. In comparison, -15 °C nor 4 °C larvae were observed to have the ability to inhibit ice recrystallization (Fig. 5.1B). Some ice shaping was also observed in autumn larvae, as seen with the formation of a hexagonal ice crystal compared to the disk shape seen with negative controls (Figure 5.1C). Low TH was also detected in the 15 °C larvae, with a mean value of 0.03 °C. There was no TH activity in any of the other larval samples.

### **5.4.2 *Ice-binding activity in larval hemolymph***

Hemolymph was collected from October-sampled larvae and assayed for IBP-associated activity. IR inhibition activity was observed (Fig. 5.2A), along with ice shaping (Fig. 5.2B). A mean TH reading of  $0.15 \pm 0.01$  °C was assessed. No INA was detected in any of the hemolymph samples (data not shown).



**Figure 5.1** Representative graphs of ice nucleation and ice-binding assays in acclimated larvae. Ice nucleation (A) is shown as the logarithm of the cumulative number of ice nuclei per mL. Samples include INP preparations from *P. syringae* (blue diamonds), +15 °C larvae (red squares), +4 °C larvae (green triangles), and -15 °C larvae (purple circles). Representative ice crystals from ice recrystallization (IR) inhibition assay (B) before and after an 18 h annealing period, as well as typical morphology of single ice crystals (C). Data shown are from 1:5 dilutions for INA and ice morphology, 1:15 dilution for IR inhibition. All assays were done in triplicate; phosphate buffered saline (PBS) and purified IBP from *L. perenne* (*LpAFP*) represent negative and positive control samples, respectively.



**Figure 5.2** Representative results from an ice recrystallization (IR) inhibition and ice morphology assays in mid-autumn hemolymph samples from October-collected larvae. IR inhibition was assessed using a 1:15 dilution (A), while ice morphologies were captured using samples which had not been diluted (B). The c-axis is indicated on one of the ice crystals; negative (PBS) and positive (*LpAFP*) controls are as described in Fig. 5.1. All assays were performed in triplicate; magnifications are shown by the scale bars.

#### **5.4.3 Ice affinity purification of goldenrod leaves**

*Solidago* leaf extracts were observed to show ice-binding activity, including an apparent increase in IR inhibition when extracts were assessed in collections made from mid-summer (July) to mid-fall (October), as well as mild ice shaping (Fig. 5.3). In an attempt to purify the biomolecule responsible for this activity, IAP was performed, since this method has been successfully used previously (Basu *et al.*, 2015). Solutes are generally excluded from becoming incorporated into the growing ice hemisphere, and thus the resulting ice fraction should be enriched for cellular components capable of binding ice. Following three rounds of IAP, IR inhibition and ice shaping was observed (Fig. 5.4); however, no TH was detected. *Eurosta* extracts purified by IAP also yielded IR inhibition, ice shaping, and TH activity (data not shown).

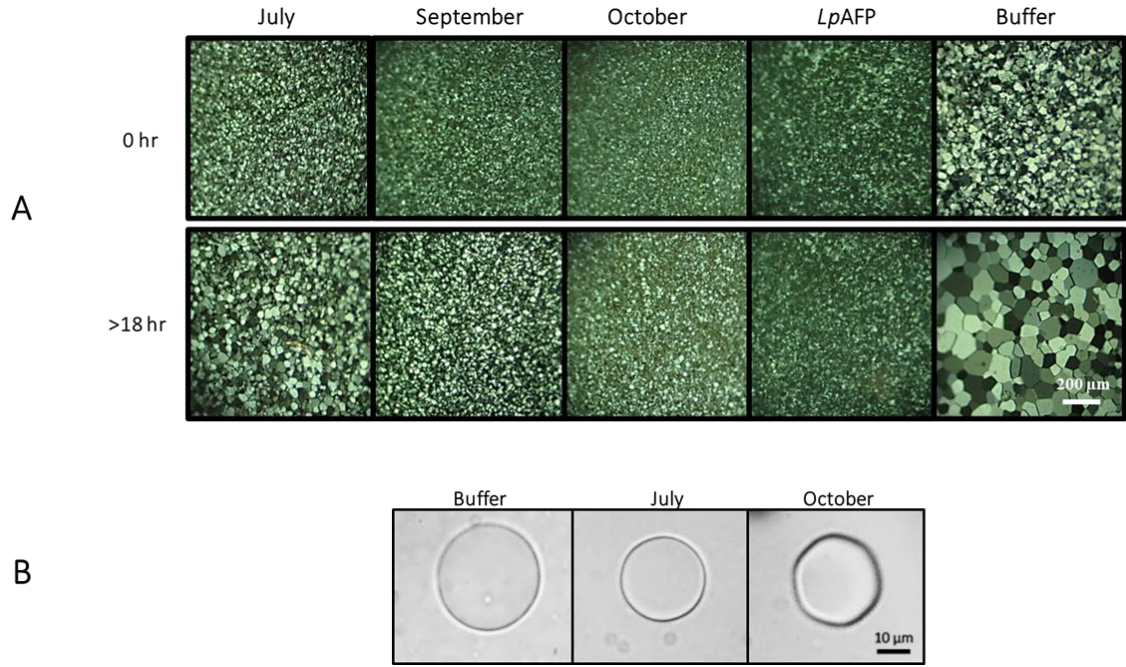
#### **5.4.4 LC MS/MS**

Database searches using sequenced *Solidago* peptide fragments provided several candidate proteins with possible ice-associating activities. A total of 505 proteins were identified; 428 with a confidence level > 95%. Within these proteins, three pathogenesis-related (PR) proteins were present that are known to have ice-binding activity in several different cold-tolerant plant species (Table 5.1; Griffith *et al.*, 2004; Zhang *et al.*, 2011; Gupta and Deswal, 2012). Peptide sequences were also obtained after LC MS/MS analysis of IAP-purified *Eurosta* extracts. These have not yet been properly mined (data not shown).

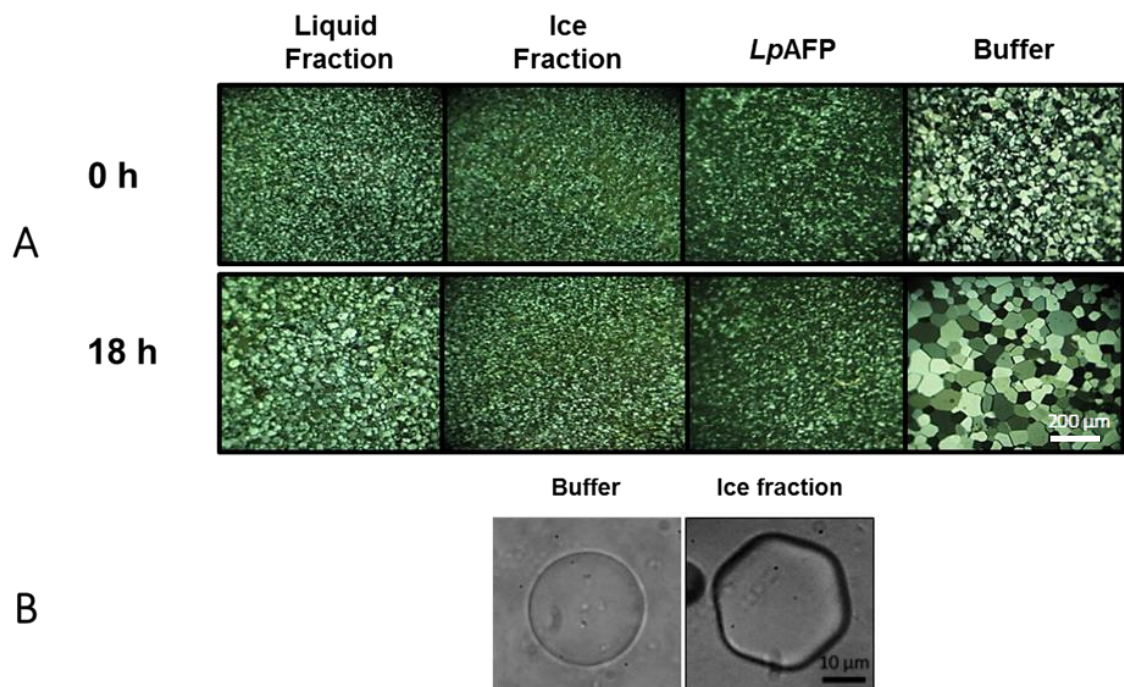
### **5.5 Discussion**

#### **5.5.1 Ice-binding activity in *E. solidaginis* larvae**

*E. solidaginis* larvae can tolerate freezing over the winter while housed within galls on the stems of goldenrod. Yet, it was unknown whether they produce IBPs as part of their survival strategy. Since IBPs have been reported in other freeze-tolerating species, mainly perennial plants, we initially explored this possibility by assessing larvae acclimated to three temperatures representing the autumn to winter transition.



**Figure 5.3** Representative results from an ice recrystallization inhibition assay (A) and ice morphology (B) in goldenrod leaf extracts collected July, September and October 2013. Negative (PBS) and positive (*LpAFP*) controls are described in Fig. 5.1. All assays were performed 3-5 times; magnification is indicated by the scale bars.



**Figure 5.4** Representative results from an ice recrystallization inhibition (A) and ice morphology (B) assays of ice-affinity purified *Solidago* leaf extracts. Positive (*LpAFP*) and negative (PBS) controls are described in Fig. 5.1. All assays have been done in triplicate; magnification is shown by the scale bars.

**Table 5.1** Putative PR proteins identified from LC MS/MS analysis in ice-affinity purified *Solidago* extracts

Putative protein domain	Plant identity	Biological function
Thaumatococcal-like proteins	<i>Ziziphus jujuba</i>	antifungal defense
	<i>Mikania micrantha</i>	
	<i>Cynara cardunculus</i> <sup>1</sup>	
	<i>Cajanus cajan</i> <sup>2</sup>	
Chitinases	<i>Ricinus communis</i>	antifungal defense
	<i>Cynara cardunculus</i> <sup>1</sup>	
Endo-1,3-beta-glucosidases	<i>Nicotiana tabacum</i>	antifungal defense
	<i>Oryza brachyantha</i>	

<sup>1</sup>salt tolerant (Gonzalez et al., 2005)

<sup>2</sup>drought tolerant (Flower and Ludlow, 1986)

Ice-binding activity was detected in larvae acclimated to 15 °C, a temperature that represented the mid-autumn season (Fig. 5.1), with no activity detected in larvae kept at 4 °C or -15 °C. While this may initially seem counter intuitive, there would be no need for IBPs later in the season given the high levels of cryoprotectants that accumulate over the winter. In addition, *E. solidaginis* larvae also produce uncommon acetylated triacylglycerols during the winter, which have been suggested to show “antifreeze-like” functions by lowering the melting points of aqueous solutions, and so assist with the maintenance of intracellular liquid states (Marshall *et al.*, 2014). To date, it has not been shown if these fats also show classic IBP characteristics, including IR inhibition, ice shaping, and significant TH activity.

The acceptance of the hypothesis that *Eurosta* larvae express an IBP during the autumn is further substantiated with experiments that showed October-sampled hemolymph displayed both TH activity and ice shaping (Figure 5.2). Only low levels of TH activity were detected. This is in contrast to the observation that most insect and arthropod IBPs characterized to date have hyperactive IBPs. The IBP from spruce budworm (Tyshenko *et al.*, 1997) and yellow mealworm (Graham *et al.*, 1997; Liou *et al.*, 2000) both display high levels of TH activity at low concentrations (e.g. ~ 2.5 °C at 1 mg/mL). Even the newly characterized midge IBP displays moderate activity (Basu *et al.*, 2014). However, in all these cases, IBPs are used as part of a freeze-avoidance mechanism, permitting the supercooling of bodily fluids below 0 °C. Low TH activity is generally seen in freeze-tolerant plants, where their primary function is to inhibit the formation of large crystals (Marentes *et al.*, 1993) and depress the nucleation activity of pathogenic epiphytes (Tomalty and Walker, 2014). It is conceivable that *E. solidaginis* larvae express an IBP during the early autumn when temperatures can periodically drop below the freezing point, functioning as a protective measure against IR damage and to inhibit ice nucleator activity in their midgut (Olsen and Duman, 1997) or their hemolymph (Mugnano *et al.*, 1996).

Our observations of IBP activity in October-collected *Eurosta* larvae are consistent with observations on the effect of desiccation on drought-induced rapid cold hardening (RCH) of *E. solidaginis* larvae (Gantz *et al.*, 2014). Short periods of desiccation led to improved cold tolerance in autumn-collected larvae compared to hydrated controls. Interestingly, hemolymph osmolality following desiccation was higher than expected based upon known solute concentrations. The TH readings from our hemolymph samples could explain this discrepancy.

Insect RCH is observed during brief periods of exposure to subzero temperatures. Initiating metabolic and physiological adjustments shortly following exposure, RCH mechanisms include polyol, sugar, and free amino acid accumulation, and modifications to cellular membranes (Michaud and Denlinger, 2007; Michaud and Denlinger, 2006). Additionally, microarray analysis in *Drosophila* has shown the upregulation of stress proteins (Hsp23, Hsp26, Hsp83, and Frost) as well as membrane-associated proteins, all of which may also contribute to this RCH response (Qin *et al.*, 2005). Other aspects of RCH appear to be influenced by temperature-dependent cellular calcium flux (Teets *et al.*, 2008). Indeed, some IBPs require Ca<sup>2+</sup>-binding for proper activity (Ewart *et al.*, 1996; Gilbert *et al.*, 2005), and calcium signaling also plays a role in the cold acclimation of plants (Knight, 1999). While speculative, it is possible fall larvae are expressing an IBP as part of their RCH response, dependent upon calcium levels for full activation.

### **5.5.2 Ice-binding activity in *S. canadensis***

Prior to overwintering, some insects evacuate gut contents to remove any potential ice nucleators (Sømme, 1982). It is possible that larvae collected mid-autumn would still have plant material present within their gut track. Thus, it was important to also assay the larvae's host plant, goldenrod, for ice-binding activity in case the phenotype was derived from the plant host. Indeed, IR inhibition and minimal ice shaping was observed in goldenrod leaf samples (Fig. 5.4). This is the first time ice-binding activity has been reported in this plant. LC MS/MS analysis of ice-

affinity purified leaf extracts resulted in the identification of several candidate proteins that could be responsible for this observed activity. In particular, three pathogenesis-related (PR) protein families were identified: thaumatin-like, chitinases,  $\beta$ -1,3-glucanases (Table 5.1).

These three families represent highly conserved protein classes PR proteins that have been shown to inhibit fungal growth *in vitro*, and are postulated to function as antifungal defense within plants (Stintzi *et al.*, 1993). Similarly, while PR proteins can be induced by a variety of environmental stresses, it has been observed that some PR proteins are upregulated during low temperature exposure including those found in potato (Zhu *et al.*, 1993), barley (Tronsmo *et al.*, 1993), bermudagrass (Gatschet *et al.*, 1995), and winter rye (Hon *et al.*, 1995). Similarly, we saw no evidence of IR inhibition until later in the season (Fig. 5.3). Ice-binding activity in members of these three different PR protein classes has also been previously documented in winter rye. Thaumatin-like, chitinases, and  $\beta$ -1,3-glucanases PR proteins accumulate in the apoplast during cold acclimation (Hon *et al.*, 1995), and also show TH activity and IR inhibition (Griffith *et al.*, 1992; Griffith *et al.*, 1997; Yeh *et al.*, 2000).

While our goldenrod extracts did not show any measureable TH, the TH activity for winter rye IBP-PR proteins is very low and only measured 0.33 °C at 60 mg/mL (Griffith *et al.*, 1992), compared to fish (~ 0.6 °C at 1 mg/mL for Type III fish IBP; Chapter 1), insects (~ 2.5 °C at 1 mg/mL for *Tenebrio molitor*; Liou *et al.*, 2000), and even overwintering plant IBPs (~0.2 °C at 1 mg/mL for *LpAFP*; Chapter 1). It is possible that TH activity is not functionally important for IBP-PR proteins. To the best of our knowledge, no modelling studies have been done on IBP-PR plant proteins to demonstrate the arrangement of water molecules along the ice-binding face (Smolin and Daggett, 2008; Nutt and Smith, 2008), which could provide further explanation for the low TH activity.

Well characterized examples of ice-binding activity in PR proteins occur within freeze-tolerant grasses; however, similar activity has also been observed in a few dicotyledonous

species. An IBP-chitinase was isolated from apoplastic extraction of wintersweet flowers (Zhang *et al.*, 2011), while in cold-acclimated seabuckthorn seedlings, two purified class I chitinases showed ice-binding activity (Gupta and Deswal, 2014). Within goldenrod, it is possible these PR proteins have similar dual functionality, providing protection from IR and disease resistance. As a perennial, the roots of goldenrod overwinter in the ground underneath the snow cover. While providing insulation from harsher winter temperatures, the snow cover does create an environment where temperatures can fluctuate close to 0 °C, providing ample opportunity for damaging IR (Knight and Duman, 1986) and allowing psychrophilic plant pathogens, such as snow molds, to thrive (Snider *et al.*, 2000). It is plausible goldenrod synthesizes IBP-PR proteins to inhibit both IR while the vegetative portion of the plant is still green, as well as protect against pathogenic fungi present in the soil during the winter months.

## 5.6 References

- Atici, O., & Nalbantoglu, B. (2003). Antifreeze proteins in higher plants. *Phytochemistry*, *64*: 1187-1196.
- Basu, K., Graham, L. A., Campbell, R. L., & Davies, P. L. (2015). Flies expand the repertoire of protein structures that bind ice. *PNAS*, *112*(3), 737-742.
- Baust, J. G., & Lee, R. E. (1982). Environmental triggers to cryoprotectant modulation in separate populations of the gall fly, *Eurosta solidaginis* (Fitch). *Journal of Insect Physiology*, *28*: 431-436.
- Bennett, V. A., Pruitt, N. L., & Lee Jr, R. E. (1997). Seasonal changes in fatty acid composition associated with cold-hardening in third instar larvae of *Eurosta solidaginis*. *Journal of Comparative Physiology B*, *167*: 249-255.
- Benlloch-González, M., Fournier, J. M., Ramos, J., & Benlloch, M. (2005). Strategies underlying salt tolerance in halophytes are present in *Cynara cardunculus*. *Plant Science*, *168*: 653-659.
- Chakrabarty, A., & Hew, C. L. (1991). The effect of enhanced  $\alpha$  - helicity on the activity of a winter flounder antifreeze polypeptide. *European Journal of Biochemistry*, *202*: 1057-1063.
- Davies, P. L. (2014). Ice-binding proteins: a remarkable diversity of structures for stopping and starting ice growth. *Trends in Biochemical Sciences*, *39*: 548-555.
- Duman, J. G. (2001). Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annual Review of Physiology*, *63*: 327-357.
- Ewart, K. V., Yang, D. S., Ananthanarayanan, V. S., Fletcher, G. L., & Hew, C. L. (1996). Ca<sup>2+</sup>-dependent antifreeze proteins modulation of conformation and activity by divalent metal ions. *Journal of Biological Chemistry*, *271*: 16627-16632.
- Flower, D. J., & Ludlow, M. M. (1986). Contribution of osmotic adjustment to the dehydration tolerance of water - stressed pigeon pea (*Cajanus cajan* (L.) millsp.) leaves. *Plant, Cell & Environment*, *9*: 33-40.
- Gantz, J. D., & Lee, R. E. (2015). The limits of drought-induced rapid cold-hardening: extremely brief, mild desiccation triggers enhanced freeze-tolerance in *Eurosta solidaginis* larvae. *Journal of Insect Physiology*, *73*: 30-36.
- Gatschet, M. J., Taliaferro, C. M., Porter, D. R., Anderson, M. P., *et al.* (1996). A cold-regulated protein from bermudagrass crowns is a chitinase. *Crop Science*, *36*: 712-718.
- Gilbert, J. A., Davies, P. L., & Laybourn-Parry, J. (2005). A hyperactive, Ca<sup>2+</sup>-dependent antifreeze protein in an Antarctic bacterium. *FEMS Microbiology Letters*, *245*: 67-72.
- Graham, L. A., Liou, Y. C., Walker, V. K., & Davies, P. L. (1997). Hyperactive antifreeze protein from beetles. *Nature*, *388*: 727-728.
- Griffith, M., Ala, P., Yang, D. S., Hon, W. C., & Moffatt, B. A. (1992). Antifreeze protein produced endogenously in winter rye leaves. *Plant Physiology*, *100*: 593-596.

- Griffith, M., Antikainen, M., Hon, W. C., Pihakaski - Maunsbach, K., Yu, X. M., Chun, J. U., & Yang, D. S. (1997). Antifreeze proteins in winter rye. *Physiologia Plantarum*, *100*: 327-332.
- Griffith, M., & Yaish, M. W. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. *Trends in plant science*, *9*: 399-405.
- Gupta, R., & Deswal, R. (2012). Low temperature stress modulated secretome analysis and purification of antifreeze protein from *Hippophae rhamnoides*, a Himalayan wonder plant. *Journal of Proteome Research*, *11*: 2684-2696.
- Hamilton, M. D., Rojas, R. R., & Baust, J. G. (1986). Juvenile hormone: modulation of cryoprotectant synthesis in *Eurosta solidaginis* by a component of the endocrine system. *Journal of Insect Physiology*, *32*: 971-979.
- Hon, W. C., Griffith, M., Mlynarz, A., Kwok, Y. C., & Yang, D. S. (1995). Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. *Plant Physiology*, *109*: 879-889.
- Knight, H. (1999). Calcium signaling during abiotic stress in plants. *International review of cytology*, *195*: 269-324.
- Knight, C. A., & Duman, J. G. (1986). Inhibition of recrystallization of ice by insect thermal hysteresis proteins - a possible cryoprotective role. *Cryobiology*, *23*: 256-262.
- Kozloff, L. M., Schofield, M. A., & Lute, M. (1983). Ice nucleating activity of *Pseudomonas syringae* and *Erwinia herbicola*. *Journal of Bacteriology*, *153*: 222-231.
- Liou, Y. C., Thibault, P., Walker, V. K., Davies, P. L., & Graham, L. A. (1999). A complex family of highly heterogeneous and internally repetitive hyperactive antifreeze proteins from the beetle *Tenebrio molitor*. *Biochemistry*, *38*: 11415-11424.
- Liou, Y. C., Tocilj, A., Davies, P. L., & Jia, Z. (2000). Mimicry of ice structure by surface hydroxyls and water of a  $\beta$ -helix antifreeze protein. *Nature*, *406*: 322-324.
- Maki, L. R., Galyan, E. L., Chang-Chien, M. M., & Caldwell, D. R. (1974). Ice nucleation induced by *Pseudomonas syringae*. *Applied Microbiology*, *28*: 456-459.
- Marentes, E., Griffith, M., Mlynarz, A., & Brush, R. A. (1993). Proteins accumulate in the apoplast of winter rye leaves during cold acclimation. *Physiologia Plantarum*, *87*: 499-507.
- Marshall, K. E., Thomas, R. H., Roxin, Á., Chen, E. K., *et al.* (2014). Seasonal accumulation of acetylated triacylglycerols by a freeze-tolerant insect. *Journal of Experimental Biology*, *217*: 1580-1587.
- Michaud, M. R., & Denlinger, D. L. (2006). Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology*, *52*: 1073-1082.
- Michaud, M. R., & Denlinger, D. L. (2007). Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies

- (*Sarcophaga crassipalpis*): a metabolomic comparison. *Journal of Comparative Physiology B*, 177: 753-763.
- Mugnano, J., Lee, R., & Taylor, R. (1996). Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *The Journal of Experimental Biology*, 199: 465-471.
- Nutt, D. R., & Smith, J. C. (2008). Dual function of the hydration layer around an antifreeze protein revealed by atomistic molecular dynamics simulations. *Journal of the American Chemical Society*, 130: 13066-13073.
- Olsen, T. M., & Duman, J. G. (1997). Maintenance of the supercooled state in the gut fluid of overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of ice nucleators and antifreeze proteins. *Journal of Comparative Physiology B*, 167: 114-122.
- Philip, B. N., & Lee, R. E. (2010). Changes in abundance of aquaporin-like proteins occurs concomitantly with seasonal acquisition of freeze tolerance in the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology*, 56: 679-685.
- Qin, W., Neal, S. J., Robertson, R. M., Westwood, J. T., & Walker, V. K. (2005). Cold hardening and transcriptional change in *Drosophila melanogaster*. *Insect Molecular Biology*, 14: 607-613.
- Rojas, R. R., Lee, R. E., & Baust, J. G. (1986). Relationship of environmental water content to glycerol accumulation in the freezing tolerant larvae of *Eurosta solidaginis*. *Cryobiology*, 23: 564.
- Smolin, N., & Daggett, V. (2008). Formation of ice-like water structure on the surface of an antifreeze protein. *The Journal of Physical Chemistry B*, 112: 6193-6202.
- Snider, C. S., Hsiang, T., Zhao, G., & Griffith, M. (2000). Role of ice nucleation and antifreeze activities in pathogenesis and growth of snow molds. *Phytopathology*, 90: 354-361.
- Sømme, L. (1982). Supercooling and winter survival in terrestrial arthropods. *Comparative Biochemistry and Physiology Part A: Physiology*, 73: 519-543.
- Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., et al. (1993). Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie*, 75(8), 687-706.
- Storey, J. M., & Storey, K. B. (1983). Regulation of cryoprotectant metabolism in the overwintering gall fly larva, *Eurosta solidaginis*: temperature control of glycerol and sorbitol levels. *Journal of Comparative Physiology*, 149: 495-502.
- Teets, N. M., Elnitsky, M. A., Benoit, J. B., Lopez-Martinez, G., et al. (2008). Rapid cold-hardening in larvae of the Antarctic midge *Belgica antarctica*: cellular cold-sensing and a role for calcium. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 294: R1938-R1946.
- Tomalty, H. E., & Walker, V. K. (2014). Perturbation of bacterial ice nucleation activity by a grass antifreeze protein. *Biochemical and Biophysical Research Communications*, 452: 636-641.

- Tronsmo, A. M., Gregersen, P., Hjeljord, L., Sandal, T., Bryngelsson, T., & Collinge, D. B. (1993). Cold-induced disease resistance. In: Fritig, B., & Legrand, M. (eds) *Mechanisms of plant defense responses* (pp. 369-369). Springer, Netherlands, pp 369.
- Tyshenko, M. G., Doucet, D., Davies, P. L., & Walker, V. K. (1997). The antifreeze potential of the spruce budworm thermal hysteresis protein. *Nature biotechnology*, 15: 887-890.
- Vali, G. (1971). Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. *Journal of the Atmospheric Sciences*, 28: 402-409.
- Yeh, S., Moffatt, B. A., Griffith, M., Xiong, F., *et al.* (2000). Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. *Plant Physiology*, 124: 1251-1264.
- Zhang, S. H., Wei, Y., Liu, J. L., Yu, H. M., Yin, J. H., Pan, H. Y., & Baldwin, T. C. (2011). An apoplastic chitinase CpCHT1 isolated from the corolla of wintersweet exhibits both antifreeze and antifungal activities. *Biologia plantarum*, 55:141-148.
- Zhu, B., Chen, T. H., & Li, P. H. (1993). Expression of an ABA-responsive osmotin-like gene during the induction of freezing tolerance in *Solanum commersonii*. *Plant Molecular Biology*, 21: 729-735.

## Chapter 6

### General Discussion

#### 6.1 General Discussion

Best known for their interactions with ice surfaces, IBPs are a distinctive group of proteins. Originally described in polar fish (Raymond and DeVries, 1977), multiple IBPs have been discovered spanning a diverse collection of species including cold-tolerant plants (Griffiths *et al.*, 1992; Smallwood *et al.*, 1999; Sidebottom *et al.*, 2000), insects (Graham *et al.*, 1997; Tyshenko *et al.*, 1997), bacteria (Gilbert *et al.*, 2005), and fungi (Hoshino *et al.*, 2003). Despite this diversity, two omnipresent characteristics have historically been used for IBP classification. This includes the depression of the freezing point of solutions in a noncolligative manner, as well as the ability to impede the growth of large ice crystals, known as TH and IR inhibition, respectively. However, it appears that we have begun to understand some of their additional roles, or new situations where traditional roles might be useful, for these proteins.

Initial suggestions that IBPs could have hitherto uncharacterized functions can be traced by to the work done by Marilyn Griffith, who published multiple papers characterizing the dual functionality of IBPs in winter rye, displaying both ice-binding and pathogenesis-related activities (reviewed in Griffith and Yaish, 2004). More recently, IBPs have been shown as surface adhesion proteins in the marine bacteria, *Marinomonas primoryensis*, permitting the bacteria to attach onto overhead ice in oxygen- and nutrient-rich surface water (Guo *et al.*, 2012). Similarly, IBPs secreted by certain psychrophilic microorganisms are postulated to structure the sea ice around them, creating brine pockets critical for survival within this extreme environment (Raymond *et al.*, 2007; Bayer-Giraldi *et al.*, 2011; Krembs *et al.*, 2011; Raymond, 2011). This thesis has explored additional functionalities and uses of IBPs, including the protection against the ice nucleation activity of pathogenic epiphytes, the improvement of hypothermic storage of organs

for transplant purposes, as well as possible RCH mechanism in a freeze-tolerant insect (summarized in Table 6.1).

### **6.1.1 Plant IBPs can function as a defense mechanism against bacterial ice nucleation**

In this thesis, Chapters 2 and 3 concern the characterization of the interaction between two different plant IBPs from *L. perenne* and *B. distachyon*, and an INP from a pathogenic epiphyte, *P. syringae*. INP preparations were mixed with recombinant IBPs encoded by each of these plants and ice-binding activities assessed as described. The INP did not decrease the activity of the plant IBPs, including TH and IR inhibition. In both cases, the plant IBPs were able to depress the freezing points of the bacterial INP. Furthermore, the incubation of *P. syringae* and *P. borealis* cultures, both known INA<sup>+</sup> bacteria, with GFP-tagged *Lp*IBP showed the plant IBP binding to the pole of some bacterial cells where INPs are believed to localize (Vanderveer *et al.*, 2014). Given that the new conferred freezing point depression of the INPs seen with both plant IBPs is greater than their known TH, along with observation of the GFP-tagging, we have suggested that the depression of bacterial ice nucleation is due to direct protein-protein interactions.

The concept of an IBP being able to depress the activity of heterogenous ice nucleators has been previously explored. Within the freeze-avoiding fire-coloured beetle, *Dendroides canadensis*, hemolymph and gut-extracted IBPs were able to reduce ice nucleation activity, including the activity of the INA<sup>+</sup> *P. fluorescens*. This depression was suggested to be due to the insect IBPs masking active ice nucleation sites, and not a direct binding of the IBPs to INPs (Olsen and Duman, 1997a; Olsen and Duman, 1997b). However, within this thesis, this is the first time this interaction has been characterized as contributing to a freeze-tolerant scenario. Interestingly, attempts to use other IBPs to depress bacterial INA were unsuccessful. This includes the Type III fish AFP, which seemed to enhance nucleation at certain concentrations

**Table 6.1** Summary of biological and industrial applications of IBPs investigated

<b>Biological function/Industrial application</b>	<b>Type of IBP</b>	<b>Species</b>	
Defense against pathogenic INA <sup>+</sup> bacteria and IR inhibition	Plant	<i>Lolium perenne</i> <sup>1</sup>	
		<i>Brachypodium distachyon</i> <sup>2</sup>	
Putative antifungal and IR inhibition	Plant	<i>Solidago canadensis</i> <sup>3</sup>	
		<i>Secale cereal</i> <sup>4</sup>	
		<i>Chinmonanthus praecox</i> <sup>5</sup>	
		<i>Hippophae rhamnoides</i> <sup>6</sup>	
Rapid cold hardening	Insect	<i>Eurosta solidaginis</i> <sup>3</sup>	
Non-frozen hypothermic storage of organs	Insect	<i>Tenebrio molitor</i> <sup>7</sup>	
		Fish (Type I)	<i>Pleuronectes americanus</i> <sup>8,9</sup>
		Fish (Type III)	<i>Macrozoarces americanus</i> <sup>8</sup>
		Fish (AFGP)	<i>Dissostichus mawsoni</i> <sup>10</sup>

<sup>1</sup> Chapter 2; <sup>2</sup> Chapter 3; <sup>3</sup> Chapter 5 ; <sup>4</sup> reviewed in Griffith and Yaish, 2004; <sup>5</sup> Zhang *et al.*, 2011; <sup>6</sup> Gupta and Renu, 2014; <sup>7</sup> Chapter 4; <sup>8</sup> Amir *et al.*, 2004; <sup>9</sup> Soltys *et al.*, 2001; <sup>10</sup> Wang *et al.*, 1994

(Tomalty and Walker, 2014) and an insect IBP from *T. molitor*, which only showed a moderate depression at high concentrations (Appendix I & II).

Within temperate North America, *P. syringae* is considered the most abundant INA<sup>+</sup> bacterium (Lindow *et al.*, 1978), having been isolated from the leaves of a variety of plants including corn, snap peas, oats, and almonds (Arny *et al.*, 1976; Lindow *et al.*, 1978; Lindow *et al.*, 1982). Using their INP to wound plants at high subzero temperatures, these bacteria are commonly regarded as a plant pathogen. Given the evolutionary history of known plant IBPs, it is not surprising that they could also be active against pathogenic INA<sup>+</sup> microorganisms. As discussed with goldenrod's putative IBPs in Chapter 3, certain plant IBPs have evolved from antifungal, PR proteins (reviewed in Griffith and Yaish, 2004). It is not known if they would display similar action against bacterial INPs as we observed with *LpIBP* and *BdIRI*; however, since some fungi are known to be INA<sup>+</sup> (Pouleur *et al.*, 1992), it is plausible that similar “anti-ice nucleation” activity could be present within antifungal plant IBPs.

The two plant IBPs tested in Chapter 2 and 3 have likely both evolved from comparable precursor LRR protein-kinases (Sandve *et al.*, 2008; Li *et al.*, 2012), ancestrally involved in protein-protein binding and recognition (Kobe and Kajava, 2001; Bella *et al.*, 2008.). Protein modelling and X-ray crystallography analysis of the ice-active portions of these proteins show domains that fold into  $\beta$ -rolls, with two relatively flat, hydrophobic faces on either side with one side involved in ice-binding activity (Middleton *et al.*, 2009; Middleton *et al.*, 2012; Chapter 1, Fig. 1.2) or responsible for the majority of activity (M. Bredow, 2016, unpublished). Interestingly, as indicated in Chapter 2, the *LpIBP*-INP interaction seems to involve the non-ice-binding face of the protein, as determined through mutagenesis assessments. It is currently unknown if *BdIRI* interacts with bacterial INPs using the less important face or even the relatively flat His-rich sides of the protein, although studies further characterizing this interaction are ongoing.

### **6.1.2 Hyperactive insect IBPs can improve the hypothermic storage of organs**

IBPs have been investigated for their use in the cryopreservation of cell and tissues, often with contradictory results. While some studies saw improvement (Arav *et al.*, 1993; Rubinsky *et al.*, 1994; Younis *et al.*, 1998), others saw significant loss in cell or tissue viability (Mugnano *et al.*, 1994; Langeaux *et al.*, 1997). One area of concern appears to be the IBP-mediated shape of ice crystals during the freezing process. By binding onto different planes of ice, IBPs facilitate the direction of ice growth once the TH limit has been reached. Without the presence of IBPs, ice crystals typically grow in a flat, circular shape along the a-axis (Figure 1.1). In the presence of fish IBPs, growth occurs along the c-axis, resulting in needle-like projections that could easily pierce and damage cells (Davies *et al.*, 2002). As investigated in Chapter 4, a promising alternative for IBP use would be in the hypothermic storage of cells and tissues, permitting storage below 0 °C but in a non-frozen state.

Chapter 4 examined the preservation of rat kidneys at temperatures below 0 °C in a non-frozen state, using a recombinant IBP from the freeze-avoiding yellowmeal worm, *T. molitor*. The addition of *TmIBP* to a proprietary preservation solution (CrS) allowed the kidneys to be stored at -4.4 °C for 72 h without freeze damage to any organs. After 72 h, CrS + *TmAFP* samples showed comparable tissue viability and similar levels of ischemic markers compared to kidneys preserved with the current industry standard, UW, for only 24 h. Indeed, the use of an insect IBP was able to extend the storage time 3-fold without the accumulation of cold-induced injury generally associated with prolonged preservation times.

The concept of using IBPs to extend organ preservation times has been investigated before. Both the Type I and Type III fish AFPs have been studied for use in heart transplants. Rat hearts stored at -1.3 °C in UW amended with Type III AFP showed improved viability, better functional output scores, and lower levels of apoptosis compared to controls (Amir *et al.*, 2004a, 2004b; Amir *et al.*, 2005). While heterotrophic transplants of UW + Type III hearts into recipient rats were done to determine the organ viability after transplantation, long-term survival of

recipient rats was not assessed. Similarly, Type I fish AFP was assessed for its suitability in the hypothermic storage of rat livers. Livers perfused with UW+ 2,3-butanediol + Type I AFP survived storage at -4 °C without freezing, however, they were not successfully able to extend storage past 24 h compared to +4 °C UW controls (Soltys *et al.*, 2001).

Within these studies, the common theme has been to use fish AFPs which offer only moderate levels of TH activity (Davies and Hew, 1990). The premise behind storing organs in a nonfrozen state below 0 °C is to reduce metabolic demands of the tissue, since every 10 °C decrease in temperature results in a 50% reduction in metabolic activity, thus possibly decreasing related ischemic injuries. The degree of ischemic injury has been shown to correlate with the success of organ function following transplantation (Salahudeen *et al.*, 2004). To further decrease the temperature, it would be sensible to use effective, nontoxic freezing point depressors. This premise was shown in rat liver preservation, where storage at -6 °C for 4 days using UW supplemented with PEG-35kD and 3-O-methyl-D-glucose resulted in a substantially increased survival rate of recipient rats (Berendsen *et al.*, 2014). While an achievement, this technique also required the use of machine perfusion (MP) during initial stages of storage and prior to transplantation. MP is advantageous for organ preservation by providing artificial circulation to counteract the effects of prolonged ischemia; however, this method also has major drawbacks, including equipment cost and tendencies to malfunction (reviewed in Guibert *et al.*, 2011; Timsit and Tullius, 2011). As such, cold static storage is still widely used.

Given our results showing an insect IBP extending the preservation time, future studies investigating the use of different IBP combinations would be beneficial. Combining IBP isoforms from the beetle, *D. canadensis*, resulted in a synergistic effect, with higher than expected TH values recorded (Wang and Duman, 2005). Similarly, glycerol and a thaumatin-like protein also were shown to function as enhancers, increasing the TH activity of certain *Dc*IBPs (Wang and Duman, 2006). Assessing organ preservation using hyperactive insects IBPs combined with TH-

activity enhancers could further decrease the storage temperatures, leading to improved organ quality and better long-term function following transplantation.

### **6.1.3 IBP function within a freeze-tolerant insect**

To date, IBP function within freeze-tolerant plants has been well characterized. This includes protection against the damaging effects of ice crystal growth at temperatures close to 0 °C (IR inhibition), counteracting bacterial ice nucleation (Tomalty and Walker, 2014), as well as functioning as antifungal agents during periods of low temperature exposure (reviewed in Griffith and Yiash, 2004). However, within freeze-tolerant animals, the benefits of IBP expression is not fully understood. In these instances, it has been suggested animal IBPs function as IR inhibitors, and may also associate with cell membranes to prevent the propagation of extracellular ice into the cell (reviewed in Duman, 2015).

In Chapter 5, IBP-activity was identified within the freeze-tolerant larvae of *E. solidaginis*. Showing the hallmark characteristics of IBP function, including TH and IR inhibition, these observations are of interest since expression appears only to be in mid-autumn larvae, as opposed to during the winter months. Similar results have been previously observed in the freeze-tolerant centipede, *Lithobius forficatus*, with TH activity only occasionally observed in autumn field-collected samples, while IR inhibition was present throughout the winter months (Tursman *et al.*, 1994). It is plausible the IBPs are used as part of the insects' RCH mechanisms as they prepare to overwinter, providing protection during autumn months when temperatures can sporadically drop below the freezing point. Still, the exact function of IBPs in this capacity is unclear.

TH values for the October-collected larval hemolymph were low (~ 0.1 °C) compared to TH activity generally seen in other insects. Low TH readings have been recorded previously in freeze-tolerant insect hemolymph (Duman *et al.*, 2004), although the biomolecules responsible for this activity have not been identified. However, given the survival strategy of these insects,

this low activity is not unexpected, but the benefits of producing an IBP are not entirely understood. Freeze-tolerant insects produce endogenous ice nucleators, such as calcium phosphate crystals, to reduce supercooling and allow the controlled formation of extracellular ice (Mugnano *et al.*, 1996). Producing an IBP with high TH activity may be counterproductive, especially since some IBPs have been shown to depress ice nucleation activity (Olsen and Duman, 1997a, 1997b; Tomalty and Walker, 2014). In this case, IBP expression would promote further supercooling, resulting in the rapid, uncontrollable ice crystal growth once the TH range has been exceeded. In freeze-tolerant insects, there has been two main purposes suggested for IBP expression. First is to function as an IR inhibitor, where tissue damage can result from uncontrolled extracellular ice growth (Mazur, 1984). Secondly, these IBPs are postulated to associate with cellular membranes during subzero temperatures to prevent intracellular ice inoculation, similar to the proposed membrane stabilization seen with fish IBPs (Duman, 2015; Tomczak *et al.*, 2002; Kun *et al.*, 2008).

To date, most TH-active biomolecules characterized have been IBPs (reviewed in Davies, 2014). However, TH activity has been described in a diverse range of species, with the majority of biomolecules responsible not being isolated or characterized (Duman *et al.*, 2004; Appendix III). Recently, several TH-active molecules have been identified within freeze-tolerant animals consisting of very little to no protein composition, resulting in new grouping of “IBP-like” molecules named antifreeze glycolipids (AFGLs). Thus far, AFGLs have been identified in several different species including a freeze tolerant beetle, *Upis ceramoides* (Walters *et al.*, 2009), two freeze-tolerant frogs, *Rana lessonae* and *Lithobates sylvatica* (Walters *et al.*, 2011; Larson *et al.*, 2014), and a freeze-tolerant plant, *Solunum dulcamara* (Walters *et al.*, 2011). AGLPs are suggested to associate with cellular membranes, possibly to prevent extracellular ice from infiltrating the cytosol (Duman, 2015). It is possible the activity seen within *E. solidaginis* larvae is not due to a classic “IBP”, but could be in the form of another TH-active biomolecule,

such as an AGLP. Further analysis of LC MS/MS data could assist with the identification of the biomolecule responsible for the activity observed in these larvae, as well as provide further insight into the function of this unknown molecule.

#### **6.1.4 How can IBPs accomplish so many different functions?**

As we start to understand the alternative functions of IBPs, an unanswered question exists. Why are these additional roles present? What is special about IBPs that allow them to accomplish multiple functions? While we understand the evolutionary history of several IBPs, as noted within several sections this thesis, multiple functionalities in their precursor proteins have not been reported. The answer may lie in the highly repetitive structure of IBPs, particularly within their ice-binding faces (Davies, 2014).

The highly repetitive, hydrophobic motifs present on ice-binding faces are hypothesized as the mechanism behind known IBP activities. Indeed, their interactions with both ice, gas hydrates, and silica matrices have all been suggested to be directly related to IBPs' repeating structures (Liou *et al.*, 2000; Sun *et al.*, 2015; Zeng *et al.*, 2007). Modelling studies have further demonstrated how repetitive, ice-like alignments of water molecules form along ice-binding faces, plausibly facilitating the absorption on these various surfaces (Smolin and Daggett, 2008; Nutt and Smith, 2008).

This repetitive nature also could explain why some IBPs are hypothesized to associate with cellular membranes (Tomczak *et al.*, 2002; Duman, 2015). Cellular membranes are distinguishable for their repetitive patterns of a phospholipid bilayers interspersed with membrane-associated proteins and channels. Similar situations of repetitive motifs involvement in cellular membrane association can be found with certain pathogenic bacteria. Adhesion molecules seen in certain strains are also characterized with repetitive adhesion domains that can interact with cellular barriers. Examples include the P.69 surface protein in *Bordetella pertussis*,

the invasin protein InIA in *Listeria monocytogenes*, as well as fibronectin-binding proteins in *Staphylococcus aureus* and *Streptococcus pyogenes* (reviewed in Niemann *et al.*, 2004).

Aside from the ability to interact with cellular membranes, repetitive motifs have also been postulated to be involved in protein-protein interactions, as demonstrated in the progression of several neurodegenerative diseases. Transmissible spongiform encephalopathies, including “mad-cow” disease seen in cattle and Creutzfeldt-Jakob disease in humans, results from protein-protein interactions. In these instances, the cellular protein PrP<sup>C</sup> is converted to abnormally-folded form PrP<sup>Sc</sup> through a direct interaction with the diseased-associated protein. In its normal form, PrP<sup>C</sup>, an alpha helical glycoprotein, associates with cellular membranes and is easily digested by proteases. PrP<sup>Sc</sup>, while having an identical primary structure to PrP<sup>C</sup>, has been modelled with a predominantly beta sheet secondary structure (Pan *et al.*, 1993), a similarity observed in the adhesion domains of some pathogenic bacteria, as well as the ice-binding face of certain IBPs (Niemann, 2004; Davies, 2014). This structure of PrP<sup>Sc</sup> is also suggested to confer high resistance to protease degradation (Kocisko *et al.*, 1994). Likewise, in Huntington’s disease, the *HTT* gene encodes for huntingtin, a protein required from normal development and brain function. Normal huntingtin proteins consist of 10-35 glutamine repeats, while the diseased protein (Htt) is characterized as containing 40+ glutamine repeats. This glutamine expansion creates aggregation of Htt proteins, resulting in the neurological dysfunction attributed to this disease (Li and Li, 2004). As observed with PrP<sup>Sc</sup>, Htt aggregates inhibit proteasome function, preventing degradation and resulting in aggregate accumulation and eventual neuron death (Bence *et al.*, 2001). A similar pathology is also seen in Alzheimer amyloid beta plaques, where aggregates are again characterized as protease resistant (Söderberg *et al.*, 2005). While speculative, it is possible IBP’s abilities to interact with different protein, such as INPs, and cellular membranes also results in a resistance against protease degradation, salt denaturation, and

enthalpically-driven low temperature denaturation. This resistance would be beneficial, especially given the importance of IBPs during periods of cold-induced stress.

Thus, this thesis has described nonconventional functions of IBPs as a defensive strategy against plant pathogens, for the storage of organs under hypothermic conditions, and for protection of freeze-tolerant organisms on the cusp of winter adaptations. Furthermore, this thesis has suggested that these additional properties could be due to the repetitive structures of IBPs.

## 6.2 References

- Amir, G., Rubinsky, B., Horowitz, L., Miller, L., *et al.* (2004a). Prolonged 24-hour subzero preservation of heterotopically transplanted rat hearts using antifreeze proteins derived from arctic fish. *The Annals of Thoracic Surgery*, 77: 1648-1655.
- Amir, G., Horowitz, L., Rubinsky, B., Yousif, B. S., Lavee, J., & Smolinsky, A. K. (2004b). Subzero nonfreezing cryopreservation of rat hearts using antifreeze protein I and antifreeze protein III. *Cryobiology*, 48: 273-282.
- Amir, G., Rubinsky, B., Basheer, S. Y., Horowitz, L., *et al.* (2005). Improved viability and reduced apoptosis in sub-zero 21-hour preservation of transplanted rat hearts using anti-freeze proteins. *The Journal of Heart and Lung Transplantation*, 24: 1915-1929.
- Arav, A., Rubinsky, B., Fletcher, G., & Seren, E. (1993). Cryogenic protection of oocytes with antifreeze proteins. *Molecular Reproduction and Development*, 36: 488-493.
- Army, D. C., Lindow, S. E., & Upper, C. D. (1976). Frost sensitivity of *Zea mays* increased by application of *Pseudomonas syringae*. *Nature*, 262: 282-284.
- Bayer-Giraldi, M., Weikusat, I., Besir, H., & Dieckmann, G. (2011). Characterization of an antifreeze protein from the polar diatom *Fragilariopsis cylindrus* and its relevance in sea ice. *Cryobiology*, 63: 210-219.
- Bella, J., Hindle, K. L., McEwan, P. A., & Lovell, S. C. (2008). The leucine-rich repeat structure. *Cellular and Molecular Life Sciences*, 65: 2307-2333.
- Bence, N. F., Sampat, R. M., & Kopito, R. R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. *Science*, 292: 1552-1555.
- Berendsen, T. A., Bruinsma, B. G., Puts, C. F., Saeidi, N., *et al.* (2014). Supercooling enables long-term transplantation survival following 4 days of liver preservation. *Nature medicine*, 20: 790-793.
- Davies, P. L., & Hew, C. L. (1990). Biochemistry of fish antifreeze proteins. *The FASEB Journal*, 4: 2460-2468.
- Davies, P. L., Baardsnes, J., Kuiper, M. J., & Walker, V. K. (2002). Structure and function of antifreeze proteins. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 357: 927-933.
- Davies, P. L. (2014). Ice-binding proteins: a remarkable diversity of structures for stopping and starting ice growth. *Trends in Biochemical Sciences*, 39: 548-555.
- Duman, J. G., Bennett, V., Sformo, T., Hochstrasser, R., & Barnes, B. M. (2004). Antifreeze proteins in Alaskan insects and spiders. *Journal of Insect Physiology*, 50: 259-266.
- Duman, J. G. (2015). Animal ice-binding (antifreeze) proteins and glycolipids: an overview with emphasis on physiological function. *Journal of Experimental Biology*, 218: 1846-1855.

- Gilbert, J. A., Davies, P. L., & Laybourn-Parry, J. (2005). A hyperactive, Ca<sup>2+</sup>-dependent antifreeze protein in an Antarctic bacterium. *FEMS Microbiology Letters*, 245: 67-72.
- Graham, L. A., Liou, Y. C., Walker, V. K., & Davies, P. L. (1997). Hyperactive antifreeze protein from beetles. *Nature*, 388: 727-728.
- Griffith, M., Antikainen, M., Hon, W. C., Pihakaski - Maunsbach, K., *et al.* (1997). Antifreeze proteins in winter rye. *Physiologia Plantarum*, 100: 327-332.
- Griffith, M., & Yaish, M. W. (2004). Antifreeze proteins in overwintering plants: a tale of two activities. *Trends in plant science*, 9: 399-405.
- Guibert, E. E., Petrenko, A. Y., Balaban, C. L., Somov, A. Y., *et al.* (2011). Organ preservation: current concepts and new strategies for the next decade. *Transfusion Medicine and Hemotherapy*, 38: 125-142.
- Guo, S., Garnham, C. P., Whitney, J. C., Graham, L. A., & Davies, P. L. (2012). Re-evaluation of a bacterial antifreeze protein as an adhesin with ice-binding activity. *PloS one*, 7: e48805.
- Gupta, R., & Deswal, R. (2012). Low temperature stress modulated secretome analysis and purification of antifreeze protein from *Hippophae rhamnoides*, a Himalayan wonder plant. *Journal of Proteome Research*, 11: 2684-2696.
- Hoshino, T., Kiriaki, M., Ohgiya, S., Fujiwara, M., *et al.* (2003). Antifreeze proteins from snow mold fungi. *Canadian Journal of Botany*, 81: 1175-1181.
- Kobe, B., & Kajava, A. V. (2001). The leucine-rich repeat as a protein recognition motif. *Current opinion in Structural Biology*, 11: 725-732.
- Kocisko, D. A., Come, J. H., Priola, S. A., Chesebro, B., Raymond, G. J., Lansbury, P. T., & Caughey, B. (1994). Cell-free formation of protease-resistant prion protein. *Nature*, 370: 471-474.
- Krembs, C., Eicken, H., & Deming, J. W. (2011). Exopolymer alteration of physical properties of sea ice and implications for ice habitability and biogeochemistry in a warmer Arctic. *PNAS*, 108: 3653-3658.
- Kun, H., Minnes, R., and Mastai, Y. (2008) Effects on antifreeze peptides on the thermotropic properties of a model membrane. *Journal of Bioenergetics and Biomembranes*, 40: 389-396.
- Lagneaux, D., Huhtinen, M., Koskinen, E., & Palmer, E. (1997). Effect of anti - freeze protein (AFP) on the cooling and freezing of equine embryos as measured by DAPI - staining. *Equine Veterinary Journal*, 29: 85-87.
- Larson, D. J., Middle, L., Vu, H., Zhang, W., Serianni, A. S., Duman, J., & Barnes, B. M. (2014). Wood frog adaptations to overwintering in Alaska: new limits to freezing tolerance. *Journal of Experimental Biology*, 217: 2193-2200.
- Li, S. H., & Li, X. J. (2004). Huntingtin–protein interactions and the pathogenesis of Huntington's disease. *TRENDS in Genetics*, 20: 146-154.

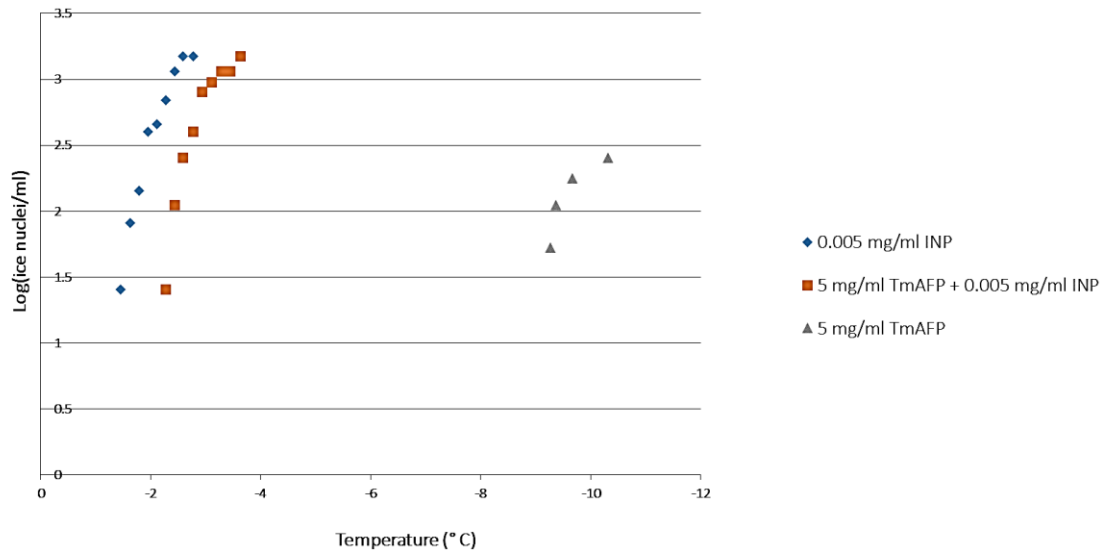
- Li, C., Rudi, H., Stockinger, E. J., Cheng, H., *et al.* (2012). Comparative analyses reveal potential uses of *Brachypodium distachyon* as a model for cold stress responses in temperate grasses. *BMC Plant Biology*, *12*: 65.
- Lindow, S. E., Arny, D. C., & Upper, C. D. (1978). Distribution of ice nucleation-active bacteria on plants in nature. *Applied and Environmental Microbiology*, *36*: 831-838.
- Liou, Y. C., Tocilj, A., Davies, P. L., & Jia, Z. (2000). Mimicry of ice structure by surface hydroxyls and water of a  $\beta$ -helix antifreeze protein. *Nature*, *406*: 322-324.
- Mazur, P. (1984). Freezing of living cells: mechanisms and implications. *American Journal of Physiology-Cell Physiology*, *247*: C125-C142.
- Middleton, A. J., Brown, A. M., Davies, P. L., & Walker, V. K. (2009). Identification of the ice-binding face of a plant antifreeze protein. *FEBS letters*, *583*: 815-819.
- Middleton, A. J., Marshall, C. B., Faucher, F., Bar-Dolev, M., Braslavsky, I., Campbell, R. L., ... & Davies, P. L. (2012). Antifreeze protein from freeze-tolerant grass has a beta-roll fold with an irregularly structured ice-binding site. *Journal of Molecular Biology*, *416*: 713-724.
- Mugnano, J. A., Wang, T., Layne, J. R., DeVries, A. L., & Lee, R. E. (1995). Antifreeze glycoproteins promote intracellular freezing of rat cardiomyocytes at high subzero temperatures. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *269*: R474-R479.
- Mugnano, J., Lee, R., & Taylor, R. (1996). Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *The Journal of Experimental Biology*, *199*: 465-471.
- Niemann, H. H., Schubert, W. D., & Heinz, D. W. (2004). Adhesins and invasins of pathogenic bacteria: a structural view. *Microbes and infection*, *6*:101-112.
- Nutt, D. R., & Smith, J. C. (2008). Dual function of the hydration layer around an antifreeze protein revealed by atomistic molecular dynamics simulations. *Journal of the American Chemical Society*, *130*: 13066-13073.
- Olsen, T. M., & Duman, J. G. (1997a). Maintenance of the supercooled state in the gut fluid of overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of ice nucleators and antifreeze proteins. *Journal of Comparative Physiology B*, *167*: 114-122.
- Olsen, T. M., & Duman, J. G. (1997b). Maintenance of the supercooled state in overwintering pyrochroid beetle larvae, *Dendroides canadensis*: role of hemolymph ice nucleators and antifreeze proteins. *Journal of Comparative Physiology B*, *167*: 105-113.
- Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., *et al.* (1993). Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *PNAS*, *90*: 10962-10966.

- Pouleur, S., Richard, C., Martin, J. G., & Antoun, H. (1992). Ice nucleation activity in *Fusarium acuminatum* and *Fusarium avenaceum*. *Applied and Environmental Microbiology*, 58: 2960-2964.
- Raymond, J. A., & Devries, A. L. (1977). Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *PNAS*, 74: 2589-2593.
- Raymond, J. A., Fritsen, C., & Shen, K. (2007). An ice-binding protein from an Antarctic sea ice bacterium. *FEMS microbiology ecology*, 61: 214-221.
- Raymond, J. A. (2011). Algal ice-binding proteins change the structure of sea ice. *PNAS*, 108: E198-E198.
- Rubinsky, B., Arav, A., Hong, J. S., & Lee, C. Y. (1994). Freezing of mammalian livers with glycerol and antifreeze proteins. *Biochemical and Biophysical Research Communications*, 200: 732-741.
- Salahudeen, A. K., Haider, N., & May, W. (2004). Cold ischemia and the reduced long-term survival of cadaveric renal allografts. *Kidney international*, 65: 713-718.
- Sandve, S. R., Rudi, H., Asp, T., & Rognli, O. A. (2008). Tracking the evolution of a cold stress associated gene family in cold tolerant grasses. *BMC Evolutionary Biology*, 8: 1.
- Sidebottom, C., Buckley, S., Pudney, P., Twigg, S., *et al.* (2000). Phytochemistry - heat-stable antifreeze protein from grass. *Nature*, 406: 256-256.
- Smallwood, M., Worrall, D., Byass, L., Elias, L., *et al.* (1999). Isolation and characterization of a novel antifreeze protein from carrot (*Daucus carota*). *Biochemical Journal*, 340: 385-391.
- Smolin, N., & Daggett, V. (2008). Formation of ice-like water structure on the surface of an antifreeze protein. *The Journal of Physical Chemistry B*, 112: 6193-6202.
- Söderberg, L., Dahlqvist, C., Kakuyama, H., Thyberg, J., *et al.* (2005). Collagenous Alzheimer amyloid plaque component assembles amyloid fibrils into protease resistant aggregates. *FEBS Journal*, 272: 2231-2236.
- Soltys, K. A., Batta, A. K., & Koneru, B. (2001). Successful nonfreezing, subzero preservation of rat liver with 2, 3-butanediol and type I antifreeze protein. *Journal of Surgical Research*, 96: 30-34.
- Sun, T., Davies, P. L., & Walker, V. K. (2015). Structural Basis for the Inhibition of Gas Hydrates by  $\alpha$ -Helical Antifreeze Proteins. *Biophysical Journal*, 109:1698-1705.
- Timsit, M. O., & Tullius, S. G. (2011). Hypothermic kidney preservation: a remembrance of the past in the future? *Current opinion in organ transplantation*, 16: 162-168.
- Tomalty, H. E., & Walker, V. K. (2014). Perturbation of bacterial ice nucleation activity by a grass antifreeze protein. *Biochemical and Biophysical Research Communications*, 452: 636-641.

- Tomczak, M. M., Hinch, D. K., Estrada, S. D., Wolkers, W. F., *et al.* (2002) A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. *Biophysical Journal*, 82: 874-881.
- Tursman, D., Duman, J. G., & Knight, C. A. (1994). Freeze tolerance adaptations in the centipede, *Lithobius forficatus*. *Journal of Experimental Zoology*, 268: 347-353.
- Tyshenko, M. G., Doucet, D., Davies, P. L., & Walker, V. K. (1997). The antifreeze potential of the spruce budworm thermal hysteresis protein. *Nature biotechnology*, 15: 887-890.
- Vanderveer, T. L., Choi, J., Miao, D., & Walker, V. K. (2014). Expression and localization of an ice nucleating protein from a soil bacterium, *Pseudomonas borealis*. *Cryobiology*, 69: 110-118.
- Walters, K. R., Serianni, A. S., Sformo, T., Barnes, B. M., & Duman, J. G. (2009). A nonprotein thermal hysteresis-producing xylomannan antifreeze in the freeze-tolerant Alaskan beetle *Upis ceramboides*. *PNAS*, 106: 20210-20215.
- Walters Jr, K. R., Serianni, A. S., Voituron, Y., Sformo, T., Barnes, B. M., & Duman, J. G. (2011). A thermal hysteresis-producing xylomannan glycolipid antifreeze associated with cold tolerance is found in diverse taxa. *Journal of Comparative Physiology B*, 181: 631-640.
- Wang, T., Zhu, Q., Yang, X., Layne, J. R., & Devries, A. L. (1994). Antifreeze glycoproteins from antarctic notothenioid fishes fail to protect the rat cardiac explant during hypothermic and freezing preservation. *Cryobiology*, 31: 185-192.
- Wang, L., & Duman, J. G. (2005). Antifreeze proteins of the beetle *Dendroides canadensis* enhance one another's activities. *Biochemistry*, 44: 10305-10312.
- Wang, L., & Duman, J. G. (2006). A thaumatin-like protein from larvae of the beetle *Dendroides canadensis* enhances the activity of antifreeze proteins. *Biochemistry*, 45: 1278-1284.
- Younis, A. I., Rooks, B., Khan, S., & Gould, K. G. (1998). The effects of antifreeze peptide III (AFP) and insulin transferrin selenium (ITS) on cryopreservation of chimpanzee (*Pan troglodytes*) spermatozoa. *Journal of andrology*, 19: 207-214.
- Zeng, H., Walker, V. K., & Ripmeester, J. A. (2007). Approaches to the Design of Better Low - Dosage Gas Hydrate Inhibitors. *Angewandte Chemie*, 119: 5498-5500.
- Zhang, S. H., Wei, Y., Liu, J. L., Yu, H. M., Yin, J. H., Pan, H. Y., & Baldwin, T. C. (2011). An apoplastic chitinase CpCHT1 isolated from the corolla of wintersweet exhibits both antifreeze and antifungal activities. *Biologia plantarum*, 55:141-148.

## Appendix I

### Effect of *Tm*IBP on INP activity



**Figure A.1** Representative graph for INA combining recombinant *Tm*IBP with INPs from *P. syringae* shown as the logarithm of the cumulative number of ice nuclei per mL with *Tm*IBP (5 mg/mL). Samples included an INP preparation from *P. syringae* (blue squares), INPs combined with *Tm*IBP (orange squares) and *Tm*IBP alone (purple triangles).

**Appendix II**  
**Mean freezing temperature differences**

**Table A.1** Mean differences in freezing temperatures for *P. syringae* INP preparations in the presence of *TmIBP*.

Protein Addition		[INP]	
		0.05 mg/ml	0.005 mg/ml
<i>TmIBP</i> *	2 mg/ml	-0.21 °C	-0.41°C
	1 mg/ml	-0.14 °C	-0.39°C

\* No significant difference existed between *TmIBP* and cytochrome c controls

## Appendix III

### **IBP-activity in bacterial isolates collected from the Spring Mountain range (Las Vegas, Nevada, USA)**

*(Note: this appendix is an excerpt from my NSERC activity report following a three month visit to the lab of Dr. James Raymond, University of Nevada, Las Vegas)*

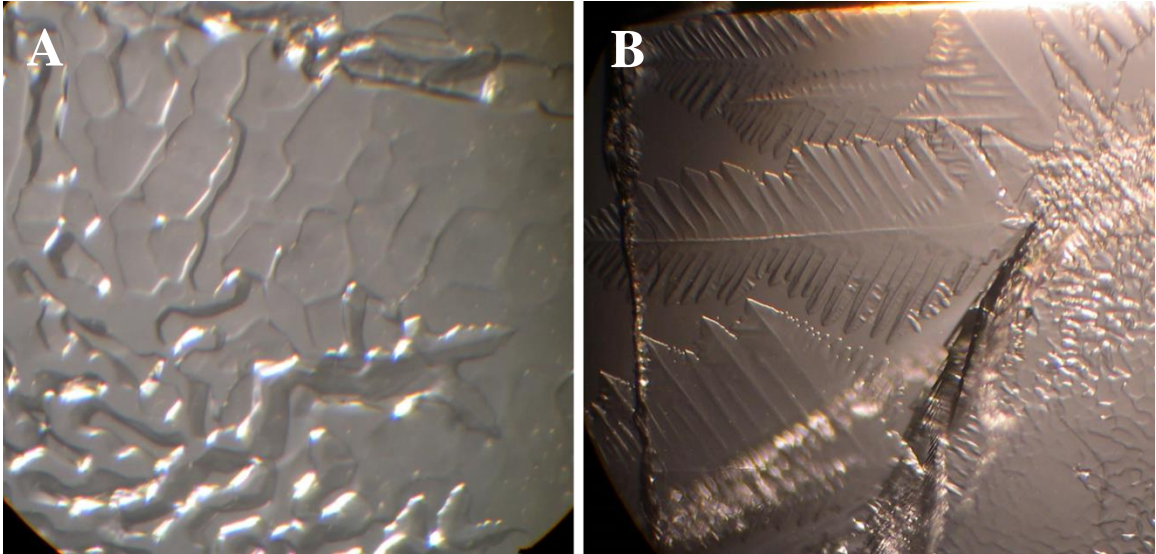
#### Objectives

In response to subzero temperatures, some microorganisms have evolved unique properties that allow them to survive these extreme conditions. One such adaptation is the production of ice-binding proteins (IBPs) which provide protection from the damaging effects of uncontrolled ice crystal growth. These macromolecules have already been isolated from a variety of plants and animals found in temperate and polar climates; however, current research has been directed towards finding microorganisms from similar climates which use IBPs as a survival mechanism. The purpose of my proposed project involved isolating ice-binding (IBP+) bacteria from Antarctic sea ice samples, followed by protein purification and analysis in hopes of further elucidating the roles these proteins play. Unfortunately, due to unforeseen time delays in sea ice being received from Antarctica, IBP+ microbes were instead isolated from soil and snow samples obtained from Mt. Charleston, the highest peak in the Spring Mountain range just outside of Las Vegas, NV.

#### Methodology and Results

Bacterial and fungal cultures (32) were isolated from soil and snow samples originally collected from Mt. Charleston, Nevada. These cultures were easily grown in LB media, with growth occurring at 10°C. Once an optimal density was reached, the cultures were assayed for the presence of IBPs. A single ice crystal was submerged into cooled culture medium and allowed to anneal for a short time period. Cultures considered to be IBP+ exhibited the ability to pit the surface of the growing ice crystal, indicating the bacteria were binding onto the ice crystal planes. Out of 32 cultures, 25 isolates were obtained and of these two cultures isolated from the snow sample displayed pitting on the ice crystal's basal plane, along with the growth of jagged dendrites indicating the presence of a possible IBP (Figure A.2). Several isolates from the soil sample displayed faint rippling on the basal plane of the ice crystal; however, no further work was pursued with these samples since minimal ice-binding activity was displayed. The two active snow cultures which were positively identified for IBP activity were sequenced using universal

16S rRNA primers to determine putative species identity. One culture (Snow #102) was identified as the yeast *Leucosporidium sp.* This yeast has already previously been isolated from Arctic water samples and is known to produce an IBP (Cryobiology 60: 222-228, 2010). The second isolate (Snow #2) was identified as *Sphingomonas sp.* This species has not yet been reported to display IBP activity.

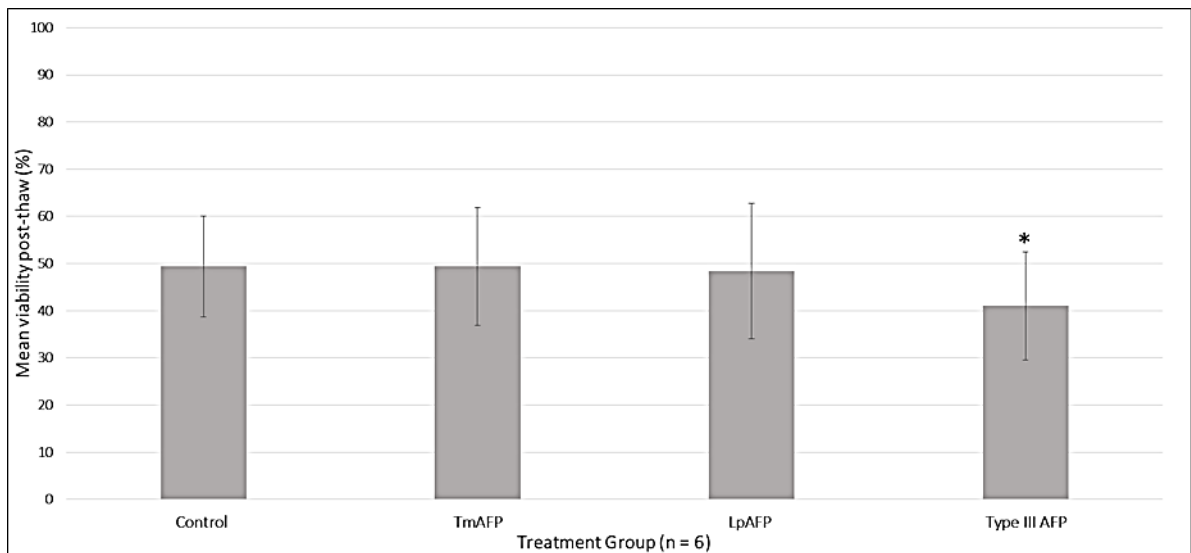


**Figure A.2** Representative images from ice pitting assay in the presence of culture supernatant from *Sphingomonas sp.* Ice pitting along the basal plane (A) and the formation of jagged dendritic growth (B) indicates the presence of a possible IBP.

## Appendix IV

### Cryopreservation of bovine sperm in the presence of IBPs

(Note: this appendix contains data collected in collaboration with Dr. Matthew Utt from Select Sires Ltd.)



**Figure A.3** Graphical representation of mean percent viability post-thaw of bovine sperm frozen in the presence IBPs (10  $\mu\text{g}/\text{mL}$ ) from either an insect (*TmAFP*), plant (*LpAFP*), or fish (Type III AFP). The asterisk represents a significant difference (one-way ANOVA;  $p < 0.05$ ).