Ice Active Proteins From Cold Tolerant Organisms

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Abstract

The Antarctic nematode *Panagrolaimus davidi* is the only animal known to survive both intracellular and extracellular freezing. Homogenates made from *P. davidi* freeze at the usual temperature (approximately 0 °C) but are able to maintain the small size of the ice crystals formed i.e. inhibit recrystallization. This ability is thought to be key to their unique ability to survive intracellular freezing as it prevents large ice crystals forming that may damage intracellular structures and membranes. The aim of this research was to further our understanding of how *P. davidi* achieves this control over the ice crystal structure by identifying the proteins responsible for the recrystallization inhibition. This would have wide ranging implications not only in the basic understanding of freeze tolerance, but huge potential in the biotechnology field e.g. frost resistant crops.

Initially the aim was to identify any proteins that bind to ice using a cold finger ice binding technique. This involved freezing a solution very slowly and trapping any ice binding proteins within the ice while all others were excluded. Interestingly no proteins were found to bind to the ice and using a grass extract that is also known to inhibit recrystallization it was found that these proteins could exert their effect on the ice crystal structure without binding to ice. This led to the proposal of a mechanism in which the recrystallization inhibition proteins situate themselves in the liquid phase between the ice crystals and prevent the movement of water molecules between ice crystals, which in turns prevents recrystallisation. This is very different to the mechanism of antifreeze proteins, which bind to the ice and provide an energy barrier to ice growth.

To help in the isolation of recrystallisation inhibiting proteins it was necessary to develop a quick reliable screen to detect recrystallization inhibition activity. This was achieved using an optical recrystallometer. This is capable of measuring the intensity of light passing through a sample and we proposed that as the ice crystal structure changed so would the intensity of light passing through a sample. Ultimately it was shown that the change in intensity of light passing through samples with recrystallisation inhibition activity was a good indication of recrystallisation inhibition activity, in that samples with high activity showed very small changes in transmittance, whereas those with low activity showed a large change.
An attempt was made to isolate the protein responsible for the recrystallisation inhibition activity. As part of this, several other basic properties were also investigated. It was shown that the activity was sensitive to proteinase K, did not require metal ions, was heat stable and likely required more than one component to be active. Several types of chromatography were not successful at isolating any proteins responsible for this activity and so a proteomic approach was then attempted. It was shown that there was a difference in the recrystallisation inhibition activity of acclimated and non-acclimated *P. davidii* (known to have different freezing survival rates) and that these differences were also matched with a difference in the protein profile of the samples. Sixty one spots across the two gels were identified as differentially expressed and excised and are awaiting mass spectrometry analysis to identify them.
Acknowledgements

Firstly I would like to think both of my supervisors; Dr. Craig Marshall (Biochemistry) and Prof. David Wharton (Zoology). Without either of you I would not have got this far and your ideas, help and mostly patience have been invaluable throughout this project and have helped shaped me into the scientist I am today.

After spending such a long time in the lab there are numerous members who I have worked with and they all deserve thanking, but several stand out, mainly due to their prolonged presence or enthusiasm, these include Melianie, Sam, James, Nik and Abishek. Dan and Tracy also fit into this category, although their help through some pretty rough times was even more important.

Next up is my family (Mum, Dad, Christopher, Amy and Nana), as with my previous projects I still don’t think you have much of an idea exactly what I do, but you always ask and act interested, also when I hear you telling other people about me I at least know you like bragging about me. Also worth mentioning here are Richard, Adele, Kayla and Zaria, without you guys I doubt I would have finished and will probably never be able to repay or express how much your support meant.

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<th>Abbreviation</th>
<th>Description</th>
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<td>ΔAU.min⁻¹</td>
<td>Change in absorbance units per minute</td>
</tr>
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<td>2-Mercapothanol</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
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<tr>
<td>AFGP</td>
<td>Antifreeze glycoprotein</td>
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<td>BCA</td>
<td>Bicinchoninic acid</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CFIB</td>
<td>Cold finger ice binding</td>
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<td>INP</td>
<td>Ice nucleating protein</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>M</td>
<td>mole.L^{-1}</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode growth medium</td>
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<tr>
<td>P. davidii</td>
<td><em>Panagrolaimus davidii</em></td>
</tr>
<tr>
<td>PK</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>RI</td>
<td>Recrystallisation inhibition</td>
</tr>
<tr>
<td>RIP</td>
<td>Recrystallisation inhibition protein</td>
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<tr>
<td>Rpm</td>
<td>revolutions per minute</td>
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<td>SDS</td>
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<td>Type I water</td>
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Chapter One

Introduction

1.0 Introduction

Poikilotherms are organisms that cannot maintain their own body temperature and assume the temperature of the surrounding environment (Atici and Nalbantoglu, 2003). This can have serious implications for the organism if it lives in an area where it is exposed to subzero temperatures e.g. high latitudes, both on land and in water (both North and South), and high altitudes. If the surrounding environment is below 0 °C then it is plausible that the internal temperature of the organism will also be below 0 °C. An example of this are the Antarctic teleost fishes, which live in water that has a constant temperature of close to -1.8 °C (significantly below their osmotic freezing point) but which nonetheless remain unfrozen (Ewart et al., 1999; Kristiansen and Zachariassen, 2005).

Organisms that inhabit these environments must have evolved methods of surviving these subzero temperatures. These methods fall into two broad classes; freeze avoidance or freeze survival. Freeze avoidance relies on avoiding freezing, which may be via behavioural methods e.g. burrowing into the ground or sheltering under cover where the microclimate does not reach freezing temperatures, or it may be via evolutionary molecular methods e.g. antifreeze proteins (DeVries et al., 1970; Bale, 1996; Hoshino et al., 2003; Hiiesaar et al., 2006; Bayer-Giraldi et al., 2011). Regardless of how these organisms avoid freezing, it must be successful because if they do freeze it is usually fatal e.g. Antarctic teleost fish, which can tolerate supercooling to -4°C, but which die once ice formation occurs within the body.

Freeze survival involves the organism freezing and being able to survive the stresses this creates. Most of the animals that utilise freeze survival only tolerate the formation of ice in extracellular spaces e.g. in the gut (Storey and Storey, 1985; Sformo et al., 2009; Costanzo and Lee, 2013; Storey and Storey, 2013). A wide variety
of organisms are able to survive freezing in this way including several species of beetle, New Zealand weta and some species of wood frog (Storey and Storey, 1985; Ramløv et al., 1996; Graham et al., 2007). In almost all cases intracellular freezing is considered lethal, with the notable exception of the Antarctic nematode Panagrolaimus davidi (Wharton and Ferns, 1995).

Intracellular freezing is damaging for several different reasons. The most obvious of these is that water is one of the few compounds that expands as it freezes and frozen water (ice) takes up more space than liquid water, which puts pressure on cellular structures and membranes (Barrett, 2001). A second reason is that different compartments of the organism or cell may freeze at different rates, which can create large osmotic gradients due to the solutes concentrating in the unfrozen parts of the organism or cell (Wharton et al., 2000; Costanzo and Lee, 2013). This can result in a number of problems including cell shape changes, damage associated with high salt concentrations and protein denaturation due to pH changes. Thirdly, having a solid in a cell compartment that is meant to be liquid can prevent movement of vital metabolites. Finally, if ice is held at high subzero temperatures (between 0 and -20 °C) then the ice crystals will rearrange themselves from many small crystals to fewer large crystals, a process known as recrystallization (Wharton et al., 2005b). These larger crystals are believed to be damaging to the cell structures as they may pierce or crush cell membranes or organelles.

Extracellular freezing is a widespread phenomenon and despite the problems it creates, it is not lethal to many organisms, which appear to have evolved mechanisms to survive it (Karlsson et al., 1993; Wharton and Ferns, 1995). Over the past decades there has been much research into what these mechanisms are that allow organisms to survive in subzero environments. One method is the production of high levels of simple molecules such as trehalose, glycerol and glucose (Layne and Stapleton, 2009). In some cases these can prevent the organism from freezing due to colligative depression of the freezing point, but they may also aid freeze survival by stabilising membranes and preventing large osmotic stresses (Wharton et al., 2000).
In addition to colligative effects, there is a protein component to freezing tolerance. Proteins associated with freeze tolerance and survival were first discovered in Antarctic fishes in 1970 and have since been discovered in a wide variety of other organisms including plants, other fish, nematodes, insects and diatoms (DeVries et al., 1970; Graether et al., 2000; Atici and Nalbantoglu, 2003; Wharton et al., 2005b; Bayer-Giraldi et al., 2011). These proteins have been given the general classification of ice active proteins (IAPs). Ice active proteins can be divided into three subclasses depending on their function; some lower the freezing point of the solution (antifreeze proteins or AFPs), others initiate ice formation (ice nucleating proteins or INPs), and the final class stabilise ice crystals after they have formed (recrystallisation inhibition proteins or RIPs).

1.1 Ice

Ice is the solid state of water and can be found in many places throughout the world. Both polar regions (the Arctic and Antarctica) are covered with large amounts of ice; many mountains are covered in ice for at least some part of the year and many regions of the world experience frost at some point during the winter (and sometimes in the summer). It is often assumed that ice is very well understood and to produce it simply requires cooling liquid water to 0 °C. This, however, is not the case and it is relatively easy to supercool water to temperatures of about -14 °C without freezing; microlitre amounts can be cooled to as low as -36 °C without freezing (Bédécarrats et al., 2010). Ice can form in 16 different types depending on the pressure and temperature at which it forms (Abascal et al., 2009). The most common form of ice found on Earth is $I_h$ (hexagonal ice), with most other forms requiring extreme temperature and/or pressure e.g. the only other form of ice present in the atmosphere is cubic ice ($I_c$) which requires temperature lower than -100 °C (Abascal et al., 2009).

The transition into a new phase e.g. solid (ice) from liquid (water), in a metastable (supercooled) state occurs via nuclei (or germ crystals) which have a molecular
structure that approximates the new solid phase. These nuclei are in equilibrium with the liquid phase and when they are small they are likely to lose molecules and ‘melt’. However, once the nuclei reach a critical size they will become stable, which means the chance of growth is much greater than the chance of decay (Matsumoto et al., 2002). Once nuclei reach this critical size they are then able to initiate a phase change. There is still much debate about what the critical size is for nuclei to induce a phase change. If the nuclei change the structure from within the liquid phase it is known as homogenous nucleation (Figure 1.1). However, it is also possible that some other surfaces may be able to act as nuclei e.g. contaminants in the water (AgI) or INPs, and this is known as heterogeneous nucleation (Matsumoto et al., 2002; Zobrist et al., 2008).

Figure 1.1: The hydrogen bond network of water during the freezing process based on a molecular dynamics simulation. A) t = 256 ns B) t = 290 ns C) t = 320 ns D) t= 500 ns. Lines indicate hydrogen bonds between water molecules and intermolecular bonds of water participating in those hydrogen bonds. Brighter blue bonds indicate longer lasting bonds with the brightest blue bonds lasting > 2 ns. The initial nucleation site is circled in yellow in A). Notice how the long lasting bonds spread out from this point and form a highly structured, long-lasting ice lattice with the typical hexagonal pattern characteristic of ice (from Matsumoto et al., 2002).
In pure water, the highest temperature at which ice can form is 0 °C. However, nuclei form very reluctantly or at least last for very short periods at this temperature. Solutions that remain free of ice below temperatures at which ice can form are said to be supercooled. The maximum amount of supercooling for pure water is approximately -36 °C, but ice will usually form between -5 °C and -15 °C due to either homogenous or heterogenous nucleation (Matsumoto et al., 2002; Bédécarrats et al., 2010). Ice formation is characterised by the release of the latent heat of crystallisation and this ‘freezing exotherm’ can be used to monitor the temperature at which freezing is initiated.

As water freezes it expands and the solid form is less dense than the liquid form, consequently ice floats in water (Akyurt et al., 2002). Water is one of the few substances in which this occurs. The shape of ice crystals is determined by the behaviour of the water molecules. Normal ice (I_h) is hexagonal because six water molecules bind to each other to form sheets (Radhakrishnan and Trout, 2003). This growth pattern results in an ice crystal with two effective axes, the a-axis (parallel to the basal face) and the c-axis (parallel to the prism face) (Figure 1.2). These two faces have a different arrangement of water molecules, which results in differently shaped crystals depending on which plane they bind to. Water molecules will bind to the prism face more easily and this results in ice growing in flat sheets and explains why the surface of water is covered with ice before the rest of the water freezes e.g. the tops of puddles freezing (Harding et al., 2003; Radhakrishnan and Trout, 2003).
Figure 1.2: Schematic view of an ice crystal. Looking down the c-axis (top view) we can see the characteristic hexagonal shape of the ice crystal with the a-axes extending outward. The side view shows the relative flatness of the ice crystal (c-axis/prism plane). Water molecules find it energetically more favourable to bind to the prism planes and this leads to faster growth in the a-axis direction than the c-axis, resulting in flat ice crystals (from Lake Ice, 2011).

Along with the significant structural change between liquid and solid water, there are also some major changes in the chemical properties. The conductivity and the hydrophilicity of ice are both much lower than liquid water and this results in the two phases having very different properties. These different properties are exploited by all IAPs to enable each class to interact with ice. INPs are proposed to form large complexes that mimic nuclei and are very hydrophilic to attract new water molecules (Duman and Patterson, 1978). Conversely AFPs are believed to be quite hydrophobic proteins that are able to form tight bonds to an ice surface and interfere with addition of water molecules and ice growth (Doucet et al., 2000; Liou et al., 2000; Davies, 2014)
1.2 Panagrolaimus davidi

*Panagrolaimus davidi* (Figure 1.3) is a free living nematode that was first isolated from the McMurdo Sound region of Antarctica (Wharton and Brown, 1989). It is associated with coastal sites, especially penguin colonies, where it is assumed that penguin debris boosts the nutrient availability of the soil. *Panagrolaimus davidi* is a very useful model organism for cold tolerance for several reasons. Firstly, it is relatively easy to grow. A laboratory culture was set up in 1989 in which the nematodes were added to nutrient agar plates, fed on bacteria grown from the original isolates and grown at 20°C (Wharton and Brown, 1989). It has been maintained ever since. Secondly *P. davidi* is transparent, which means it is easy to visualise the formation of ice in the body on a microscope cold stage (Wharton and Ferns, 1995). Finally, and most importantly, *P. davidi* is the only intact animal that has been shown to survive intracellular freezing, which in all other animals is considered lethal (Wharton and Ferns, 1995; Wharton *et al.*, 2005a).

Figure 1.3: Scanning Electron micrograph of the Antarctic nematode *P. davidi* (photo D.A. Wharton)
*Panagrolaimus davidi* is essentially an aquatic animal that needs at least a film of water for movement and is likely to be exposed to inoculative freezing i.e. ice from the surrounding water travelling through a body opening to cause freezing in the animal (Wharton and Ferns, 1995; Wharton *et al*., 2005a). The ability to survive intracellular freezing is therefore very important to its survival.

When *P. davidi* is frozen under conditions where there is a relatively rapid spread of ice through the sample, the ice enters the body via body openings, in particular, the excretory pore. After inoculative freezing is initiated, the freezing process is very fast, taking about 0.2 seconds (Figure 1.4) (Wharton and Ferns, 1995). This is much faster than the spread of ice in other freeze tolerant organisms and may aid in survival because less osmotic stress is created as gradients have insufficient time to form (Wharton *et al*., 2005a).

When *P. davidi* is acclimated to low temperatures there is an increase in the production of trehalose which may play a role in freeze tolerance through membrane stabilisation and/or by supercooling the internal liquid allowing rapid freezing (Wharton *et al*., 2000). There is also a protein component to the freezing tolerance of *P. davidi*. This protein is believed to be a recrystallisation inhibitor that is thought to aid survival by maintaining small ice crystals and therefore preventing the growth of large ice crystals that damage cellular structures and membranes (Wharton *et al*., 2005b).
Figure 1.4: The freezing of *P. davidii* photographed during cooling on a cold stage. Freezing is initiated in the space between the oesophagus and the body wall. Frames A-J show how the ice (darker areas) spreads throughout the nematode to eventually freeze all compartments including intracellular spaces. Scale bar = 100 µm (from Wharton and Ferns, 1995).

The second mechanism *P. davidii* can use to survive freezing temperatures is cryoprotective dehydration. This process is driven by a difference in the vapour pressure between supercooled liquid water and ice at the same temperature (Wharton *et al.*, 2005a). Using freeze substitution and transmission electron microscopy to visualise ice within the ultrastructure, it has been shown that an increase in the freezing rate i.e. a faster rate of temperature decrease, leads to a change from cryoprotective dehydration to extracellular freezing to intracellular freezing (Wharton *et al.*, 2005a).
1.3 Ice Active Proteins

Ice active proteins (IAPs) are proteins that affect the formation and/or stability of ice and can be divided into three classes – antifreeze proteins (AFPs), ice nucleating proteins (INPs) and recrystallisation inhibition proteins (RIPs). The distinction between each type of IAP is somewhat arbitrary and often a protein may fit into 2 (or even 3) of the categories. This is especially the case between AFPs and RIPs as all AFPs have RI activity and most RIPs have thermal hysteresis activity (although often to a very small, biologically insignificant degree).

1.3.1 Antifreeze Proteins

Antifreeze proteins (AFPs) are characterised by their thermal hysteresis activity, an ability to lower the freezing point of a solution in the presence of an ice crystal without altering the melting point significantly (Raymond and DeVries, 1977; Ewart et al., 1999; Barrett, 2001; Davies, 2014). The amount by which the AFP is able to decrease the freezing point relative to the melting point is known as the hysteresis gap. Solutions containing AFPs e.g. fish blood, are able to stay liquid below the normal freezing temperature of the solution so the organism is able to continue living without freezing (DeVries et al., 1970). Antifreeze proteins do this in a non-colligative manner i.e. the decrease in freezing point is more than would be expected from the increase in osmolarity of the solution caused by the addition of the AFP (DeVries et al., 1970; Fletcher et al., 2001). Antifreeze proteins were first identified in Antarctic teleost fishes in 1970 and have since been found in a wide range of species that inhabit regions where freezing temperatures are likely to be encountered (DeVries et al., 1970; Davies and Sykes, 1997; Ewart et al., 1999). The level of thermal hysteresis of AFPs (the amount that the AFP suppresses freezing), varies widely depending on the species and the environment that is likely to be encountered. For example, the AFPs of Antarctic fish are able to lower the freezing point by about 2 °C, whereas some insect AFPs can lower the freezing point by more than 10 °C (DeVries et al., 1970; Chao et al., 1996; Graether et al., 2000; Pertaya et al., 2008).
The first fish AFP was found to be a glycoprotein and this family is known as antifreeze glycoproteins (AFGPs). Since their discovery, other AFPs have been identified in many different organisms including plants, invertebrates, vertebrates, fungi, diatoms and bacteria (DeVries et al., 1970; Atici and Nalbantoglu, 2003; Hoshino et al., 2003; Gilbert et al., 2004; Qin and Walker, 2006; Bayer-Giraldi et al., 2011). Because of this wide source of AFPs, many different families of AFPs have been reported. However between each family there appears to be very little homology and no common ancestor: even within the insects there are evolutionary distinct AFPs (Barrett, 2001).

1.3.1.1. Structure

A wide range of structures have been reported for AFPs. Five classes of AFPs have been identified in Antarctic and Arctic fishes; AFP types I, II, III and IV and an AFGP, and numerous other classes are found in other organisms (Barrett, 2001).

The AFGPs have a repeating tripeptide unit (Ala-Ala-Thr) with galactosamine linked to the threonine via a 1→3 galactosyl linkage (Fletcher et al., 2001). Eight different AFGP isoforms have been identified that range in size from 2.7 to 32 kDa and differ primarily in the number of tripeptide repeats (4-50) (Barrett, 2001; Evans et al., 2012). The AFGPs appear to exist as an extended left-handed three-fold helix in solution. This formation allows the disaccharide groups to be positioned on one side of the molecule where they can form a stable hydrophilic face capable of interacting with the ice, while the hydrophobic alanine groups are on the opposite side of the helix (Mimuar et al., 1992). The spacing of the tripeptide repeats (9.31 Å) is approximately twice the spacing of the a-axes of ice and hydrogen bonding can presumably occur at the hydroxyl groups of the threonine linked galactosamine repeats, which may allow the AFGPs to interact with the ice and exert their thermal hysteresis effect (Knight et al., 1991). Larger AFGPs are more active than small AFGPs, suggesting that the size of ice interaction is important for activity (Barrett, 2001).
Type I AFPs are long, alanine-rich single α-helices found mainly in flounders and sculpins (Figure 1.5). The helices are stabilised by complex N and C termini cap structures with complementary charged groups and internal salt bridges (Chakrabarty and Hew, 1991). The type I AFPs from flounder have an 11 amino acid repeat, TxxNxxxxxx, where x is usually alanine and N is either aspartate or asparagine (Chao et al., 1996). X-ray studies have shown that the Thr and Asp/Asn are on one side of the helix and form a ‘flat’ surface with a spacing of the hydroxyl groups (16.5 Å) that very closely matches that of ice (16.7 Å). The structure of the winter flounder type I AFP has suggested a strong dependence on both the hydrophobic environment and the high level of surface complementarity at the ice binding surface as being important for AFP activity (Sicheri and Yang, 1995; Leinala et al., 2002a).

**Figure 1.5: Structure of the type I AFPs.** A) Molecular surface of the type I AFP from winter flounder ice binding region. The threonine and alanine residues form a regular pattern that allows the AFP to interact with ice. The complex N and C terminal caps are not shown (from Leinala et al., 2002a). B) Ribbon structure of shorthorn sculpin type I AFP, based on NMR data, showing the α-helical ice binding domain (from Kwan et al., 2004). Both structures are shown in the same orientation with the N terminal at the top.
Type II AFPs are found in many different species including sea raven, smelt and Atlantic herring. The main feature of this class is that they are globular and cysteine rich (up to 9%) (Barrett, 2001; Kandaswamy et al., 2011). This results in a protein with a relatively large number of disulfide bonds and cleavage of these bonds can lead to a significant loss of antifreeze activity (Barrett, 2001). Type II AFPs appear to be homologs of the carbohydrate recognition domain of calcium-dependent C-type lectins, without the carbohydrate binding activity. However, some have retained a dependency on calcium for activity (Ewart et al., 1992; Loewen et al., 1998). Type II AFPs have a very limited α-helical content e.g. sea raven AFP is 18% α-helix, 38% β-sheet and 44% random coil (Figure 1.6) (Sönnichsen et al., 1995). One model of the sea raven AFP suggests that the exposed part of the peptide chain is composed mainly of hydrophilic residues that are capable of forming hydrogen bonds (Ng and Hew, 1992).

![A) Structural model of herring AFP obtained by homology modelling B) Solution structure of sea raven AFP determined by NMR (from Ewart et al., 1992).](image)

**Figure 1.6: Type II AFPs shown as ribbon models.** A) Structural model of herring AFP obtained by homology modelling B) Solution structure of sea raven AFP determined by NMR (from Ewart et al., 1992).
Type III AFPs are found in Antarctic eelpout and wolf fish. These are essentially globular proteins not dominated by any particular amino acid. The overall structure has a very compact fold with very few secondary structural elements; they essentially contain seven antiparallel β-strands, three $3_{10}$ helices, eight β-turns and interconnecting coils (Jia et al., 1996; Sönnichsen et al., 1996; Yang et al., 1998; Kandaswamy et al., 2011). Type III AFPs also have a unique fold that was named the “pretzel fold” (Figure 1.7) (Yang et al., 1998). This fold gives the type III AFPs a two-fold symmetry with the main chain backbone of the two 19-residue loops varying by only 0.59 Å (RMSD) (Yang et al., 1998). Remarkably, despite this high level of structural homology, the two loops share only 33 % sequence identity. One of these loops (loop 1) is the putative ice-binding site and the second loop (loop 2) is thought to be important in providing rigid support for the ice-binding site (Yang et al., 1998).

![Figure 1.7: View perpendicular to the pretzel fold of the type III AFP from ocean pout. Notice the high level of symmetry between the two loops and unique pretzel fold. green = helix, blue = β-strand, orange = turn and black = coil (from Yang et al., 1998).](image)

Type IV AFPs, found in the Longhorn sculpin, are four-helix bundles that have 22 % sequence identity with the low-density lipoprotein receptor-binding domain of
human apoliprotein E3. The four helices are amphipathic and may be arranged as an antiparallel bundle with the hydrophobic faces on the inside and the hydrophilic faces on the outside (Deng et al., 1997).

As well as these five groups of fish AFPs there are many AFP structures from other species, especially insects (Liou et al., 2000; Doucet et al., 2000; Mok et al., 2010; Kristiansen et al., 2011). The insect AFPs so far characterised are all small proteins (usually <15 kDa) that exist as a series of isoforms. AFPs from both *Tenebrio molitor* (meal worm) and *Dendroides canadensis* (fire beetle) have very high cysteine and threonine contents and an almost identical 12-13 amino acid repeat (Liou et al., 2000; Leinala et al., 2002a). Both NMR and circular dichroism spectra indicate extensive β-sheet structure (70-80 %) and little or no α-helix (Figure 1.8A and 1.8B). Additionally, these proteins do not have any close similarity to other proteins, and other insect AFPs also appear completely unrelated. The AFP from *Choristoneura fumiferana* (spruce budworm) has a high serine and threonine content and an unusual β-helix structure (Figure 1.8C). A theoretical model of the *Lolium perenne* (perennial ryegrass) AFP shows two putative ice binding faces, one on each side of a β-roll with eight loops, and each loop is made up of a 14-15 amino acid repeat (Figure 1.8D) (Kuiper et al., 2001).
Figure 1.8: Insect and plant AFP structures. A) End-on view of the *Tenebrio molitor* AFP highlighting the flat surface created by the β-sheets (green) also showing the alignment of the threonines (oxygen atoms in red) and the internal disulfide bonds (yellow) (from Liou et al., 2000) B). Ribbon representation of the *Dendroides canadensis* AFP looking onto the ice-binding face. Blue arrows are β-sheet and yellow are the disulphide bonds (from Leinala et al., 2002a). C) Looking onto the proposed ice-binding face of the *Choristoneura fumiferana* AFP with the threonines shown as ball and sticks (from Doucet et al., 2000). D) Theoretical structure of the *Lolium perenne* AFP. The two parallel β-sheet regions (yellow) both form flat surfaces that may be able to interact with ice (from Kuiper et al., 2001).
1.3.1.2 Mechanism

To understand the mechanism of action for AFPs it is necessary to have a basic understanding of the underlying physics of thermal hysteresis. From a thermodynamic point of view, if two phases are at equilibrium then their free energies per quantity are equal (Kristiansen and Zachariassen, 2005). Below the bulk melting point the free energy of the solid phase (ice) is less than that of the liquid phase and this difference increases with decreasing temperature, this should lead to an increase in the amount of ice. It can be shown thermodynamically that the difference in free energy of ice and water increases with the degree of supercooling (Figure 1.9) (Kristiansen and Zachariassen, 2005). However, since ice crystals do not grow within the hysteresis gap, the antifreeze proteins must cause the energy equilibrium to be maintained throughout the hysteresis gap. Because ice crystals do not show any visible growth within the hysteresis gap there cannot be any net transfer of water molecules between the ice and liquid. This means that the AFPs must affect vapour pressure equilibrium during thermal hysteresis. The vapour pressure of a solid or liquid phase refers to the tendency of a molecule to leave that phase. The vapour pressure is lowered by a reduction in temperature and different phases of a substance have different vapour pressures. At the melting point there is ice-water energy equality. Therefore, at this temperature there is vapour pressure equilibrium and the rate of molecules joining the crystal is equal to the rate that they leave (Kristiansen and Zachariassen, 2005). This means that in the hysteresis gap, the vapour pressure of ice must be elevated to that of the surrounding solution or the vapour pressure of the water must be lowered to prevent ice growth.
Figure 1.9: Vapour pressure as a function of temperature. Within the hysteresis gap the AFP elevates the vapour pressure of ice to match that of the supercooled liquid. At the hysteresis freezing point the AFP can no longer maintain the vapour pressure and the liquid freezes. Within the hysteresis gap the AFP compensates exactly for the vapour pressure difference between water and ice (from Kristiansen and Zachariassen, 2005).

No single mechanism has yet been proposed that adequately explains how AFPs work (Davies and Sykes, 1997). One proposed mechanism is that AFPs structure or somehow immobilise water in a way that reduces the amount available for freezing. Although AFPs may bind slightly more water than other proteins of a similar size, it is has not been shown that they affect the structure of bulk water (Barrett, 2001). This means that AFPs must work by preventing ice nucleation or by preventing crystal growth. From a practical point of view, the prevention of ice nucleation is the most plausible since it removes any chance of uncontrolled (and damaging) ice growth. However, there is little evidence that AFPs have any ice nucleation inhibition activity (Hew and Yang, 1992). For organisms living at sub-zero temperatures, ice nucleation across cell membranes is an ever present danger and under these conditions it may be easier to control the growth of ice crystals rather than prevent nucleation (Barrett, 2001).
The most common explanations of how AFPs could control the growth of ice crystals rely on some form of adsorption inhibition mechanism. Raymond and DeVries proposed that by adsorbing irreversibly to the ice crystal surface the AFPs force the ice to grow out as convex fronts between them and it is this growth pattern that causes the antifreeze effect (Raymond and DeVries, 1977; Kristiansen and Zachariassen, 2005; Drori et al., 2014). There are two fundamentally different explanations as to how this convex ice growth elevates the free energy and therefore allows the supercooling of the solution. The first may be regarded as a consequence of the addition of high energy surface water molecules per unit of ice and the second as a consequence of the pressure build-up due to the convexity of the ice-water interface. Raymond and DeVries attributed the antifreeze effect of the convex interface to the increased surface area of the interface. If we assume that the surface water molecules obtain a greater free energy per unit than those in the bulk phase then the addition of ice at the convex surface may account for the ice-water energetic difference described previously (Raymond and DeVries, 1977; Kristiansen and Zachariassen, 2005). Another way of explaining this is using the Gibbs-Thomson model. This model uses an equation that shows that freezing hysteresis is directly proportional to the binding energy and inversely proportional to the radius of the ice crystal (Inada and Modak, 2006; Nada and Furukawa, 2008; Drori et al., 2014). By binding to the ice surface, the AFP prevents ice growth at that point and so further growth will have a radius that is less than the rest of the crystal i.e. the ice face becomes more curved (Figure 1.10). Because of the smaller radius of curvature, the growth front will have a freezing temperature that is lower than the bulk system (Barrett, 2001). Slight variations of this model have been proposed where the AFP adsorption is considered reversible and where the protein is treated as a polymer (Celik et al., 2013). Other models, where binding to different planes of ice results in different levels of hysteresis, have been proposed (Hew and Yang, 1992; Wilson, 1994).
**Figure 1.10: Possible arrangement of AFPs at an ice-water interface.** Where the AFPs bind to the ice surface they prevent further growth which results in the intervening spaces becoming more curved and therefore less likely to have water molecules bind to the ice phase (from Barrett, 2001).

One significant consequence of this model is that the binding of AFPs to the ice must be essentially irreversible (Kristiansen and Zachariassen, 2005). If the binding is reversible it will mean that there will be periods, even if they are very brief, when the water will be able to join the ice crystal at a lower energy level. This is because the greater distance between the AFPs will result in a larger radius of curvature and consequently less energy is required for the water to bind to the ice crystal and this will result in ice growth and this growth could potentially cover the other bound AFPs rendering them ineffective. However, if the binding is irreversible then an obvious problem is that all the AFP molecules will eventually end up in the ice and fewer and fewer will be left to prevent further freezing on new ice crystals (Kristiansen and Zachariassen, 2005). There is also not a linear relationship between AFP concentration and thermal hysteresis and beyond a certain AFP concentration there is very little increase in thermal hysteresis activity (Graham et al., 1997; Kristiansen and Zachariassen, 2005). Assuming the initial concentration of AFP is
above this upper concentration for maximum activity the loss of some AFP into the ice will not lower the AFP concentration enough to cause a noticeable change in thermal hysteresis (Graham et al., 1997; Kuiper et al., 2001; Marshall et al., 2004. In addition to this it has been shown that the AFPs are able to prevent freezing even at depleted concentrations (Celik et al., 2013).

There has been a great deal of research into identifying binding surfaces of AFPs. There are a two of common characteristics to most AFPs: they are all stable at or near 0 °C, and all putative ice binding sites are relatively flat, relatively hydrophobic and have a regular spacing of polar amino acids (Davies and Sykes, 1997; Barrett, 2001; Kandaswamy et al., 2011). Due to the wide variety of AFP sequences and structures these are only general features and not all are applicable to all AFPs. In many cases, the spacing of the polar residues matches the spacing of the water molecules along the particular ice plane that the AFP binds to, and in many cases a single side chain mutation leads to significant loss of antifreeze activity (Wen and Laursen, 1993; Graether et al., 2000; Hakim et al., 2013). The energetics of adsorption are not yet understood, but kinetic studies on AFP binding are consistent with an adsorption-mediated mechanism (Chapsky and Rubinsky, 1997). Several studies have shown a strong complementarity between the 2D array of polar amino acids (usually threonine) and the ice-binding face, suggesting that hydrogen bonding plays an important role in the ice-binding (Figure 1.11) (Sönnichsen et al., 1996; Pertaya et al., 2008; Hakim et al., 2013).
Figure 1.11: AFP structures displaying potential hydrogen bonds with the ice surface

A) Type III AFP bound to ice. A view nearly parallel to the ice face (on the left) the protein backbone is shown in red with the amino acid side chains of the ice binding face also displayed. The potential hydrogen bonds between the ice binding surface and the ice are shown in yellow (from Sönnichsen et al., 1996). B) Schematic diagram of type I AFP from winter flounder showing the potential hydrogen bonds (zigzag lines) between the conserved threonine residues and the oxygen atoms of the ice lattice (from Chou, 1991).

Recent experiments have brought into question the emphasis on hydrogen bonding as the key to ice binding. These experiments showed that substituting valine for threonine in type I AFP has less effect on activity than the substitution of serine for threonine (Chou, 1991). This suggests that shape is more important than charge as valine has a similar shape to threonine but no H-bonding capabilities, whereas serine has H-bonding ability but is a different shape. These results have led to a recent move to place more emphasis on surface-surface complementarity of the AFP and ice. This surface-surface complementarity may allow intimate van der Waals or hydrophobic interactions over a large area and these interactions may be complemented (but not dominated) by hydrogen bonds (Figure 1.12) (Jia and Davies, 2002; Hakim et al., 2013).
Figure 1.12: AFPs showing the surface complementarity between the AFP and ice. A) A surface presentation of *Tenebrio* AFP with the closest rank of water molecules (red spheres) showing their regularity and how well they fit into the AFP (from Liou *et al.*, 2000). B) The *Lolium perenne* AFP with the van der Waals surfaces displayed to highlight the complementarity of the protein with the ice molecules (blue spheres). A row of water molecules have also been added to the top of the AFP to show the possible dual sidedness of this AFP (from Kuiper *et al.*, 2001).

Another proposed mechanism suggests that the irreversible binding of the AFP to ice may be a two-step process (Kristiansen and Zachariassen, 2005). The reason a two-step process has been proposed is that there is no clear boundary between ice and water, but a 1-2 nm wide transition region that does not provide enough rigidity to support permanent binding (Kristiansen and Zachariassen, 2005). It is suggested that by reversibly covering a small area of the ice surface an AFP will prevent water leaving the covered region. Then, as the temperature drops the covered region will form a solid ice surface onto which the AFP can then bind essentially irreversibly.

A more recent proposal is that the AFP binds to ice via an ‘anchored clathrate’ system (Garnham *et al.*, 2011). This involves the ice-binding surface of the AFP binding and arranging water molecules in a pattern very similar to ice. Once these ice crystals are ordered in the ice-binding plane, the entire structure is able to recognise and bind to small ice crystals and prevent their growth. The structure of the ice binding surface of the AFP will determine the arrangement of the water molecules
and will in turn determine the plane(s) of ice the AFP can bind to and the ability of the AFP to depress freezing (Garnham et al., 2011; Nada and Furukawa, 2012).

Rather than affecting ice growth it may also be possible that AFPs protect organisms from freezing damage by other mechanisms as well e.g. protecting membranes. In plants it has been reported that β-1,3-glucanase (a cryoprotective protein) can protect thylakoids against freeze-thaw injury and at least three types of protein accumulate outside the cells during cold-acclimation (Hincha et al., 1997; Atici and Nalbantoglu, 2003).

1.4.2 Ice Nucleating Proteins

INPs are active in almost the opposite way to AFPs. When small volumes of water are cooled they do not usually freeze at 0 °C but supercool, sometimes by as much as 36 °C to a point where spontaneous nucleation occurs. INPs function to limit supercooling and reduce the temperature between freezing point and nucleation temperature to ensure that ice formation occurs in the extracellular fluid at fairly high sub-zero temperatures (Duman, 2001). Extensive supercooling can result in lethal intracellular ice formation even though ice may initially form in the extracellular fluid. INPs may also allow organisms to control where and when fluid freezes. This may be important in managing the formation of osmotic pressure or gradients to allow freezing in some compartments but not others e.g. only freezing in the gut but not the rest of the body (Duman, 2001). Most INPs that have been studied have come from bacteria and insects and their efficiency can vary considerably.

The first insect INP was isolated from a hornet Vespula maculata and is a 74 kDa protein (Duman and Patterson, 1978). Very little is known about this protein other than its amino acid composition, which indicates that it is a hydrophilic protein containing about 20 % glutamate and glutamine. Many of the other INPs that have been isolated are also large proteins that are predicted to be relatively hydrophilic.
It is believed that the hydrogen bonding abilities of the hydrophilic side chains could be important for organising water into embryo crystals on the surface of the INP that are then capable of causing the rest of the solution to freeze (Duman, 2001). Often it is necessary for the INPs to aggregate to be able to function effectively (Duman, 2001; Kawahara, 2002; Hartmann et al., 2013). This may be because only a small portion of the surface of the INP is able to bind/arrange water, so it may take many INPs working together to arrange enough water molecules to act as a seed crystal and freeze the solution.

1.3.3 Recrystallisation Inhibition Proteins

When ice initially forms it is a fairly homogenous structure consisting of a large number of small crystals (Figure 1.13A); however, over time when ice is held at high subzero temperatures the ice restructures itself resulting in a smaller number of large crystals (Figure 1.13B) in a process is known as recrystallization (Mazur, 1970; Knight et al., 1984). Recrystallisation occurs due to thermodynamics. It is energetically more favourable to have a smaller number of large crystals than a larger number of small crystals, so over time some of the water molecules will leave the smaller crystals and move to the larger crystals. This results in the larger crystals growing and the smaller crystals shrinking and disappearing (Knight et al., 1988; Wharton et al., 2007; Budke et al., 2009).

Figure 1.13: Effect of recrystallisation on crystal size. A) 25 mM Tris buffer (pH 8.0) solution immediately after freezing B) the same solution after 30 min at -8 °C. Scale bar = 100 µm
As their name suggests recrystallisation inhibition proteins (RIPs) prevent recrystallisation occurring and have only been identified as a separate class of IAPs recently (Wharton *et al.*, 2005). All AFPs also have recrystallisation inhibition activity. Before their classification, RIPs were considered to be AFPs with very low levels of thermal hysteresis. More recently it has been acknowledged that the very low level of thermal hysteresis of these proteins (usually < 0.1 °C) is likely to have no relevance to their biology since the temperatures (and hence the level of supercooling) that these organisms would encounter is much lower than what the protein can protect against (Tursman *et al.*, 1994; Yamashita *et al.*, 2002; Hoshino *et al.*, 2003). Despite the lack of thermal hysteresis, these proteins are very potent inhibitors of recrystallisation (hence the name). Recrystallisation inhibition proteins are capable of preventing the change in crystal structure over time and the ice maintains its small crystal size almost indefinitely (Wharton *et al.*, 2005). Recrystallisation inhibition proteins are thought to enhance survival by preventing the growth of large ice crystals that are likely to damage cell structures and membranes and they may also be important in controlling the shape and location of ice crystals (Ramløv *et al.*, 1996).

Natural RIPs have so far only been reported in plants and *P. davidii* (Kuiper *et al.*, 2001; Wharton *et al.*, 2005). However, this category can be expected to grow as many proteins with low thermal hysteresis previously classified as AFPs are reclassified as RIPs. Because of the low number of RIPs so far identified, they have yet to be purified and nothing is known about their structure. Their mechanism of action is also a mystery; it had been assumed that it would be similar to that of AFPs and involve some variation of the proposed adsorption-inhibition mechanism.
1.3.3.1 Mechanism of Recrystallisation

Recrystallisation of ice is believed to occur through one of two mechanisms; grain boundary migration or Ostwald ripening.

Grain boundary migration in ice is proposed to be similar to grain boundary migration in metals, where large ice grains grow at the expense of smaller ice grains. In ice a grain consists of the crystallographic orientation of water molecules observed in ice I\textsubscript{h}. This leads to different grains having different orientations and the grain boundaries are the interfaces between these (Knight, 1966). Grain boundary migration occurs as individual water molecules migrate from grains with unfavourable orientation to those with favourable orientation. The determination of favourable or unfavourable is based on the curvature of the ice grain, with less curvature being more favourable. Smaller crystals have a higher degree of curvature (thus higher amount of surface energy), whereas large crystals have a lower degree of curvature (thus lower amount of surface energy). Due to the physical need to reduce the amount of energy in a system, water molecules will migrate from smaller crystals (high energy) to larger crystals (lower energy), resulting in an overall decrease in the energy in the system (Alley et al., 1986). The mechanism assumes the water molecules move from the shrinking ice grain to growing grain, which generally ignores the presence of bulk water or the quasi-liquid layer between the grains, which is an acceptable assumption if the temperature is below −10 °C, however this is not always the case in nature (Alley et al., 1986).

The second possible mechanism is Ostwald ripening. This mechanism considers the entire ice crystal/liquid water system as a whole and so accounts for the presence of bulk water and the quasi-liquid layer. Ostwald ripening is a thermodynamically driven process where large crystals grow at the expense of small crystals, resulting in an overall reduction in energy of the ice crystal/bulk water interface, but not a change in the ice volume i.e. the total amount of ice is the same throughout Ostwald ripening (assuming constant temperature) (Sutton et al., 1996; Budke et al., 2009). Due to the higher surface area to volume ratio of smaller ice crystals they have a higher surface free energy, which means that water molecules on the surface of
these crystals are less stable. This means that these water molecules are more likely to leave these small crystals and join the quasi-liquid layer and from here migrate to larger ice crystals, which due to the lower surface area to volume ratio are more stable (Sutton et al., 1996; Hagiwara et al., 2006)). Overall this results in an increase in the average ice crystal size, a decrease in the total number of ice crystals and an overall reduction in the free energy of the system (Budke et al., 2009).

Because of the high sub-zero freezing temperatures generally encountered in nature there will be a quasi-liquid layer in biological systems and therefore Ostwald ripening provides the better explanation of how recrystallisation could occur in these systems.

1.3.3.2 Recrystallisation Inhibition

The mechanism of recrystallisation is still not understood despite a recent increase in the discovery of compounds with this function. Initially it was assumed that there was a link between the thermal hysteresis and recrystallisation inhibition activities of AFPs and the both properties were related to their ability to bind to ice and early studies supported this link (Wen and Laursen, 1992, Knight et al., 1995). However, many plant AFPs have a low level of thermal hysteresis and high levels of recrystallisation inhibition and many insect AFPs have high thermal hysteresis activity with minimal recrystallisation inhibition, both of which suggest these activities are distinct even if they share a common mechanism (Sidebottom et al., 2000; Pudney et al., 2003; Yu et al., 2010).

Further studies looking at the relationship between glycopeptide/glycoprotein mass on thermal hysteresis and recrystallisation inhibition activity using synthetic AFGPs showed that activities were able to be partially separated (Wilkinson et al., 2012). This study created a family of synthetic AFGPs with varying numbers of tripeptide repeats, that, depending on the number of repeats, increased or decreased both thermal hysteresis and recrystallisation inhibition activity, although not always together; i.e. some combinations had lower thermal hysteresis and higher
recrystallisation inhibition activity when compared to other combinations. This provides further support to the hypothesis that these activities are not as closely linked as initially thought and further more may lead to the development of ‘custom-tailored’ compounds (Liu and Ben, 2005; Czechura et al., 2008; Wilkinson et al., 2012).

The ability to separate the thermal hysteresis and recrystallisation activities of AFPs is interesting, but does very little to explain the mechanism of recrystallisation inhibition. The first insights into a possible mechanism came when small molecules (carbohydrates) were investigated for recrystallisation inhibition activity (Tam et al., 2008). Further work on this found a link between the hydration number of the carbohydrate and recrystallisation inhibition activity. The hydration number of a carbohydrate is related to the number of tightly bound water molecules in an aqueous solution (Tam et al., 2008). This has led to a proposed mechanism for recrystallisation inhibition based on the compatibility of the solute with bulk water. As mentioned previously for recrystallisation to occur water molecules need to move from the quasi-liquid layer into the bulk water and then onto a growing quasi-liquid layer. The proposed mechanism suggests that the recrystallisation inhibitor occupies space in the bulk water and disrupts the hydrogen-bonding network of the bulk water (the amount of disruption depends on the hydration number, with a higher value resulting in more disruption). Due to the disruption of this network the amount of energy required for a water molecule to move across the bulk water layer is increased and is therefore energetically unfavourable, thus preventing recrystallisation (Tam et al., 2008). While further work is required to confirm this mechanism, it does explain how recrystallisation inhibition could occur without the molecule binding to ice, as observed for these carbohydrates.
1.4 Biotechnology

There is considerable interest in the possible medical and commercial applications of IAPs. Amongst their many possible uses are as protective agents in cryogenic and/or hypothermic storage of whole organs, tissues or cell lines; improvement of cold tolerance in plants; maintenance of the texture of frozen food; and targeting tissues during cryo-surgery (Barrett, 2001; Lillford and Holt, 2003; Prathalingam, 2006; Bang et al., 2013). Much of the early work with cryogenic preservation involved the use of small molecules e.g. glycerol or dimethyl sulfoxide. However, these often caused damage themselves and were only effective to temperatures of about -20 °C (Bang et al., 2013). More recently there has been interest in looking at using AFPs as cryoprotective agents. AFPs may be more suitable for this function because they are proteins and therefore more ‘natural’ than some of the other cryoprotectants currently in use i.e. some of the problems of the currently used small molecules e.g. dimethyl sulfoxide will be overcome using AFPs. They also may be able to be used at much lower concentrations and this may limit damage caused by the cryoprotectant itself. Despite all the possible advantages in using AFPs, there is still much basic research that needs to be carried out, especially in the complex areas of whole organ preservation and improving cold-hardiness.

Many attempts at using AFPs to improve cold tolerance so far have involved injecting or surrounding the tissue or organ with exogenous AFP, which has led to some very mixed results (Cutler et al., 1989; Fletcher et al., 1997; Wu et al., 1998). Several studies have shown an improvement in organ storage due to the addition of AFPs. These include rat livers being preserved by AFGP perfusion before storage and hearts being stored at sub-zero temperatures using either Type I or Type III AFPs (Lee et al., 1992; Magnano et al., 1995; Amir et al., 2003).

The failure of other AFPs to confer freeze resistance may be for a number of reasons. The membrane lipid composition may be different and limit protein/membrane interactions; the protein may be acting as an ice nucleator; or the protein may need some cofactor or potentiating protein that is not present in the new host (Larese et al., 1996; Barrett, 2001; Bang et al., 2013).
More recently, advancements in cloning and identification of IAP genes has led to transgenic expression of IAPs becoming a favoured way of introducing them into new organisms (Kenward et al., 1993; Wallis et al., 1993; Nicodemus et al., 2006). In some cases the introduction of an IAP has resulted in an increase in freeze tolerance. A beetle (*D. canadensis*) AFP was introduced into *Drosophila* and this led to a thermal hysteresis value as high as 6.2 °C in the *Drosophila* hemolymph. This increase in hemolymph thermal hysteresis resulted in greater supercooling values i.e. ice nucleation occurred at lower temperatures, and more importantly, the insect was able to prevent inoculative freezing across the cuticle and freezing initiated by endogenous ice nucleators. Together these led to an increase in cold tolerance of the transgenic *Drosophila* (Nicodemus et al., 2006). While the thermal hysteresis gap caused by the *D. canadensis* AFP in *Drosophila* is not as large as in *D. canadensis* itself, it clearly shows that a functional AFP is being produced in the transgenic *Drosophila* and that this approach has potential as a method for introducing IAPs into organisms where they are not normally found.

While the introduction of the *D. canadensis* AFP gene into *Drosophila* was successful, there have been other cases where there has been no effect on cold tolerance (Duncker et al., 1996). There may be a number of reasons for the lower or lack of activity of the AFP; the gene dosage may be much lower than in the native source; it may be that multiple types of IAP complement each other to produce the native level of activity; the new organism may lack the necessary processing enzymes to produce a functional final protein; or the location of expression may be important e.g. tissue specific (Duncker et al., 1996; Barrett, 2001).
1.5 Aims of this Research

This research is important due to the lack of understanding about cold tolerance in general and in particular intracellular freezing. Currently, *P. davidi* is the only animal known to be able to survive this stress and intracellular freezing is considered lethal to all other animals. There must be something very special about the biology of this organism. While general details about how *P. davidi* is able to survive freezing are known e.g. how cooling rate affects cold tolerance mechanisms, very little is known about the underlying processes. By investigating the proteins involved in freeze tolerance of *P. davidi* a better understanding of how freezing tolerance works may be developed.

From a more general point of view, RIPs have only recently been recognised as a class of IAPs, and because of this very little is known about them. So far no RIPs have been successfully purified and nothing is known about either their structure or mechanism of action. All that is certain is that they impart negligible thermal hysteresis and are able to inhibit recrystallisation. Because of this anything we are able to find out about RIPs would be valuable in understanding how they work and their roles in freeze survival.

It is very likely that freeze survival is a multi-faceted process involving many different levels of protection and the interaction of many different proteins and other compounds. By comparing acclimated and non-acclimated proteomes we may be able to get some clues to the extent of the changes required for freeze tolerance and the number of processes and proteins involved.
1.5.1 Overall Aims of this Research

The overall aim of this research was to increase our understanding of the proteins responsible for freeze tolerance in *P. davidii* and in particular to find out more about the proteins involved in the freeze survival of *P. davidii* and in particular the proteins responsible for recrystallisation inhibition.

1.5.2 Specific Aims of this Research

1. To characterise the ice binding substances in *P. davidii* (Chapter 3)
2. To develop a method suitable for screening a large number of samples for putative RI activity simultaneously (Chapter 4)
3. To determine some basic properties of the protein(s) responsible for RI activity and attempt to purify them using column chromatography (Chapter 5)
4. To Compare the proteome of acclimated and non-acclimated *P. davidii* (Chapter 6)
Chapter Two
Materials and Methods

2.1 Materials

2.1.1 Chemicals

*Product information is listed according to manufacturer’s name at the time of use.*

Tris-hydroxymethylaminomethane (Tris), glycine *AppliChem, Germany*

Sodium dodecyl sulfate (SDS), *Fisher Scientific, USA*

Sodium chloride, ammonium sulfate, glycerol, acetic acid, ethylenediaminetetraacetic acid (EDTA), methanol, *Scharlau, Spain*

Complete™ protease inhibitor, nicotinamide adenine dinucleotide (NADH), Proteinase K *Roche, Germany*

Imidazole, *Merck, Germany*

Pyruvate, bovine serum albumin (BSA), copper sulfate, BCA reagent, silver nitrate *Sigma, USA*

Acrylamide, ammonium persulfate, IPG Isoelectric focusing (IEF) strips, IEF buffer concentrate, *BioRad, USA*


No. 1 filter paper, *Whatman Ltd., England*

IEF buffer concentrate, *GE Healthcare, USA.*

Bromophenol blue, *Hopkin & Williams, England.*

Sodium carbonate, *Univar, USA*

All other chemicals were reagent grade, *Sigma, USA*
2.1.2 Equipment

Nanolitre osmometer and optical recrystallometer, Otago Osmometers, New Zealand.

_P. davidi_ cultures were incubated in a Contherm Thermotec 2000 oven, Contherm, New Zealand

BeadBeater and glass beads, Biospec Products Inc., USA

Cary 50 BioUV/Vis spectrophotometer, Varian Inc., USA

pH measurement with a Jenway 3540 pH meter, UK

Weighing of dry chemicals was carried out with Sartorius top pan balance. Schott Gerate, Germany.

Beckman Coulter microfuge®16 centrifuge, Beckman Coulter, USA

Medium quantities of liquid were pipetted with Gilson pipetman pipettes, Gilson, USA

Concentration of solution via a cold trap and vacuum pump was carried out with Speedvac® SC110. Savant, USA.

Type I water (18 MOhm) from a MilliQ water filtration system. Millipore, USA.

A BioRad GS-800 densitometer with Quantity One software was used to scan gel images.

Electrophoresis of mini gels was carried out with Mini-Protean II Cell tank. BioRad, USA.

Power to provide separation in all 1D electrophoresis experiments was provided by Power 300 Pac. BioRad, USA.

All samples were stored at -80 °C in a Revco ultra-freezer between experiments. Revco, USA.

Working samples were kept at -20 °C in a S300 freezer. Fisher and Paykel, New Zealand.

Separation in all large format 2D electrophoresis experiments was done by an Electrophoresis EPS 601, GE Healthcare, Sweden.

All gels were sealed in plastic for storage using a TISH-400 Impulse Sealer. TEW Electric Company, Taiwan.
Gels were more clearly visualised using a Mighty Bright Visible light box. Hoefer, USA.
Proteins were focused using an IPG phor. Pharmacia Biotech, USA.
Large format 2D electrophoresis was carried out in Ettan DALTsix, GE Healthcare, USA.
Temperature controlled water bath. Grant, USA.
Haake F3 or F8 refrigerated circulators were used for cooling samples for CFIB, splat and optical recrystallometer, ThermoScientific, USA.
A Zeiss Axiophot Photomicroscope was used for splat assays, Zeiss, USA.
Images of the splats were captured using a Powershot A640 AiAF camera, Canon, Japan.
Optical recrystallometer data were converted using a MacLab4 analog.digital converter connected to a Macintosh Classic, Apple Computer, USA.
Leica GZ6 binocular microscope, Leica, Germany.
Chromatography was carried out using the ÄKTA Prime plus chromatography system, GE Healthcare, UK.
A range of HiTrap™ Sephadex chromatography columns were used for ion exchange and hydrophobic interaction chromatography and a Superdex™ 75 HR10/30 column was used for gel permeation chromatography, GE Healthcare, UK.
Solutions were degassed using a V-700 vacuum pump. Bucchi, Switzerland.
2.2 Buffers and Solutions

All buffers and solutions were made up to volume with ASTM type I water (Type I water). Specifications of ASTM type I water are available on Wikipedia – purified water (http://en.wikipedia.org/wiki/Purified_water)

Ammonium bicarbonate 1×
\[ \text{NH}_4\text{HCO}_3 \quad 50 \text{ mM} \]

Ammonium bicarbonate 2×
\[ \text{NH}_4\text{HCO}_3 \quad 100 \text{ mM} \]

Anion exchange chromatography buffer A
\[ \text{Tris-HCl} \quad 25 \text{ mM} \]
\[ \text{pH} \; 8.0 \]

Anion exchange chromatography buffer B
\[ \text{Tris-HCl} \quad 25 \text{ mM} \]
\[ \text{NaCl} \quad 1 \text{ M} \]
\[ \text{pH} \; 8.0 \]

Artificial tap water
\[ \text{NaCl} \quad 20.45 \text{ mg} \]
\[ \text{KCl} \quad 3.28 \text{ mg} \]
\[ \text{CaCO}_3 \quad 100.09 \text{ mg} \]
\[ 4(\text{MgCO}_3)\text{.Mg(OH)}_2\text{.5H}_2\text{O} \quad 45.98 \text{ mg} \]
Make to 1 L with Type I water and bubble CO\textsubscript{2} through the solution for > 30 min to dissolve the carbonates. Then bubble air through the solution for 30 min to oxygenate and then check pH is approximately 7.0.

Cation exchange chromatography buffer A
\[ \text{Tris-HCl} \quad 25 \text{ mM} \]
\[ \text{pH} \; 6.0 \]
Cation exchange chromatography buffer B

- Tris-HCl 25 mM
- NaCl 1 M
- pH 6.0

Colloidal Coomassie stain

- $\text{Al}_2(\text{SO}_4)_3$ 50 g
- Dissolved in 50 mL ortho-phosphoric acid then
- Ethanol 50 mL
- Coomassie G-250 1.2 g
- Type I water 900 mL
- Mix overnight

Gel permeation buffer

- Tris-HCl 25 mM
- NaCl 100 mM
- pH 8.0

Hydrophobic interaction chromatography buffer A

- $(\text{NH}_4)_2\text{SO}_4$ 1 M
- Tris-HCl 25 mM
- pH 8.0

Hydrophobic interaction chromatography buffer B

- Tris-HCl 25 mM
- pH 8.0

Homogenisation buffer

- 1 Complete™ Protease Inhibitor tablet dissolved in 10 mL Tris-HCl pH 8.0

Isoelectric focusing rehydration buffer

- Urea 7 M
- Thiourea 2 M
- CHAPS 2 %
- DTT 50 mM
- TCEP 4 mM
Isoelectric focusing strip alkylation solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>3.6 g</td>
</tr>
<tr>
<td>IAA</td>
<td>250 mg</td>
</tr>
<tr>
<td>SDS-PAGE resolving buffer</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Glycerol (50 %)</td>
<td>4 mL</td>
</tr>
<tr>
<td>Type I water</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

Isoelectric focusing strip reducing solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>3.6 g</td>
</tr>
<tr>
<td>DTT</td>
<td>200 mg</td>
</tr>
<tr>
<td>SDS-PAGE resolving buffer</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Glycerol (50 %)</td>
<td>4 mL</td>
</tr>
<tr>
<td>Type I water</td>
<td>1 mL</td>
</tr>
</tbody>
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LDH assay cocktail

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>80 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>100 mM</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>2 mM</td>
</tr>
<tr>
<td>NADH</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
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</tbody>
</table>

NGM Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>25.5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>4.5 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>3.75 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.5 L</td>
</tr>
</tbody>
</table>

Potassium citrate pH 6.0

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid monohydrate</td>
<td>20 g</td>
</tr>
<tr>
<td>Tri-potassium citrate monohydrate</td>
<td>293.5 g</td>
</tr>
</tbody>
</table>

Make to 1 L with Type I water and check pH is 6.0. Sterilise by autoclaving

S Basal

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5.85 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1.0 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Cholesterol (5 mg·mL$^{-1}$)</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

Make to 1 L with Type I water and sterilise by autoclaving
S Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S Basal</td>
<td>1 L</td>
</tr>
<tr>
<td>Potassium citrate pH 6.0</td>
<td>10 mL</td>
</tr>
<tr>
<td>Trace metal solution</td>
<td>10 mL</td>
</tr>
<tr>
<td>CaCl₂ (1 M)</td>
<td>3 mL</td>
</tr>
<tr>
<td>MgSO₄ (1 M)</td>
<td>3 mL</td>
</tr>
</tbody>
</table>

Add all components using sterile technique and do not autoclave.

2× SDS-PAGE Sample buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>250 mM</td>
</tr>
<tr>
<td>SDS (w/v)</td>
<td>4 %</td>
</tr>
<tr>
<td>Glycerol (v/v)</td>
<td>20 %</td>
</tr>
<tr>
<td>pH 6.8</td>
<td></td>
</tr>
</tbody>
</table>

To make 1× sample buffer with 2 MCE mix 100 µL of 2× sample buffer, 90 µL Type I water and 10 µL 2MCE.

SDS-PAGE electrode buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>25 mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>250 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 %</td>
</tr>
</tbody>
</table>

SDS-PAGE loading buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>62.5 mM</td>
</tr>
<tr>
<td>Imidazole</td>
<td>62.5 mM</td>
</tr>
<tr>
<td>Glycerol (v/v)</td>
<td>10 %</td>
</tr>
<tr>
<td>SDS (w/v)</td>
<td>2 %</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>0.0025 %</td>
</tr>
<tr>
<td>pH 6.8</td>
<td></td>
</tr>
</tbody>
</table>

SDS-PAGE resolving gel buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>3 M</td>
</tr>
<tr>
<td>pH 8.9</td>
<td></td>
</tr>
</tbody>
</table>

SDS-PAGE stacking gel buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>62.5 mM</td>
</tr>
<tr>
<td>Imidazole</td>
<td>62.5 mM</td>
</tr>
</tbody>
</table>
Both solutions are adjusted to pH 6.8 and then combined 1:1 to make the final SDS-PAGE stacking gel buffer.

Silver nitrate solution
\[ AgNO_3 \quad 0.1 \% \]

Silver stain developing solution
- Formaldehyde 0.04 %
- \( Na_2CO_3 \) 2 %

Silver stain fixing solution
- MeOH 50 %
- HAc 5 %

Silver stain sensitising solution
- \( Na_2S_2O_3 \) 0.02 %

Trace metal solution
- Disodium EDTA 1.86 g
- \( FeSO_4.7H_2O \) 0.69 g
- \( MnCl_2.4H_2O \) 0.2 g
- \( ZnSO_4.7H_2O \) 0.29 g
- \( CuSO_4.5H_2O \) 0.025 g

Make to 1 L with Type I water and sterilise by autoclaving
2.3 Methods

2.3.1 *Panagrolaimus davidi* Culturing and Collection

The *P. davidi* used throughout this project were all cultured in the Department of Zoology, Otago University, Dunedin, New Zealand. The original wild samples were collected from McMurdo Sound region in Antarctica (Wharton and Brown, 1989). There were two different culture techniques employed; egg plates and liquid culture. In both cases *P. davidi* were grown at 20 °C and fed *E. coli* OP50.

2.3.1.1. Egg Plates

Sterilised NGM agar was poured into a Nalgene plastic plate (approximate dimensions 50 cm × 10 cm × 10 cm) to a depth of approximately 1 cm (this was approximately 375 mL of agar) and left to set. Once the agar was set, 3 chicken eggs (Farmer Brown, size 6) were sterilised by spraying and wiping with ethanol. The eggs were broken and the contents added to 100 mL of boiling water and mixed vigorously for 5 min on medium heat. After 5 min the mixture was left to cool. This mixture was then spread evenly over the set NGM agar (4 plates were done at each time). Once the egg mixture had cooled further, 10 mL of overnight *E. coli* OP50 culture and approximately 25,000 *P. davidi* were added to each plate. The seeded cultures were then incubated at 20 °C for 2 weeks.

2.3.1.2 Liquid Cultures

The liquid culture technique uses a method adapted from Sulston and Hodgkin (1988). The culture consisted of a wild-type strain of *E. coli* (as a food source) at a concentration of approximately $10^7$ cfu·mL$^{-1}$ in 100 mL of S medium. To this approximately 100,000 *P. davidi* (final concentration 1000 nematodes·mL$^{-1}$) were added and the cultures were incubated at 20 °C with shaking at 300 rpm. The cultures were checked regularly and if the culture appeared clear further *E. coli* were added to ensure that the *P. davidi* had adequate food (Wharton, 2000).
2.3.1.3 *P. davidi* Collection

For both egg plates and liquid cultures *P. davidi* were collected in a similar way. However, due to the larger volumes with the egg plates, the method was scaled up. For egg plates, the agar was tipped into tissue paper that was surrounded by tap water. The nematodes migrate through the tissue paper and into the tap water when left overnight. After sitting overnight, the water was transferred to large measuring cylinders where the *P. davidi* were then left to settle (for not longer than 4 h). The excess liquid was removed using a suction pump. Once the volume was reduced to approximately 200 mL, *P. davidi* were separated by allowing them to migrate through tissue paper into fresh tap water. The *P. davidi* were then further concentrated by centrifugation (1,500 ×g, 5 minutes, three times) and washed using artificial tap water. Nematodes were either used immediately, stored at -18 °C for short term storage, or at -80 °C for longer term storage.

2.3.2 *Tenebrio molitor* Collection and Acclimation

*T. molitor* larvae were supplied by the Department of Zoology, University of Otago, where they had been reared on wholegrain flour with small pieces of fruit to provide the necessary moisture. Third instar larvae were collected from this main population using tweezers. The selected larvae were then transferred to a smaller plastic container that contained wholegrain flour and a damp tissue paper to provide moisture. This container was then kept in a 4 °C incubator for 2 weeks to allow the larvae to acclimate before use.

2.3.3 Homogenate Production

2.3.3.1 *Panagrolaimus davidi*

*Panagrolaimus davidi* were harvested and placed in a 15 mL polypropylene tube (total volume of *P. davidi* was approximately 4 mL) and made up to 10 mL with ATW. The nematodes were washed with 40 mL homogenisation buffer and transferred to a 50 mL BeadBeater (BioSpec Products Inc.) chamber half filled with 0.1 mm diameter glass beads. The chamber was topped up with 25 mM Tris-HCl pH 8.0 to exclude all
The chamber was surrounded with ice and the nematodes were beaten for three 1 min bursts with 1 min between each burst. The beads were allowed to settle and the supernatant collected. The total volume was made up to 140 mL with 25 mM Tris-HCl pH 8.0. The supernatant was then centrifuged at 10 000 × g for 5 min. The supernatant from this centrifugation was the *P. davidii* homogenate.

For smaller volumes of nematodes, the *P. davidii* homogenate was produced using a glass homogeniser. The *P. davidii* (typically about 1 mL) were placed in a glass homogeniser and then 1 mL homogenisation buffer was added. The *P. davidii* were then homogenised on ice for 15 min. The solution was collected and the homogenisation chamber rinsed with the rest of the homogenisation buffer. The solution was then centrifuged at 10,000 ×g for 5 min. The supernatant was collected and used as the *P. davidii* homogenate.

### 2.3.3.2 Tenebrio molitor

Third instar *Tenebrio molitor* larvae were acclimated at 4 °C for 2 weeks with sufficient food and water (Graham *et al.*, 2000). The front section of each larva including the head and legs was removed, the remaining section of the larva was homogenised in 1 mL homogenisation buffer using a glass homogeniser. Once all larvae had been homogenised the glass homogeniser was washed with the remaining homogenisation buffer. The homogenate was centrifuged at 10 000 ×g for 5 min. After centrifugation the supernatant from below the fat layer was collected. This is the *Tenebrio* homogenate.

### 2.3.3.3 Grass Extract

Grass extracts were prepared from a mixture of *Festuca nigrescens*, *Agrostis tenuis* and *Lolium* sp. collected from a suburban lawn in Dunedin, New Zealand (45.90 ° S, 170.48 ° E). Chlorophyll was removed from freeze-dried grass with cold acetone and the soluble proteins extracted with 25 mM ascorbic acid, 50 mM ammonium bicarbonate, pH 8.0 (20 mL per gram of dried grass). The filtered supernatant was heated to 95 °C for 15 min and the soluble fraction was then freeze-dried and stored.
at -20 °C (Wharton et al., 2007). A solution of this extract could then be made up and filtered through Whatman No 1 filter paper. The filtrate is the grass extract.

### 2.3.4 Recrystallization Inhibition (Splat) Assay

Recrystallization inhibition was tested using a splat freezing assay (Knight et al., 1988; Ramløv et al., 1996). This involved dropping a 10 µL drop of test solution approximately 3 metres onto an aluminium block cooled to −78 °C by dry ice. A section of the resulting thin layer of ice was then transferred to a microscope cold stage (approx. −20 °C), mounted on a Zeiss Axiophot Photomicroscope (Zeiss). The temperature was raised to −8 °C for 30 min and then rephotographed. The largest diameter of the 10 most prominent (which were usually the largest) ice crystals were measured using Image J photo analysis software (Rasband 1997-2005).

The recrystallization inhibition of the extract was determined as the mean ice crystal diameter of the 10 most prominent ice crystals after annealing at −8 °C for 30 min.

Previous work in the Marshal lab has shown that to confirm RI activity it is necessary to carry out this assay on a 2-fold dilution series of the sample. To prevent false positives a sample must show activity at 4-fold dilution to be considered as having RI activity.
Figure 2.1: Equipment for splat assay. A) Aluminium block, scalpel and tweezers cooled to -78 °C in dry ice. B) Dropping sample (10 µL) down the tube (approximately 3 m) onto an aluminium block to generate thin layer of ice that can be transferred to cold stage microscope. C) Cold stage microscope with nitrogen (red tube on left) entering the sleeve surrounding the cold stage itself (middle) that is temperature controlled by a refrigerated circulator (black tube on right). Images were captured using a Powershot A640 AiAFcamera (Canon) (not shown).
Figure 2.2: Typical photos of various solutions obtained using the splat assay. A) Tris buffer (25 mM pH 8.0) B) P. davidii homogenate C) 4-fold dilution of P. davidii homogenate D) 16-fold dilution of P. davidii homogenate. Scale bar is 100 µm.

2.3.5 Cold Finger Ice Binding

Cold finger ice binding (CFIB) was performed using a cold finger first described by Kuiper et al., (2003) and connected to a Haake F8-C35 refrigerated circulator (Thermo Scientific). The initial temperature of the sample was brought close to 0 °C by placing on ice. The fraction before freezing is termed the starting material (SM). Once the sample was ready for freezing the cold finger was cooled to −0.5 °C and coated with a thin layer of ice by immersing it in cold distilled water and seeding with ice crystals. Once a thin ice layer had formed, the cold finger was placed into 100 mL of the starting material, in a plastic beaker insulated with polystyrene, on a magnetic stirrer. The refrigerated circulator lowered the temperature from −0.5 °C to −2.5 °C over a 20 h period. After the 20 h freezing period, the remaining liquid portion was
removed for a second round of fractionation. It was made up to 100 mL with 25 mM Tris-HCl pH 8.0 and subjected to a second round of freezing under the same conditions. The liquid portion left after the second round of ice binding was kept and labelled as first liquid fraction (LF1). The ice portions were melted, combined and made to 100 mL (using 25 mM Tris pH 8.0), if necessary. This combined fraction was then subjected to another round of ice binding using the same conditions as previous rounds of ice binding (−0.5 °C to −2.5 °C over 20 h). The liquid fraction from this round was labelled as second liquid fraction (LF2) and the ice fraction was labelled second ice fraction (IF2).

**Figure 2.3: Schematic diagram of the cold finger ice binding set up.** The cold finger (left) is a hollow brass tube with a smaller brass tube inside it that allows the fluid from a refrigerated circulator to cycle through it. The cold finger is lowered into an insulated beaker of sample on a stirrer (right) and the temperature slowly lowered over 20 h (from Kuiper et al., 2003).
Figure 2.4: Schematic overview of the cold finger ice binding procedure highlighting the fractions kept for analysis.

Figure 2.5: Ice hemisphere generated using CFIB after second round of ice binding (IF2)
2.3.6 Protein Assay

The BCA assay was used to determine the protein concentration of samples (Smith et al., 1985). Protein standards across the range 0 – 1.0 mg·mL$^{-1}$ were made using BSA. Fifty microliters of the unknown or 50 µL of standard was placed into microcentrifuge tubes, 950 µL of BCA standard working reagent was added to each tube and mixed. This mixture was incubated at 37 °C for 30 min and the $A_{562}$ was recorded. The concentrations of the unknowns could then be calculated from the standard curve.

2.3.7 Intact Mass Spectrometry

The sample was prepared for mass spectrometry analysis by drying and resolubilisation in 30 % (v/v) acetonitrile and 0.1 % (v/v) trifluoroacetic acid (TFA) in water. One microlitre of sample was pre-mixed with 1 µL of matrix (10 mg·mL$^{-1}$ alpha cyano-4-hydroxycinnamic acid dissolved in 65 % (v/v) aqueous acetonitrile containing 0.1 % TFA). A 0.8 µL aliquot was spotted onto a MALDI sample plate (Opti-TOF 384 well plate, Applied Biosystems MA) and air dried. Samples were analysed on a 4800 MALDI tandem Time-of-flight Analyzer (MALDI TOF/TOF, Applied Biosystems). All MS spectra were acquired in positive-ion mode with 800-1000 laser pulses per spot. The 15-20 strongest precursor ions of each sample spot were used for MS/MS collision-induced dissociation analysis. Collision-induced dissociation spectra were acquired with 2000-4000 laser pulses per selected precursor using the 5 kV mode and air as the collision gas at a pressure of 1×10$^{-6}$ torr.

2.3.8 PAGE Protein Analysis

Two different types of PAGE analysis were used. The first was 1D SDS-PAGE that separated proteins based on size. The second was large format 2D-PAGE. This separated proteins based on isoelectric point (pI) in the first dimension and then on size in the second dimension.
2.3.8.1 1D SDS-PAGE Gel Preparation

The gels were made in batches of two using the BioRad Protean II mini gel system. The 10 % resolving gel was prepared by combining 3.12 mL of 40 % Bis/acrylamide (BioRad), 1.43 mL of resolving gel buffer, 0.13 mL 10 % SDS and 6.73 mL Type I water. The resolving gel was mixed and the polymerising agents, 40 µL of freshly made 10 % APS and 10 µL TEMED, were added. The resolving gel solution was pipetted between glass plates until the solution reached approximately 20 mm from the top of the plates. The gel was then overlaid with approximately 1 mL water-saturated n-butanol to remove the meniscus effect and exclude oxygen. The gel was left at room temperature until it was set (approximately 30 min). The n-butanol overlay was rinsed off with distilled water prior to adding the stacking gel.

The 5 % stacking gel was prepared by combining 0.5 mL of 40 % Bis/acrylamide (BioRad), 2.0 mL stacking gel buffer and 1.5 mL Type I water. The stacking gel was mixed and the polymerising agents, 20 µL of freshly made 10 % APS and 10 µL TEMED, added. The stacking gel solution was pipetted onto the resolving gel until reaching the top of the plates. Immediately after pouring, a 10 well comb was inserted in the top of each gel. The gel was left at room temperature until set (approximately 20 min). The gels were either used immediately or stored at 4 °C in a sealed container containing a damp paper towel until use.

2.3.8.2 1D SDS-PAGE Analysis of Protein Samples

The samples for analysis by SDS-PAGE were prepared in one of two ways depending on the amount of protein in the sample. If the amount of protein was high, 10 µL of sample was mixed with 10 µL of 1× sample buffer with 2MCE and heated at 95 °C for 5 min and allowed to cool.

If the amount of protein in the sample was expected to be low, a volume of the sample was freeze-dried until dry. The pellet was resuspended in 20 µL of 1× sample buffer with 2MCE, heated at 95 °C for 5 min and allowed to cool. After the sample had cooled to room temperature 5 µL of loading buffer was added and 15 µL of this
was loaded onto the gel. If the sample had a higher protein concentration 10 µL of sample was mixed with 10 µL of 1x sample buffer with 2MCE, heated at 95°C for 5 min and allowed to cool. After the sample had cooled to room temperature, 5 µL of loading buffer was added and 15 µL of this was loaded onto the gel. The samples were initially electrophoresed at 15 mA per gel until the dye front migrated into the resolving gel (approximately 20 min). The rest of the electrophoresis was carried out at 20 mA per gel until the dye front reached the bottom of the gel. Once the electrophoresis had finished, the gel apparatus was disassembled and the gels fixed by soaking in 50 % MeOH, 5 % HAc for 20 min followed by 10 min in 50 % MeOH and then washing for at least 2 h in Type I water. Proteins were visualised by silver staining.

2.3.8.3 Silver staining
The gel was sensitised in Na₂S₂O₃ (0.02 %) for 1 min with two further washes in Type I water for 1 min each. The gel was then incubated with cold (4 °C) AgNO₃ (0.1 %) for 20 min, washed twice with Type I water for 1 min each and transferred to a clean container. Developing solution (0.04 % formaldehyde, 2 % Na₂CO₃) was then added and left until bands were clearly visible. Development was stopped by rinsing three times with 5 % acetic acid and the gel(s) were stored in 1 % acetic acid (Shevchenko et al., 1996). Gels were imaged using a CanoScan LiDE 600F scanner (Canon).

2.3.8.4 2D-PAGE Sample Preparation
*Panagrolaimus davidi* samples to be analysed by 2D PAGE analysis were first subjected to a clean-up procedure using a commercial 2D Gel Clean Up Kit (GE Healthcare) to remove lipid contaminants. This method involved the phase separation and precipitation of protein from possible contaminants using commercial solutions and was performed according to the manufacturer’s instructions.

The protein pellets from the 2D Gel Clean Up procedure were dissolved initially in 100 µL of isoelectric focusing (IEF) rehydration buffer (7 M urea, 50 mM
dithiothreitol (DTT), 4 mM tris carboxyethylphosphine (TCEP), 2 M thiourea, 2 % cholamidopropyl dimethylammonio-hydroxy-propanesulfonate (CHAPS), containing 5 µL of IEF buffer concentrate (GE Healthcare) per mL of rehydration buffer. Protease inhibitor and tributyl phosphine were also included (2 µL each). The protein pellet was then resuspended and solubilised using a mini pestle, followed by vortexing and sonication on ice for 20 min. The sample was then centrifuged at 12,000 ×g for 5 min and the supernatant transferred to a new 1.5 mL microcentrifuge tube and kept on ice until used.

2.3.8.5 First Dimension Isoelectric Focusing
Protein samples were made to a final volume of 330 µL with IEF rehydration buffer. The sample was pipetted into a well of an IPG strip rehydration plate (BioRad). The plastic overlay cover of a BioRad IEF IPG 18 cm pH 3-10 non-linear dry strip was removed and the strip was laid dry-gel side down in the well. This was performed with care to prevent bubble formation under the strip so the whole strip was in contact with the protein sample solution. The IEF strip was left to rehydrate for at least 10 h at room temperature with the rehydration plate sealed to prevent evaporation and subsequent recrystallisation of the urea.

Each side of the rehydrated gel strip was rinsed with 150 µL of Type I water to remove rehydration buffer solution from the surface of the strip and then placed gel side up in an IPGphor isoelectric focusing ceramic manifold (GE Healthcare) and filled with mineral oil. Paper wicks (GE Healthcare) wetted with 150 µL of Type I water were overlayed on each end of the strip and the electrode bars were positioned on the wicks to enable electrical contact. Isoelectric focusing (IEF) was conducted over 24 h to achieve a recommended volt-hour total for the IEF. The running protocol was; 2 h at 200 V, 3 h at 500 V, 4 h at 1000 V, 3 h at 2000 V, 6 h at 4000 V and finally 7 h at 8000 V (total volt-hour 91900). The current was limited to 50 µA. After IEF, IPG strips were processed for the second dimension SDS-PAGE.
2.3.8.6 Second Dimension Large Format SDS-PAGE

Second dimension, 12.5 % acrylamide, large format gels were made in batches of six using an Ettan™ DALT Six (GE Healthcare) gel casting mould. The acrylamide solution (186.3 mL 30 % acrylamide/0.8 % bisacrylamide, 112.5 mL resolving Tris-HCl buffer pH 8.8, 50.3 mL Type I water and 100 mL 50 % glycerol) was prepared in a 2 L Buchner flask and degassed using a Buchii V300 pump for approximately 1 h. Freshly made 10 % APS (1.4 mL) and TEMED (190 µL) were added and mixed and the gel solution degassed for a further 3 min. The acrylamide solution was carefully poured into the gel mould over a period of 5 min. Each gel unit in the gel mould was overlayed with $2 \times 2.5$ mL aliquots of degassed SDS-PAGE electrode buffer to exclude oxygen and produce a flat surface. The gels were covered with a plastic bag and left at room temperature to set overnight. Once set, the mould was dismantled, excess acrylamide removed and the individual gel units were rinsed and stored in a plastic bag at 4 °C.

Prior to second dimension electrophoresis, the proteins focused on the IPG strip were reduced with DTT and alkylated using iodoacetamide (IAA). The IPG strip was incubated for 10 min in a 1.8 mL aliquot of a IEF strip reducing solution (3.6 g urea (6M), 200 mg DTT (130 mM) in 2.5 mL SDS-PAGE resolving buffer, 4 mL 50 % glycerol and 1 mL Type I water followed by the addition of 200 µL saturated bromophenol blue solution in Type I water).

The focussed strip was transferred to a clean well of a BioRad IPG strip rehydration plate and incubated for a further 10 min in a 1.8 mL aliquot of IEF strip alkylation solution (250 mg IAA and 3.6 g urea (6M) in 2.5 mL SDS-PAGE resolving buffer, 4 mL 50 % glycerol and 1 mL Type I water followed by the addition of 200 µL saturated bromophenol blue solution in Type I water). The IEF strip was transferred to the top of a second dimension acrylamide gel so that the IEF strip was in direct contact with the SDS-PAGE gel. Melted 0.5 % agarose in SDS-PAGE electrode buffer was then overlayed to secure the IEF strip in place.
The second dimension SDS-PAGE was performed in an Ettan™ DALT Six electrophoresis tank (GE Healthcare) filled with SDS-PAGE electrode buffer and coupled to an Electrophoresis Power Supply-EPS 601 (GE Healthcare) unit. Electrophoresis was first conducted for 1 h at 2 W/gel to assist protein transfer from the IEF strip into the SDS-PAGE gel. Power was then increased to 15-20 W/gel until the dye front had reached the bottom of the gel. Typically, the second dimension SDS-PAGE was conducted over 6-7 h. The glass plates were then dismantled and the gel soaked in a fixing solution (10 % methanol, 7 % acetic acid, 500 mL with two changes of 30 min each) on a rocking table (Stuart® SSL3 Gyro Rocker, USA).

2.3.8.7 Staining of Large Format 2D-PAGE Gels

The gels were stained using a home-made colloidal Coomassie stain based on that of Neuhoff et al. (1998) and later modified by Candiano et al. (2004). The stain was prepared by dissolving 50 g of aluminium sulfate in 50 mL of o-phosphoric acid and then adding 100 mL ethanol, 1.2 g Coomassie G-250 and 900 mL Type I water with mixing overnight. Gels were stained in 250 mL of colloidal Coomassie at room temperature on a rocking table overnight. Gels were destained in distilled water for 48 h to remove background staining to visualise the protein display. After destaining the gels were imaged using a CanoScan LiDE 600F scanner (Canon).

2.3.9 LDH Activity Assay

LDH assays were carried out by adding 20 µL of appropriately diluted LDH (Sigma) to 980 µL of LDH assay cocktail (80 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM pyruvate, 0.3 mM NADH). The change in absorbance at 340 nm was recorded.

2.3.10 LDH Protection Assay

This assay is based on the method described by Carpenter and Crowe (1988) and modified by Wisniewski et al. (1999). LDH was mixed with the solution being tested, e.g. *P. davidi* homogenate. This mixture was then cooled to the holding temperature
(either 4 °C or -18 °C) and held for 4 h. The solution was then defrosted and tested for LDH activity using the LDH assay described above (see 2.3.9). The change in absorbance was taken as a proxy for LDH activity i.e. actual pyruvate change was not calculated. The fresh unfrozen LDH dilution was used as the 100 % activity and all activities were compared to this value and expressed as percentages of starting activity.

2.3.11 Nanolitre Osmometer Screen

The surface of the nanolitre osmometer was covered with a heat sink paste and overlaid with aluminium foil. Two microlitre drops of solution were placed on the aluminium foil and then covered with circle of cellulose acetate (approximately 5 mm in diameter, made using a hole punch). The temperature of the nanolitre osmometer was then dropped rapidly to – 40 °C to freeze the samples quickly. Once all the samples had been frozen the temperature of the nanolitre osmometer was raised to the holding temperature. After a holding time of 4 h the stage of the nanolitre osmometer was placed under a microscope and the ice crystals were observed. The level of RI was determined by comparing the ice crystal morphology to both buffer (low RI) and grass or P. davidi extracts (high RI). To make the viewing of the ice crystal boundaries easier, carbon powder was added (0.5 % w/v) to the samples before they were frozen.

2.3.12 Optical Recrystallometer Screen

An Al block (dimensions approximately 20 cm × 12 cm × 7 cm) with 3 mm diameter holes drilled to a depth of 5 cm was cooled to –20 °C in a refrigerated circulator (Haake F8-C35). The Al block was chilled for at least 1 h. Two hundred microlitres of sample were pipetted into a glass sample tube designed to fit snugly in the holes in the Al block. The sample was snap frozen by immersing the sample tube in an ethanol/dry ice slurry (approximately –70 °C) for 30 s and was then placed in the Al block. Multiple samples could be frozen at the same time provided dry ice was added to the ethanol slurry to maintain the temperature. Once all samples had been frozen and transferred to the Al block the refrigerated circulator was warmed to the holding
temperature. As soon as the temperature of the Al block reached the holding temperature (measured using a thermocouple in an empty sample tube in the Al block) the time zero (t=0) readings were taken and a timer started.

The readings were taken by removing the sample tube from the Al block, wiping with tissue paper to remove any liquid on the outside of the tube, then placing the tube in the OR (set at -5°C) for 3-5 s. The OR was calibrated so that 0 units equalled no transmittance and 100 units equalled full transmittance of light. Sample readings were recorded using an A/D converter connected to a MacIntosh Classic computer. The sample was then removed and placed back in the Al block trying to minimise the time spent out of the block, usually < 15 s. Samples were also visually checked to ensure they were not melting. All samples were measured at each time point trying to get through them as quickly as possible, usually <5 min.

### 2.3.13 Proteinase K Digestion

Five mirolitres of 20 mg·mL\(^{-1}\) proteinase K (PK) (Roche) was added to 1 mL of *P. davidi*, grass or *Tenebrio* homogenate to give a final concentration of 0.1 mg·mL\(^{-1}\) of PK. The samples were then incubated for 1, 6 or 24 h at 37 °C. After incubation the reaction was stopped by the addition of 150 µL of 7× Complete™ protease inhibitor (Roche). As controls, 1 mL samples of *P. davidi*, grass and *Tenebrio* homogenate were also incubated at 37 °C with 5 µL of Type I water for 24 h before addition of 150 µL of 7× Complete™ protease inhibitor (Roche). One mL of Type I water was also incubated with 5 µL of 20 mg·mL\(^{-1}\) PK at 37 °C for 24 h before addition of 150 µL of 7× Complete™ protease inhibitor (Roche) to ensure that PK did not have RI activity.

### 2.3.14 Ammonium Sulfate Precipitation

The amount of ammonium sulfate required was determined using the table shown in Figure 2.6. The amount of ammonium sulfate (Scharlau) was then accurately weighed and added to the sample. The ammonium sulfate was dissolved on a rotating mixer at 4 °C. After all the ammonium sulfate was dissolved the sample was placed on ice (~0 °C) for 6 h with occasional inversion. The sample was centrifuged at
13,000 rpm for 30 min at 4 °C. As much of the supernatant as possible was transferred to a clean 1.5 mL microcentrifuge tube. The pellet was resuspended in the original volume of buffer (25 mM Tris-HCl, pH 8.0) and both pellet and supernatant were either used immediately or frozen until required.

<table>
<thead>
<tr>
<th>Initial % saturation ammonium sulfate(s)</th>
<th>Milligrams of solid ammonium sulfate to add to 1 ml of solution to achieve desired saturation at 0 °C</th>
<th>Final concentration of ammonium sulfate in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>106</td>
<td>134</td>
</tr>
<tr>
<td>25</td>
<td>79</td>
<td>108</td>
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<td>0</td>
</tr>
<tr>
<td>100</td>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 2.6:** Ammonium sulfate saturation table. Used for calculating the amount of ammonium sulfate required to reach a desired level of saturation. The initial saturation is listed on the left and the desired saturation level is along the top, the number where the two intersect is the weight (mg) of ammonium sulfate to be added per 1 mL of sample to achieve the desired level of ammonium sulfate saturation at 0 °C.
2.3.15 Chromatography

All chromatography was performed using the ÄKTAPrime™ plus chromatography system. The data were recorded on an Acer laptop and analysed using PrimeView Evaluation software.

2.3.15.1 Hydrophobic Interaction Chromatography

Three different types of hydrophobic interaction chromatography (HIC) columns were used – HiTrap™ Butyl Sepharose FF, HiTrap™ Phenyl Sepharose FF (low substitution) and HiTrap™ Octyl Sepharose FF (GE Healthcare).

The HIC protocol involved running 100 % HIC buffer A for 10 min to equilibrate the column. One millilitre of sample was injected, followed by a further 5 min of 100 % HIC buffer A, this was followed by 5 min of 50 % HIC buffer B, followed by 5 min of 100 % HIC buffer B and finally 24 min of 100 % HIC buffer A. The entire protocol was run at a flow rate of 1 mL.min⁻¹ and 1 mL fractions were collected from the injection time (t=0) until the end of the run. An overview of this method is shown in Figure 2.7.

![Figure 2.7: Theoretical elution profile for the hydrophobic interaction chromatography.](image)

The line indicates the proportion of buffer B.
2.3.15.2 Ion Exchange Chromatography

A 1 mL HiTrap™ SP Sepharose XL column (GE Healthcare) was used for the cation exchange chromatography and a 1 mL HiTrap™ Q Sepharose XL column (GE Healthcare) was used for the anion exchange chromatography. The running conditions were the same for both anion and cation exchange chromatography with the only alteration being the different pH of the buffers – 8.0 for cation exchange and 6.0 for anion exchange.

The ion exchange protocol involved running 25 mL of 100 % ion exchange (IEX) buffer B (flow rate 10 mL.min\(^{-1}\)) followed by 15 mL of 100 % IEX buffer A (flow rate 10 mL.min\(^{-1}\)). One millilitre of sample (dialysed against appropriate buffer overnight) was injected followed by a further 10 mL of 100 % IEX buffer A (flow rate 1 mL.min\(^{-1}\)). A step to 100 % IEX buffer B for 10 mL was followed by a further 13 mL of 100 % IEX buffer A (flow rate 1 mL.min\(^{-1}\)). One mL fractions were collected from the injection time until the end of the run. An overview of this protocol is shown in figure 2.8.

Figure 2.8: Theoretical elution profile for the ion exchange chromatography. The line indicates the proportion of buffer B.
2.3.15.3 Gel Permeation Chromatography

Gel permeation chromatography was done using a Superdex 75 column (GE Healthcare). The column was equilibrated with gel permeation buffer (25 mM Tris-HCl, 100 mM NaCl, pH 8.0) at a flow rate of 0.5 mL.min\(^{-1}\) until a consistent, flat baseline was achieved (approximately 2 h). Once the column had equilibrated, the gel permeation program was started. The program was run using a constant flow rate of 0.5 mL.min\(^{-1}\) and consisted of equilibration step for 4 min followed by injection of 0.5 mL of sample and then an elution time of 60 min. One millilitre fractions were collected from the injection point until the end of the run.
Chapter Three

Cold Finger Ice Binding

3.0 Introduction

All AFPs are thought to act via some form of adsorption-inhibition mechanism where they bind to ice in an essentially irreversible manner. The cold finger ice binding method was developed to exploit this property (Kuiper et al, 2003). This requires the slow growth of ice over a long period (typically about 20 h). Molecules that are not compatible with the ice lattice (most molecules) are excluded, whereas those molecules that can bind to the ice (mainly AFPs) are incorporated into the growing hemisphere. This method was used to purify a recombinant AFP from a crude cell extract to > 95 % purity after one round of ice binding and that, after a second round of ice binding, appeared to be homogenous (Kuiper et al, 2003).

Because RIPs affect how ice crystals grow it was thought that they too would interact with ice and it would therefore be possible to purify the RIP(s) from *P. davidi* using this technique.
3.1 Cold Finger Ice Binding

Two rounds of ice binding were carried out as described (Method 2.3.5). After the second round of ice binding a clear ice hemisphere was recovered and the four fractions generated were tested for protein concentration and RI activity. Several different extracts were analysed in this way.

3.1.1 *Panagrolaimus davidi* Cold Finger Ice Binding

The cold finger ice binding (CFIB) was carried out on a *P. davidi* homogenate to try and isolate any ice binding proteins. Both the LF1 and LF2 fractions were approximately 30 mL while the IF2 fraction was 69 mL (Table 3.1). The protein concentration in LF1 (0.208 mg.mL\(^{-1}\)) was higher than the SM (0.162 mg.mL\(^{-1}\)). The LF2 fraction had a very low concentration (0.013 mg.mL\(^{-1}\)) and IF2 had a protein concentration below the detection limits of the BCA assay used (Table 3.1).

<table>
<thead>
<tr>
<th><em>P. davidi</em></th>
<th>SM</th>
<th>LF1</th>
<th>LF2</th>
<th>IF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction volume (mL)</td>
<td>100</td>
<td>31</td>
<td>29</td>
<td>69</td>
</tr>
<tr>
<td>Protein concentration (mg.mL(^{-1}))</td>
<td>0.162</td>
<td>0.208</td>
<td>0.013</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

Table 3.1: Volumes and protein concentrations of *P. davidi* fractions generated from CFIB.

The volume of each fraction was determined using a measuring cylinder (SM was set as 100 mL) after each round of CFIB. The protein concentrations were determined using the BCA assay (Methods 2.3.6). Values below 0.01 mg.mL\(^{-1}\) were recorded as < 0.01 mg.mL\(^{-1}\) as this was the limit of the sensitivity of the assay.
**Figure 3.1**: Ice crystal sizes of *P. davidii* CFIB fractions. The four fractions (SM, LF1, LF2 and IF2) were produced using the CFIB Method (Method 2.3.5). A dilution series of each fraction was then tested for RI activity using the splat freezing method (Method 2.3.4). The average diameter of the 10 most prominent ice crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 125 µm ± 13 µm.

The RI activity of the fractions was determined using the splat freezing assay. All fractions had some RI activity in the undiluted sample, with the average crystal size of SM, LF1 and IF2 close to 40 µm, while LF2 had an average crystal size of just over 60 µm. At the 2-fold dilution, the average crystal size of both SM and LF1 had increased to about 60 µm and continued to increase with dilution until it reached a plateau at approximately 120 µm. In both LF2 and IF2 the average crystal size increased to over 100 µm by the 2-fold dilution and stayed at this size for the remaining dilutions (Figure 3.1). This result was unexpected because it was hypothesised that the RI protein would bind to ice and therefore be enriched in the IF2 fraction. To determine if this was an anomaly a grass extract known to have RI proteins was also tested.
3.1.2 Grass Cold Finger Ice Binding

The grass extract was made as described in the methods (Method 2.3.3.3). The CFIB was carried out using exactly the same conditions as was used for the *P. davidi* homogenate.

Both the grass SM and LF1 fractions had high protein concentrations (0.62 mg.mL\(^{-1}\) and 0.54 mg.mL\(^{-1}\) respectively). The LF2 fraction had a much lower protein concentration (0.094 mg.mL\(^{-1}\)) and the IF2 had a protein concentration below the detection limits of the BCA assay used. The fraction volumes were all very similar, between 42 and 48 mL (Table 3.2).

<table>
<thead>
<tr>
<th>Grass</th>
<th>SM</th>
<th>LF1</th>
<th>LF2</th>
<th>IF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction volume (mL)</td>
<td>100</td>
<td>48</td>
<td>42</td>
<td>47</td>
</tr>
<tr>
<td>Protein concentration (mg.mL(^{-1}))</td>
<td>0.62</td>
<td>0.54</td>
<td>0.09</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

**Table 3.2: Volumes and protein concentrations of grass fractions generated from CFIB.** The volume of each fraction was determined using a measuring cylinder (SM was set as 100 mL) after each round of CFIB. The protein concentrations were determined using the BCA assay (Methods 2.3.6). Values below 0.01 mg.mL\(^{-1}\) were recorded as < 0.01 mg.mL\(^{-1}\) as this was the limit of the sensitivity of the assay.

The fractions generated by the CFIB were tested by splat assays to assess RI activity. Both the SM and LF1 fractions started off with small ice crystals (30-40 µm) and these grew slightly as the sample was diluted. A final size of about 90 µm was reached at the 16-fold dilution. The LF2 fraction had ice crystals of about 50 µm in the undiluted sample and these grew at each dilution until a final size of just over 100 µm in the 16-fold dilution. The IF2 had large crystals in the undiluted sample (90 µm) and these only grew slightly with dilution, reaching a maximum size of nearly 120 µm in the 8-fold and 16-fold dilutions (Figure 3.2). This pattern is very similar to the unexpected pattern observed for *P. davidi*. To check that both the running conditions and the cold finger were suitable for purifying ice binding proteins from a
crude mixture the CFIB was carried out using a *Tenebrio* homogenate that is known to contain an AFP rather than an RIP (Graham et al., 1997).

![Figure 3.2: Ice crystal sizes of grass CFIB fractions.](image)

**Figure 3.2: Ice crystal sizes of grass CFIB fractions.** The four fractions (SM, LF1, LF2 and IF2) were produced using the CFIB method (Method 2.3.5). A dilution series of each fraction was then tested for RI activity using the splat freezing method (Methods 2.3.4). The average diameter of the 10 most prominent ice crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 113 µm ± 14 µm.

### 3.1.3 *Tenebrio* Cold Finger Ice Binding

When the *Tenebrio* homogenate was put through the CFIB procedure the protein concentrations followed a similar pattern to that observed with *P. davidi* CFIB (Table 3.1 and table 3.3). The LF1 fraction had a slightly higher protein concentration than the SM (0.107 mg.mL⁻¹ and 0.093 mg.mL⁻¹ respectively) and both the LF2 and IF2 had very low protein levels (0.049 mg.mL⁻¹ for LF2 and below the detection limit for IF2). The volumes of the fraction for the *Tenebrio* CFIB were all between 41 and 55 mL.
Table 3.3: Volumes and protein concentrations of *Tenebrio* fractions generated from CFIB.

The volume of each fraction was determined using a measuring cylinder (SM was set as 100 mL) after each round of CFIB. The protein concentrations were determined using the BCA assay (Methods 2.3.6). Values below 0.01 mg.mL\(^{-1}\) were recorded as < 0.01 mg.mL\(^{-1}\) as this was the limit of the sensitivity of the assay.

The splat assays show that the SM fraction had small crystals in the undiluted sample (40 µm) that grew steadily with dilution to just over 100 µm. Both LF1 and LF2 had large crystals at all dilutions tested, starting at nearly 90 µm and growing to over 120 µm by the 16-fold dilution. The IF2 fraction was similar to the SM, beginning with small crystals in the undiluted sample (30 µm) which grew at each dilution until reaching a size of nearly 140 µm in the 16-fold dilution (Figure 3.3).

**Figure 3.3: Ice crystal sizes of *Tenebrio* CFIB fractions.** The four fractions (SM, LF1, LF2 and IF2) were produced using the CFIB method (Methods 2.3.5). A dilution series of each fraction was then tested for RI activity using the splat freezing method (Methods 2.3.4). The average diameter of the 10 most prominent ice crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 130 µm ± 14 µm.
3.2 Calculated Recrystallisation Inhibition Activity

By assuming that the partitioning of the proteins that incorporate into ice is based on the amount of the sample that is frozen i.e. if half the sample freezes then half the ice binding protein will be in the ice fraction and half in the liquid fraction, the amount of activity expected in each fraction could be determined (Kuiper et al., 2003). The SM fractions were defined as having 100 arbitrary units of activity and the activity tracked through the CFIB procedure using the volumes from Tables 3.1, 3.2, and 3.3. The predicted RI activity in each fraction (in arbitrary units) based on these calculations is shown in Table 3.4. Both the *P. davidi* LF1 and LF2 had low expected activity (11 and 21, respectively) whereas IF2 would be expected to have approximately half the activity of the SM (51 units). The grass fractions followed a similar pattern; however, the differences between each fraction was not expected to be as large with LF1 having 21 units and LF2 and IF2 both very similar with 32 and 35 units, respectively. The *Tenebrio* fractions showed the lowest expected activity in the LF1 fraction (21 units) followed by LF2 (30 units) and finally IF2 as the most active (40 units).

<table>
<thead>
<tr>
<th></th>
<th>SM</th>
<th>LF1</th>
<th>LF2</th>
<th>IF2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. davidi</em></td>
<td>100 (+++)</td>
<td>11 (+++)</td>
<td>21 (-)</td>
<td>51 (-)</td>
</tr>
<tr>
<td>Grass</td>
<td>100 (+++)</td>
<td>21 (+++)</td>
<td>32 (+)</td>
<td>35 (-)</td>
</tr>
<tr>
<td><em>Tenebrio</em></td>
<td>100 (+++)</td>
<td>21 (+)</td>
<td>30 (+)</td>
<td>40 (+++)</td>
</tr>
</tbody>
</table>

Table 3.4: Predicted RI activity units in each CFIB fraction. The SM was given an arbitrary value of 100 units of activity. The activity of the other units was then calculated using the assumption that the activity will partition based on the amount of the sample frozen i.e. if half the sample freezes then half the activity will be in the frozen portion and half in the liquid portion. Shown in parenthesis is qualitative indication of observed RI activity.

From these data we can see that for each sample we would expect the SM to have the most RI activity followed by IF2, then LF2, and finally LF1 would be the least active (SM > IF2 > LF2 > LF1). If we assume that higher activity results in smaller crystal sizes i.e. the more active a sample the smaller the ice crystal will be, then the
calculated pattern does not match the pattern observed for either the *P. davidi* (*SM ≈ LF1 > LF2 ≈ IF2*) or the grass (*SM = LF1 > LF2 > IF2*) but is close to the pattern seen with *Tenebrio* (*SM = IF2 > LF2 = LF1*).

### 3.3 Protein Analysis

One-dimensional SDS-PAGE gels all showed similar patterns. In each case (Figures 3.4, 3.5 and 3.6) both the SM and LF1 lanes appear to contain a lot of protein (the lanes are very dark) over a wide range of sizes. The LF2 lane shows some variation in the protein level with the *P. davidi* (Figure 3.4) showing no bands, the grass LF2 (Figure 3.5) has some very faint bands and *Tenebrio* LF2 (Figure 3.6) appears to have several bands over a range of sizes. Both the *P. davidi* and the grass IF2 have no visible bands whereas the *Tenebrio* IF2 has a band at approximately 45 kDa. Based on the protein concentrations (Tables 3.1, 3.2, 3.3) it was expected that some of the fractions (especially LF2 and IF2) would have very low protein amounts and to enhance the chance of visualizing any proteins present silver staining was used due to its increased sensitivity compared to Coomassie staining. The silver staining method of Shevchenko *et al.* (1996) was selected as it was compatible with mass-spectrometry if necessary (Shevchenko *et al.*, 1996).
Figure 3.4: SDS-PAGE of *P. davidi* fractions produced by CFIB. One hundred microlitres of each fraction was vacuum dried. The pellet was then resuspended in 20 µL 1× sample buffer with 2MCE and heated for 5 min at 95 °C. Five microlitres loading buffer was then added and 15 µL of this was loaded onto a 10% acrylamide gel. A 5 µL sample of molecular weight marker (Mr) was also loaded in lane 1. The gel was run at 100 V and 25 mA until the dye front reached the bottom of the gel. The gel was then silver stained (Shevchenko et al., 1996). The sizes of the molecular weight markers are shown in kDa.
Figure 3.5: SDS-PAGE of grass fractions produced by CFIB. One hundred microlitres of each fraction was vacuum dried. The pellet was then resuspended in 20 µL 1 × sample buffer with 2MCE and heated for 5 min at 95 °C. Five microlitres loading buffer was then added and 15 µL of this was loaded onto a 10% acrylamide gel. A 5 µL sample of molecular weight marker (Mr) was also loaded in lane 1. The gel was run at 100 V and 25 mA until the dye front reached the bottom of the gel. The gel was then silver stained (Shevchenko et al., 1996). The sizes of the molecular weight markers are shown in kDa.
Figure 3.6: SDS-PAGE of *Tenebrio* fractions produced by CFIB. One hundred microlitres of each fraction was vacuum dried. The pellet was then resuspended in 20 µL 1 x sample buffer with 2MCE and heated for 5 min at 95 °C. Five microlitres loading buffer was then added and 15 µL of this was loaded onto a 10% acrylamide gel. A 5 µL sample of molecular weight marker (Mr) was also loaded in lane 1. The gel was run at 100 V and 25 mA until the dye front reached the bottom of the gel. The gel was then silver stained (Shevchenko *et al.*, 1996). The sizes of the molecular weight markers are shown in kDa.
3.4 Mass Spectrometry

To check the protein profiles of the IF2 fractions (*P. davidii*, grass and *Tenebrio*), all of which had protein levels below the detection limit of the BCA assay (Table 3.1, 3.2 and 3.3), intact mass spectrometry was carried out on each sample. Both the *P. davidii* and the grass IF2 had no detectable protein (Figure 3.7A and 3.7B). The *Tenebrio* IF2 had 3 peaks with a mass to charge ratio in the range of 8.258 kDa to 8.394 kDa (Figure 3.7C). For details of the mass spectrometry conditions see Methods 2.3.7.
Figure 3.7: Mass spectrometry profiles for IF2 from A) *P. davidi* B) grass and C) *Tenebrio.*
3.5 Summary

In this chapter we attempted to purify ice active proteins from *P. davidi* homogenates using the cold finger ice binding method (Kuiper *et al.*, 2003). This method slowly grows an ice crystal over an extended period of time (20 hours), which excludes any molecules that do not bind to ice, resulting in an ice hemisphere containing only molecules that bind to ice.

Using two rounds of ice binding we were able to generate four different fractions (SM, LF1, LF2 and IF2). Assuming the protein responsible for RI activity binds to the ice we would expect to see an increase in activity in fraction IF2. From the splat assay results this was clearly not the case, with IF2 having very low RI activity, unexpectedly however LF2 did have a large amount of RI activity (Figure 3.1). These results indicated that the protein(s) responsible for the RI activity in *P. davidi* was not binding to ice. To check if the CFIB method was able to isolate these proteins we used a grass extract also known to have RI activity (Kumble *et al.*, 2008).

Using the grass extract the CFIB was carried out using the same conditions used with the *P. davidi* homogenate. The results using the grass extract were exactly the same as achieved with *P. davidi* i.e. IF2 showed very low RI activity, whereas LF2 had very high activity (Figure 3.2). Again, this was an unexpected result and suggested that either the CFIB was not working correctly or RIPs do not bind to ice. To differentiate between these options, we carried out a final CFIB trial using a *Tenebrio* homogenate. *Tenebrio* contains an AFP, as opposed to a RIP (Graham *et al.*, 1997).

This round of CFIB resulted in an IF2 with very high RI activity and LF2 had a much lower level of activity (Figure 3.3). This is the pattern of activity we would expect if the AFP was binding to the ice and confirmed that the CFIB was capable of isolating proteins that bind to ice.

In addition to the activity of each fraction the protein content was also tested to confirm that no proteins were in IF2 of either *P. davidi* or grass homogenates. The protein was testing using three methods – BCA assay, 1D SDS-PAGE and intact mass spectrometry, and all of these methods were unable to detect any protein in the IF2
(Table 3.1, Table 3.2, Figure 3.4, Figure 3.5, Figure 3.7A and Figure 3.7B). However we did detect a protein using intact mass spectrometry with a size consistent with the *Tenebrio* AFP, showing that the CFIB was successfully isolating this protein and therefore working correctly (Figure 3.7C).

These results taken together indicate that the CFIB is capable of isolating proteins that bind to ice. In addition, the lack of RI activity or protein in IF2 of both *P. davidii* and grass homogenates show that the RI activity of these is not due ice binding and must be due to a mechanism that is not dependent on ice binding. If this is the case it would be unique among ice active proteins and shows that RIP and AFP activities are the result of two different mechanisms.
Chapter 4

Screen Development

4.0 Introduction

Recrystallisation inhibition (RI) activity is a very difficult property to test quickly. Over the years there have been many methods used to test RI activity but the splat assay remains the ‘gold standard’ against which other methods are compared (Knight et al., 1988). The splat assay is not a technically difficult assay to perform but it is time-consuming and requires specialised equipment including a refrigerated circulator and a cold stage microscope. In addition to the specialised equipment, each assay takes approximately 35 – 40 min to complete and only one splat can be carried out at a time. This makes doing splats very time consuming and impractical for screening a large number of samples. Here, I have attempted to develop a fast and reliable method for screening a large number of samples simultaneously to identify those that may have RI activity. The RI activity was then confirmed by splat assay.

4.1 LDH Method

The first screen method attempted was based on that of Wisniewski et al. and is contingent on the loss of activity of lactate dehydrogenase (LDH) upon freezing (Wisniewski et al., 1999). By adding an antifreeze protein (AFP) to the LDH solution, Wisniewski et al. were able to protect the LDH from freezing damage, with nearly all the LDH activity surviving the freezing process. Because the sample had to be frozen I hypothesised that the RI activity of the AFP was likely to be protecting the LDH rather than the thermal hysteresis. If this is the case it should be possible to adapt this method to screen for RI activity.
4.1.1 Determination of Optimum LDH Concentration

Initially, the optimum dilution of LDH to obtain accurate assay results had to be determined. To do this a series of LDH dilutions were tested. Both the 1 in 10 dilution (Figure 4.1A), and the 1 in 1000 dilution (Figure 4.1D) had very little change in absorbance (flat lines) over the 2 min tested. The 1 in 100 dilution (Figure 4.1B) has a gradient that is not linear (as can be seen by not aligning with the linear line of best fit overlaid). The 1 in 500 dilution (Figure 4.1C) has a linear change in absorbance ($\Delta A_{\text{min}}^{-1}$ of 0.0451) over the 2 min recorded. The aim of this approach was to determine the optimum dilution of LDH to use in the assay; from experience in our laboratory it is known that a $\Delta A_{\text{min}}^{-1}$ of between 0.02 and 0.05 is optimal for this kind of analysis. The results of this dilution clearly show that a 1 in 500 dilution gives this change. Other dilutions (1 in 10 and 1 in 100) are either too fast (1 in 10) and give no change or do not produce a linear change (1 in 100). Similarly, the 1 in 1000 dilution is too slow and does not show any change in a suitable time frame.
Figure 4.1: Activity of different dilutions of commercial LDH. The different dilutions tested A) 1 in 10 B) 1 in 100 C) 1 in 500 and D) 1 in 1000 were used to determine the optimum dilution for use in the LDH protection assay. Twenty microlitres of each dilution were added to 980 µL of LDH assay cocktail (80 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM pyruvate and 0.3 mM NADH) and the $A_{340}$ recorded for 2 min (see 2.3.10).

4.1.2 LDH Protection Trial

After the appropriate dilution of LDH had been determined the ability of samples to protect the LDH from freezing-induced loss of activity was investigated. All of the homogenates tested conferred high levels of protection to LDH when frozen (Figure 4.2). In all cases where the LDH was mixed with either a *P. davidi* or *Tenebrio* homogenate there was at least 80 % of the activity still present after 4 h of freezing. This is much greater than when LDH was mixed with Type I water where < 2 % of the activity is retained. In the case of the fresh *P. davidi* homogenate there was more LDH activity after freezing than before. Overall this looked very promising as both the *P. davidi* and *Tenebrio* homogenates appear to protect LDH from freezing induced damage.
Figure 4.2: Percent of LDH activity retained after freezing. Fifty microlitres of 1 in 250 LDH (Sigma) was mixed with 50 µL of sample (P. davidi, Tenebrio or Type I water) and frozen at -18 °C for 4 h (method 2.3.10). The mixture was then defrosted and 20 µL were mixed with 980 µL of LDH assay cocktail and the ΔAU.min⁻¹ recorded (Method 2.3.11). The ΔAU.min⁻¹ was then converted to a percentage of initial (unfrozen) LDH activity. Both P. davidi SM fresh and Tenebrio SM fresh were made fresh, whereas P. davidi SM and Tenebrio SM had been frozen prior to use. The values are averages of two duplicates. Error bars are ± standard deviation.

4.1.3 Endogenous LDH Activity

To ensure that these high LDH activity levels in both the P. davidi and Tenebrio homogenate samples after freezing were not due to native LDH activity in the homogenate, both homogenates were tested for native LDH activity after chilling or freezing. The fresh LDH had high activity, and chilling for 4 h had very little effect on this activity (approximately 95 %). Freezing the sample resulted in large loss of activity (approximately 90 %). Both P. davidi and Tenebrio SM have very low activity in the fresh samples and this did not change in the chilled or frozen samples (Figure 4.3).
Figure 4.3: Native LDH activity. To test the native LDH activity in *P. davidi* and *Tenebrio* 50 µL of sample (*P. davidi*, *Tenebrio* or LDH) was added to 50 µL Type I water and then either chilled (4 °C) or frozen (-18 °C) for 4 h (method 2.3.10). The LDH activity was then measured by adding 20 µL of defrosted sample to 980 µL of LDH assay cocktail and the ΔAₘₐₜ⁻¹ recorded (method 2.3.11). The unfrozen (fresh) samples were measured as soon as they were made up. All values were converted to a percentage of fresh LDH. Error bars are ± standard deviation.

4.1.4 Substrate Dependence

In the presence of both NADH and pyruvate (standard LDH assay cocktail) all the samples have high LDH activity and both *P. davidi* and *Tenebrio* fractions have more activity than the LDH with type I water. The *P. davidi* and *Tenebrio* samples retain some activity after freezing when tested in the presence of both NADH and pyruvate (+ NADH + pyruvate). If pyruvate is removed from the cocktail, then very little activity is detectable either fresh or after freezing (Figure 4.4).
Figure 4.4: The effect of substrate presence or absence on LDH activity. Fifty microlitres of each sample (P. davidi, Tenebrio or Type I water) were mixed with 50 µL of 1 in 250 LDH and frozen for 4 h (Method 2.3.10). Twenty microlitres of defrosted sample were then mixed with 980 µL of either the standard LDH assay cocktail (+ NADH + pyruvate) or the LDH assay cocktail without the 2 mM pyruvate (+ NADH – pyruvate) and the ΔAU.min⁻¹ measured (Method 2.3.11). Fresh samples of each mixture were also tested. The activity of the pre-frozen LDH + type I water in the standard LDH assay cocktail (+ NADH + pyruvate) was defined as having 100 % activity and all other activities are expressed as a percentage of this activity.

4.1.5 Effect of Protein Concentration

To ensure that the protection of LDH during freezing by both P. davidi and Tenebrio homogenates was due to a specific activity (such as RI activity) rather than just non-specific protection due to higher protein levels the effect of differing concentrations of BSA was also tested (Figure 4.5). The BSA concentration has very little effect on the activity of LDH either fresh or after chilling, with all concentrations tested having an activity close to 100 % of the fresh LDH + Type I water sample. When the sample is frozen the BSA appears to have a concentration-dependent effect (Figure 4.5). The 0 mg·mL⁻¹ (Type I water) sample has almost no LDH activity after freezing (~ 5 %). However, by adding 0.05 mg·mL⁻¹ of BSA to the sample nearly 50 % of the LDH activity is still present after freezing and as higher concentrations of BSA are used the...
level of freeze protection increases until at 1 mg·mL\(^{-1}\) there is approximately 100 % of the LDH activity still present after freezing.

![Graph showing the effect of BSA concentration on LDH freezing stability.](image)

**Figure 4.5: Effect of BSA concentration on LDH freezing stability.** Fifty µL of each concentration of BSA or Type I water was mixed with 50 µL LDH (1 in 250). The sample was then either frozen (-18 °C) or chilled (4 °C) for 4 h (method 2.3.10), after which the sample was defrosted and 20 µL was mixed with 980 µL of LDH assay cocktail and the ΔA\(U\).\(min\)\(^{-1}\) measured. Freshly made samples were also tested for LDH activity (method 2.3.11). The fresh LDH with 0 mg·mL\(^{-1}\) (type I water) was defined as having 100 % activity. All other activities are expressed as a percentage of this activity.

### 4.2 Nanolitre Osmometer Method

A nanolitre osmometer was also investigated as a viable screen for RI activity. This can be used for measuring the melting point and hence osmolarity of small volumes of liquid as well as determining thermal hysteresis of samples or observing ice crystal structure and growth. It was hoped to be able to use the temperature controlled stage to quickly freeze a large number of samples and then observe the samples some time later and see some differences among them.
4.2.1 Grass Extract Trials

In Figure 4.6 it can be clearly seen that there is some dependence on the concentration of the grass extract and the appearance of sample after 4 h at -8°C. As the concentration of the grass extract decreases the appearance of the solution appears less smooth, or patchier. This may be due to the size of the ice crystals increasing as the concentration decreases. Smaller ice crystals in the undiluted extract are harder to tell apart and therefore the entire sample appears more homogenous and smoother. Due to this encouraging result I used this approach on some column fractions.

Figure 4.6: Grass dilution series viewed using the nanolitre osmometer method. A) undiluted (approx. 10 mg·mL⁻¹) B) 10-fold dilution (approx. 1 mg·mL⁻¹) C) 100-fold dilution (approx. 0.1 mg·mL⁻¹). Two microlitres drops of each dilution were put on the stage of the nanolitre osmometer and then covered with a circle of cellulose acetate. The samples were quickly frozen and then raised to the holding temperature (-8°C) and observed and photographed (40x magnification) after 4 h at the holding temperature. Scale bar = 0.5 cm.

4.2.2 Grass Chromatography Fractions

Cationic ion exchange chromatography was performed on a grass extract. The gradient was from 0 M NaCl to 1 M NaCl in 25 mM Tris-HCl, pH 8.0. Five fractions were collected throughout the run. Each of the fractions and a buffer control was then subjected to the nanolitre osmometer method to determine if it had any RI activity.
Figure 4.7: Fractions from chromatography of grass extract assayed by nanolitre osmometry. Fractions were from cation exchange chromatography on a grass extract as discussed above. Five fractions were collected during the run (A-E, respectively) then viewed using the nanolitre osmometer method (see 2.3.12). Buffer A was also included in the screen (F). Scale bar = 0.5 cm

In Figure 4.7 we can see that all of the fractions (and the buffer sample) appear very similar after 4 h at -8°C. All the fractions look similar to the 100-fold dilution (Figure 4.6C) which suggests that none of the fractions has significant RI activity. The lack of RI activity is also backed up by the fractions looking similar to the buffer that has no RI activity (Figure 4.7F). This is unexpected as at least one of the fractions should have had some RI activity given the high level of RI activity in the start sample (Figure 4.6A). To confirm that there were differences in RI activity in the fractions, splat assays were carried out. These assays confirmed that fraction 2 and 3 (Figure 4.7B and 4.7C) had much smaller crystals than any of the other fractions or the buffer (not shown). These data suggest that the nanolitre osmometer cannot be used in this way to detect RI activity.
4.2.3 Addition of Graphite to Samples

To try and make the crystal boundaries clearer and aid determination of the size of the ice crystals, powdered graphite was added to the samples (0.05% w/v). The graphite will not be incorporated into the ice crystal and will therefore concentrate in the crystal boundaries after freezing (figure 4.8).

Figure 4.8: Effect of addition of graphite to nanolitre osmometer samples. Undiluted (A) and diluted (1 in 10) (B) samples of grass extract with 0.05 % w/v powdered graphite added. The grass extract containing 0.05 % w/v powdered graphite was then tested by the nanolitre osmometer method (see 2.3.12). Scale bar = 0.5 cm.

In Figure 4.8 we can see that crystal boundaries are more noticeable with the addition of the powdered graphite. It is very obvious in the diluted grass sample (figure 4.8B). The addition of the graphite has made it much easier to determine crystal size and therefore it is easier to determine which samples will have more or less RI activity. However, even with the addition of graphite, this method is very subjective and open to interpretation by the observer. Because of this, I decided to investigate other methods that may be more suitable for a RI screen and that would be less subjective.
4.3 Optical Recrystallometer Method

An optical recrystallometer was used to test a range of different samples with different RI activities to see if differences between fractions could be distinguished. *P. davidi*, grass and *Tenebrio* homogenates with high levels of RI and beef heart extract (F1) and BSA with low RI activity were used. To ensure that different amounts of protein were not responsible for different activities the protein concentrations of all samples were standardised to 1 mg·mL⁻¹. For detailed equipment and methods see Methods 2.3.12.

### 4.3.1 Recrystallisation Inhibition of Samples

To confirm RI activities, splat assays were performed on all samples (Figure 4.9). Both grass and *Tenebrio* samples have very small crystals at 1 mg·mL⁻¹ (both about 20 µm). These crystals get progressively larger as the sample is diluted and reach a diameter of 100 µm for grass and 55 µm for *Tenebrio* samples at 0.0625 mg·mL⁻¹. The *P. davidi* sample has intermediate-sized crystals (50 µm) at 1 mg·mL⁻¹ and these gradually increase to about 110 µm in the 0.0625 mg·mL⁻¹ sample. Extract F1 has an initial crystal size of about 70 µm at 1 mg·mL⁻¹ and is over 100 µm at 0.5 mg·mL⁻¹. This grows very little so that at the 0.0625 mg·mL⁻¹ it is only just over 140 µm. The solutions of BSA have crystal sizes of about 90 µm at all concentrations tested.
Figure 4.9: Ice crystals sizes of all samples measured using the splat assay. Each assay involved dropping 10 µL of sample approximately 3 m onto an aluminium block, which was cooled by dry ice (-78°C), to form a small disc of ice. Part of this disc was then transferred to a microscope cold stage and held for 30 min at the annealing temperature (-8°C) and then photographed. The average diameter of the 10 most prominent ice crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 128 ±18 µm.

4.3.2 Effect of Holding Temperature on Optical Recrystallometer Values

When the samples were held in the OR at -4 °C, all grass homogenates (high RI activity) start off with an OR reading < 20 and these went up very rapidly with the three lowest concentrations (0.25, 0.125 and 0.0625 mg·mL⁻¹) reaching a maximum value (> 90) after 4 h (Figure 4.10A). Samples of both 1 and 0.5 mg·mL⁻¹ had intermediate values at 4 h and by 24 h all five concentrations had reached maximum transmittance (> 90). The F1 (low RI activity) samples showed a similar pattern except it occurred faster with all concentrations reaching maximum transmittance at 4 h. At 1 h the 1 and 0.5 mg·mL⁻¹ samples had readings near 40 and the more dilute samples were all about 70 (Figure 4.10B).
Two hundred microlitres of each sample were snap frozen in an ethanol/dry ice slurry for 30 s and then transferred to an Al block cooled to -20 °C. Once all samples had been frozen the Al block was warmed to -4 °C. When the Al block reached -4 °C (t = 0) the transmittance was measured by placing the sample in the OR. Readings were also taken at 0, 1, 4 and 24 h.

When the holding temperature was decreased to -6 °C there was a change in the pattern of transmittance seen. The grass extract had very little change in the OR reading over time when held at -6 °C with all values starting <20 and only the most dilute samples having a value above 20 after 24 h (Figure 4.11A). All dilutions of F1 had low OR readings initially (<20) and these grew over time depending on their dilution. The three most dilute samples (0.25, 0.125 and 0.0625 mg·mL⁻¹) had
maximum OR values (>90) after 24 h while the 1 mg·mL⁻¹ sample has only reached 40 and the 0.5 mg·mL⁻¹ is between them (70) after 24 h (Figure 4.11B).

**Figure 4.11: Change in OR readings for A) grass and B) F1 over time at -6°C.** Two hundred microlitres of each sample were snap frozen in ethanol/dry ice slurry for 30 s then transferred to an Al block cooled to -20 °C. Once all samples had been frozen the Al block was warmed to -6 °C. When the Al block reached -6 °C (t = 0) the transmittance was measured by placing the sample in the OR. Readings were taken at 0, 1, 4 and 24 h.
As the holding temperature was decreased even further to -8 °C the pattern of transmittance changed again. The OR readings for the grass extract changed very little over 24 h when held at -8 °C. All concentrations had a t=0 reading < 20 with very little change with all 24 h readings still < 20 (Figure 4.12A). There was also very little change in the OR reading of F1 with all concentrations having a starting value (t=0) of < 20 and only a small increase at the 24 h reading (Figure 4.12B).

The *P. davidii* and *Tenebrio* homogenates had almost identical results as the grass homogenate and the BSA was the same as the F1. For ease of viewing these are not shown.
Two hundred microlitres of each sample were snap frozen in ethanol/dry ice slurry for 30 s then transferred to an Al block cooled to -20 °C. Once all samples had been frozen the Al block was warmed to -8 °C. When the Al block reached -8 °C (t = 0) the transmittance was measured by placing the sample in the OR. Readings were taken at 0, 1, 4 and 24 h.

By showing the 24 h OR readings at different temperatures on the same graph as the ice crystal sizes determined by splats it is possible to see how these two properties are related. When the samples are held at -4 °C for 24 h the OR readings are all at about the maximum value of transmittance that the OR reads (about 90-95) (Figure 4.13 A – E). This may be due to high recrystallisation in the sample, partial melting of the sample or a combination of the two. Similarly, when the samples are held at -
8 °C for 24 h the OR readings are very low (< 25) with very little change as the concentration decreases (Figure 4.13A-E). When both the grass and *Tenebrio* samples were held at -6 °C there is a very slight increase in the OR reading as the concentration decreases (from about 10 in the 1 mg·mL⁻¹ sample to about 30 in the 0.0625 mg·mL⁻¹ sample). This occurs for both samples even though there is a large difference in the change in crystal size over the same range; the grass crystals grow from about 25 µm (1 mg·mL⁻¹) to over 100 µm (0.0625 mg·mL⁻¹) whereas the *Tenebrio* crystals go from about 25 µm (1 mg·mL⁻¹) to nearly 60 µm (0.0625 mg·mL⁻¹) (Figure 4.13 A and B). The *P. davidi* OR readings (at -6 °C) start at about 20 and only grow slightly until the 0.125 mg·mL⁻¹ dilution (reading of about 30) before increasing in the 0.0625 mg·mL⁻¹ sample to about 80. This lags behind the crystal size growth, which starts at about 50 µm and then grows slightly until the 0.25 mg·mL⁻¹ dilution after which the crystals are over 100 µm (Figure 4.13 C). The F1 sample has an OR reading of about 40 in the 1 mg·mL⁻¹ sample and this increases to about 70 in the 0.5 mg·mL⁻¹ sample and reaches maximum for transmittance (> 90) for the subsequent dilutions. The ice crystal sizes started off relatively large (about 70 µm) and these grew at each dilution reaching a final size of 140 µm in the 0.0625 mg·mL⁻¹ sample (Figure 4.13 D). The BSA OR readings started off about 60 (1 mg·mL⁻¹) and then reached the maximum transmittance value at the 0.5 mg·mL⁻¹ dilution and stayed at this high reading for all the lower dilutions. The crystal sizes at all dilutions are between 80 and 100 µm (Figure 4.13E).
Figure 4.13: Ice crystal sizes determined using the splat assay and OR readings at different temperature. A) grass B) Tenebrio C) P. davidi D) F1 and E) BSA. The ice crystal size was determined for a dilution series of the samples using the splat assay as described (method 2.3.4). To determine OR readings, samples were snap frozen in an ethanol/dry ice slurry for 30 s and then placed in an Al block cooled to -20 °C. Once all samples had been frozen the Al block was warmed to the holding temperature (-4 °C, -6 °C or -8 °C) and held for 24 h. After 24 h the sample transmittance was measured using the OR (method 2.3.13).
It was also necessary to determine what effect salt would have on the OR values. To do this a blank ion exchange run was done and the fractions tested using the OR (-6 °C holding temperature).

**Figure 4.14: OR readings of blank ion exchange chromatography run.** A blank ion exchange chromatography run was done and the fractions collected were snap frozen in an ethanol/dry ice slurry for 30 s and placed into an Al block cooled to -20 °C. Once all samples had been frozen, the Al block was warmed to -6 °C. When the Al block reached -6 °C (t=0) the transmittance of each sample was measured by placing the sample OR. The transmittance was also measured at 1, 4 and 24 h.

Figure 4.14 shows the OR readings for fractions taken from a blank ion exchange run. The 0 h readings all had an OR reading of approximately 20 from fraction 1-11. After fraction 11 there was a very quick rise in the OR reading such that by fraction 13 the OR reading was at the maximum transmittance (>95) and stayed at this high level for the rest of the fractions. The 1 h readings had a very similar pattern to the 0 h readings; however, the readings were only low for fractions 1-8 (OR readings of approximately 25). Fractions 9-13 were more variable with OR readings between 20 and 45 and again fraction 14 onwards had maximum OR readings (>95). This presumably reflects the effect of NaCl on the behaviour of the ice crystals.
4.4 Summary

In this chapter we attempted to develop a screening method that would allow us to test samples for RI activity, without the need to do time consuming splat assays. To do this three different potential screens were investigated; LDH protection, nanolitre osmometer and optical recrystallometer.

The first of these methods (LDH protection) was based on the fact AFPs have been shown to protect LDH enzyme activity during freezing, which usually results in loss of LDH activity (Wisniewski et al., 1999). We hypothesised that RI activity also protect LDH activity during freezing. Initial results were promising, with the *P. davidi* homogenate showing protection of LDH activity during freezing. To confirm that the activity was due to protection by RIPs we carried out a series of controls to rule other causes for the apparent protection of LDH activity. Most of these (endogenous LDH, activators, endogenous co-factors) were shown not to affect the results. However using BSA we were able to show that any protein at a high enough level (0.1 mg·mL$^{-1}$) would have a protective affect, thus this method was not pursued further.

The second method used the cold stage of a nanolitre osmometer. This method involved freezing a small amount of sample (2 µL) very quickly and then holding at a high sub-zero e.g. – 6 °C for a period of time before visually inspecting the samples (using a microscope). It was expected that samples with RI activity would not change from their initial appearance, whereas those without RI would appear different/changed. Initial work on this method was promising with a difference between samples with (*P. davidi* homogenate) and without (buffer) RI activity noticeable and made more noticeable with the addition of a small amount of graphite powder to the sample prior to freezing. However, when the method was attempted on samples with unknown RI activity it was much harder to identify samples with RI activity, especially samples with an intermediate level of activity. This made this method very subjective and it was due to subjectivity that this method was not continued with. Although with more time and/or experience it should be possible to develop this method further into a suitable screen, if desired.
The final method utilised an optical recrystallometer. This equipment is able to measure the amount of light that passes (transmits) through a sample. Previous work had shown that as recrystallisation occurs in a sample, the amount of light transmitted increases. By modifying this previous method slightly we were able to adapt this to be able to measure many samples during the same run. Experiments were carried out to determine the optimum holding time and temperature (24 hours and –6 °C respectively). In addition we were able to show that samples without RI activity did not appear positive (as with the LDH method). Due to this we were able to conclude that the optical recrystallometer method was suitable for use as a quick screening method for RI activity.
Chapter Five

Protein and Chromatography

5.0 Introduction

After the failure to isolate any proteins with RI activity using the CFiB technique (Chapter 3) a more traditional chromatographic method was investigated for purifying potential RI proteins. The successful development of a screening method (Chapter 4) that allows the rapid analysis of many samples for RI activity made this a less daunting prospect. In addition to this, some basic properties of the protein(s) responsible for RI activity e.g. proteinase K sensitivity, heat stability and co-factor dependence, were also investigated.

5.1 Basic properties of the RI activity

Because I had no success in purifying RI activity some of the basic properties of the protein responsible for the activity were investigated. First, I wanted to investigate whether the RI activity was due to a protein as there have been several recent reports of other molecules with RI activity (Wu et al., 2011; Capicciotti et al., 2012). If I was able to show that a protein was responsible for activity, then I would also investigate the effect of some other basic properties that may also aid in the purification of the RIP e.g. metal ion dependence, heat stability and ammonium sulfate solubility. Panagrolaimus davidi, grass and Tenebrio samples were analysed for all of these properties because it would allow determination of whether the properties are specific to RIP (P. davidi and grass) or were more general properties of IAP (Tenebrio).

5.1.1 Proteinase K Digestion

Proteinase K is a fungal serine protease with a broad spectrum of activity. It generally cleaves the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids (Ebeling et al., 1974). Because of its relatively non-selective nature, proteinase K is
often used during DNA/RNA purification as it will eventually digest (inactivate) all proteins in the sample (Abubaker et al., 1998; König et al., 2012; Portela et al., 2013). I hoped that by incubating homogenates with proteinase K I would be able to show (via loss of RI activity) that a protein(s) was responsible for the RI activity of *P. davidi*.

Figure 5.1A shows that both *P. davidi* SM and SM + H$_2$O (control) samples have initial crystal sizes of slightly less than 40 µm and both the 1 h and 6 h PK incubations have initial crystal sizes of slightly more than 50 µm in the splat assay. Both of the 2-fold and 4-fold dilutions of these samples (*P. davidi* SM, SM + H$_2$O, 1 h and 6 h) do not show much difference with all samples exhibiting similar crystal sizes. The 24 h samples have crystal sizes close to 100 µm at all dilutions. Figure 5.1B shows a similar pattern with both the grass SM and SM + H$_2$O sample containing small crystals (about 20 µm) and the crystal size grows as the samples are diluted reaching about 60 µm at the 8-fold dilution. Both the 1 h and 6 h PK samples had intermediate sized crystals (about 30 µm and 50 µm, respectively) in the undiluted samples and grew at each dilution until reaching a size of 70 µm and 90 µm, respectively, at the 8-fold dilution. The 24 h PK sample had large crystals in the undiluted sample (90 µm) and these did not grow very much as the sample was diluted, with a final size of just over 100 µm in the 8-fold dilution. In the *Tenebrio* sample (Figure 5.1C) the SM, SM + H$_2$O and 1 h samples all have similar crystal sizes at each of the dilutions tested. The undiluted samples have a crystal size of under 20 µm and these grow slightly at each dilution reaching just under 40 µm in the 8-fold dilution. The 6 h sample also has small crystals in the undiluted sample (< 20 µm). However, the 2-fold dilution has a crystal size over 50 µm and the crystals continue to grow as the sample is further diluted reaching about 70 µm in the 8-fold dilution. The 24 h sample had the largest crystal size at each dilution tested, with the undiluted sample being about 50 µm and these continued to grow at each dilution, reaching a final size of 80 µm in the 8-fold dilution.
Figure 5.1: Effect of proteinase K on RI activity in a *P. davidi* (A), grass (B) or *Tenebrio* (C) homogenate. Five microlitres of PK (20 mg·mL⁻¹) was added to 1 mL of either *P. davidi*, grass or *Tenebrio* homogenate. This mixture was then incubated at 37 °C for 1, 6 or 24 h before the reaction was stopped by the addition of 150 µL of 7× stock solution of Complete™ protease inhibitor (Roche). A separate 1 mL aliquot of each homogenate was also incubated at 37 °C with 5 µL of Type I water for 24 h before the addition 150 µL of 7× stock solution of Complete™ protease inhibitor (Roche). All samples and the starting homogenate were tested for RI activity using the splat assay (Method 2.3.4). The average diameter of the 10 most prominent ice crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 110 µm ± 13 µm.

The effects of proteinase K on the protein profile of a *P. davidi* homogenate, a grass extract and a *Tenebrio* homogenate are shown in Figures 5.2, 5.3 and 5.4, respectively. In the *P. davidi* gel (Figure 5.2) both the SM and SM + H₂O lanes are very similar and have many bands over a wide range of sizes. The 1, 6 and 24 h and H₂O + PK lanes are also very similar to each other. There is a band at approximately 30 kDa and then a number of bands towards the bottom of the gel (< 25 kDa). In both the 1 and 6 h lanes there are some faint high molecular weight bands (approximately 100 kDa) and these are darker in the 1 h compared to the 6 h sample. The grass proteinase K gel (Figure 5.3) shows a very similar pattern to the one seen in the *P. davidi* sample with both the SM and SM + H₂O lanes having a range of bands over a range of sizes. The 1, 6 and 24 h and H₂O + PK lanes all have a band at approximately 30 kDa and in the H₂O + PK lane there is another band of approximately 75 kDa. The bands in the 1, 6 and 24 h lanes are all lower intensity than the H₂O + PK lane and are also progressively fainter in each lane i.e. 1 h is darker than the 6 h which is darker than the 24 h. The *Tenebrio* proteinase K gel (Figure 5.4) is also very similar to both the *P. davidi* and grass gels. The SM lane is a solid smear of protein and it is almost impossible to identify individual protein bands, in part due to over-staining of the gel to visualize low abundance proteins and because of the difficulty in staining *Tenebrio* AFP. The SM + H₂O lane is not as intense as the SM lane and several individual bands are visible. The 1, 6 and 24 h lanes have faint bands at approximately 30 kDa and this faint band matches the dominant band in the H₂O + PK lane suggesting this band is proteinase K. There are also some faint, low molecular weight (< 15 kDa) bands in the 1, 6 and 24 h lanes.
These results show that the RI activities of *P. davidi*, grass and *Tenebrio* homogenates are abolished by exposure to proteinase K (Figure 5.1). The exposure of the homogenates to proteinase K also resulted in protein degradation (Figures 5.2, 5.3 and 5.4). It is noticeable that the protein degrades much faster than the RI activity is lost in most cases (1 h for protein degradation vs. >6 h for loss of RI activity). This suggests that the molecule(s) responsible are protein(s) and that even small fragments are able to maintain some RI activity or that the RI proteins are more resistant to proteolysis than most of the homogenate.
Figure 5.2: SDS-PAGE gel of P. davidi fractions produced by proteinase K treatment. One milliliter of P. davidi homogenate (SM) was incubated with 5 µL 20 mg·mL⁻¹ proteinase K (final conc. 0.1 mg·mL⁻¹) for 1, 6 or 24 h at 37 °C and stopped using 150 µL Complete™ protease inhibitor. As controls 5 µL of Type I water were added to 1 mL of SM (SM + H₂O) and 5 µL of PK were added to 1 mL Type I water (H₂O + PK). Both controls were incubated at 37 °C for 24 h and then 150 µL Complete™ protease inhibitor added. One hundred microlitres of each fraction were freeze-dried until dryness. The pellet was then resuspended in 20 µL 1× sample buffer with 2MCE and heated for 5 min at 95 °C. Five microlitres loading buffer were then added and 15 µL of this was loaded onto a 10 % acrylamide gel. Five microlitres molecular weight marker (Mr) were loaded. The gel was run at 100 V and 25 mA until the dye front reached the bottom of the gel and then silver stained. The sizes of the molecular weight markers are shown in kDa.
Figure 5.3: SDS-PAGE gel of grass fractions produced by proteinase K treatment. One millilitre of grass homogenate (SM) was incubated with 5 µL 20 mg·mL⁻¹ proteinase K (final conc. 0.1 mg·mL⁻¹) for 1, 6 or 24 h at 37 °C and stopped using 150 µL Complete™ protease inhibitor. As controls 5 µL of Type I water were added to 1 mL of SM (SM + H₂O) and 5 µL of PK were added to 1 mL Type I water (H₂O + PK). Both controls were incubated at 37 °C for 24 h and then 150 µL Complete™ protease inhibitor added. Ten microlitres of each fraction were mixed with 10 µL 1× sample buffer with 2MCE and heated for 5 min at 95 °C. Five microlitres loading buffer were then added and 15 µL of this was loaded onto a 10 % acrylamide gel. Five microlitres molecular weight marker were also loaded. The gel was run at 100 V and 25 mA until the dye front reached the bottom of the gel and then silver stained. The sizes of the molecular weight markers are shown in kDa.
Figure 5.4: SDS-PAGE gel of *Tenebrio* fractions produced by proteinase K treatment. One millilitre of *Tenebrio* homogenate (SM) was incubated with 5 µL 20 mg·mL⁻¹ proteinase K (final conc. 0.1 mg·mL⁻¹) for 1, 6 or 24 h at 37 °C and stopped using 150 µL Complete™ protease inhibitor. As controls 5 µL of Type I water was added to 1 mL of SM (SM + H₂O) and 5 µL of PK were added to 1 mL Type I water (H₂O + PK). Both controls were incubated at 37 °C for 24 h and then 150 µL Complete™ protease inhibitor added. Ten microlitres of each fraction were mixed with 10 µL 1× sample buffer with 2MCE and heated for 5 min at 95 °C. Five microlitres loading buffer were then added and 15 µL of this loaded onto a 10% acrylamide gel. Five microlitres molecular weight marker were loaded. The gel was run at 100 V and 25 mA until the dye front reached the bottom of the gel and then silver stained. The sizes of the molecular weight markers are shown in kDa.
5.1.2 Effect of EDTA on RI activity

When EDTA was added to the *P. davidi* homogenate it had very little effect on the RI activity. At the dilutions tested both the SM and SM + 5 mM EDTA had very similar crystal sizes (Figure 5.5). This result suggests that the protein(s) responsible for the RI activity do not require any metal ions for activity.

![Figure 5.5: The effect of EDTA on RI activity of a *P. davidi* homogenate. EDTA was added to a *P. davidi* homogenate to give a final concentration of 5 mM. The sample was then mixed for 4 h at 4 °C. Both the original homogenate (SM) and the homogenate with EDTA added (SM + 5 mM EDTA) were tested for RI activity using the splat assay. The average diameter of the ten most prominent ice crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 118 µm ± 16 µm.](image-url)
5.1.3 Effect of heating on RI activity

To determine the effect that heating may have on RI activity a 1 ml sample of *P. davidi* homogenate (see method 2.3.3.1) was heated at 95 °C for 30 min. After 30 min the sample was centrifuged at 13,000 × *g* for 10 min at 4 °C. The supernatant was transferred to a new microcentrifuge tube (heated supernatant) and the precipitate in the tube resuspended in 1 ml Tris-HCl buffer (25 mM, pH 8.0) (heated pellet). As a control 1 ml of homogenate was kept on ice for 30 min before undergoing the centrifugation and resuspension to produce unheated supernatant and pellet.

Both the unheated and heated supernatants had similar sized crystals at all dilutions tested. In both the heated and unheated supernatant; the undiluted sample crystal sizes were approximately 50 µm, which increased slightly until the 8-fold dilution where the crystal size was over 100 µm. Both the heated and unheated pellet had crystal sizes above 100 µm at every dilution tested. This result suggests that heating has very little effect on the activity of the RI protein(s), as both heated and unheated samples have very similar crystal sizes.
Figure 5.6: Effect of heating on *P. davidi* homogenate. A 1 mL aliquot of *P. davidi* homogenate was heated at 95 °C for 30 min and another 1 mL aliquot of *P. davidi* homogenate was incubated on ice for 30 min. Both samples were then centrifuged at 13,000 × *g* for 10 min in a microcentrifuge at 4 °C. The supernatant was removed to a new microcentrifuge tube (heated or unheated supernatant) and the precipitate in the tube resuspended in 1 mL Tris-HCl buffer (25 mM, pH 8.0) (heated or unheated pellet). Each of these samples was then tested using the splat assay (Method 2.3.4) to determine RI activity and the average size of the ten most prominent ice crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 117 µm ± 12 µm.

5.1.4 Ammonium sulfate precipitation

The SM for the ammonium sulfate precipitation experiments had small crystals in the undiluted sample (30 µm) and these grew steadily as the sample was diluted until they reached just over 90 µm in the 8-fold dilution (Figure 5.7). Both the 50 % supernatant and 50 % pellet (Method 2.3.15) had large crystals in the undiluted sample (both approximately 80 µm). In the 50 % supernatant the crystal sizes grew slightly at each dilution until a final size of about 110 µm in the 8-fold dilution. The 50 % pellet crystal size changed little with dilution and had crystal sizes of between 85 and 95 µm at all dilutions tested. Equal amounts of both supernatant and pellet were combined (remix) to make a sample with a composition equal to the original homogenate. The remixed sample had intermediate sized crystals in the undiluted sample (approximately 50 µm) which grew to 75 µm in the 2-fold dilution.
dilution before growing more slowly at both the 4-fold and 8-fold dilutions (both slightly above 80 µm) (Figure 5.7). This result is very interesting and unexpected. It suggests that the RI activity can be removed and separated by ammonium sulfate precipitation. However, the activity can then be recovered by recombining the two parts in a ratio that approximates the original sample. This may mean the RI activity requires more than one component to effectively inhibit ice crystal growth.

![Figure 5.7: Ice crystal sizes of fractions obtained by ammonium sulfate precipitation.](image)

Ammonium sulfate was added to 1 mL of *P. davidi* homogenate (SM) to obtain a 50 % saturated solution (Figure 2.6). The ammonium sulfate was dissolved and the sample kept on ice with occasional mixing for 6 h. It was then centrifuged at 13,000 × g for 30 min at 4 °C. After centrifugation, the supernatant was transferred to a clean microcentrifuge tube (50 % supernatant). The pellet was then resuspended in 1 mL of 25 mM Tris-HCl, pH 8.0 buffer (50 % pellet). Both the 50 % supernatant and pellet were dialysed overnight against 25 mM Tris-HCl, pH8.0. The dialysed 50 % supernatant and 50 % pellet were mixed in equal amounts (1:1) to generate the remix sample. All samples were then tested for RI activity using the splat assay (Method 2.3.4). The average crystal size of the ten most prominent crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 124 µm ± 18 µm.
5.2 Chromatography

Having confirmed that a heat stable protein (proteinase sensitive) was responsible for the RI activity of the *P. davidi* homogenate I moved onto attempts at purifying it.

5.2.1 Ion exchange chromatography

Ion exchange chromatography was performed using an AKTA prime chromatography system. Both anion and cation exchange chromatography were performed, with the only difference between the two set ups being the column and pH of the buffer used. The columns were equilibrated in buffer A (low salt) before the sample was injected and allowed to bind, a step gradient (0-100%) of buffer B (high salt) was then carried out and fractions collected from the injection time (for more detail see Method 2.3.15.2).

In both ion exchange chromatograms there are two main peaks. In Figure 5.8A (Q Sepharose) there is a small absorbance peak near the start (approximately 2 min) followed by a larger peak between 16 and 20 min. This second absorbance peak coincides with the conductivity peak, with the end of the absorbance peak (approximately 20 min) occurring when the conductivity reached approximately 90 % of its peak value. In Figure 5.8B (SP Sepharose) the absorbance peak pattern is reversed, with a large peak near the start of the run (approximately 2 min) and a much smaller peak at approximately 16 min. This second absorbance peak again coincides with the beginning of the increase in conductivity. However, the absorbance peak only lasts until the conductivity has reached about 25 % of its peak value. All of the fractions within the absorbance peaks were retained and fractions from each peak were pooled. This resulted in four fractions; Q bound, Q flow-through (Q FT), SP bound and SP flow-through SP FT). Each of these fractions and the *P. davidi* homogenate (SM) were then tested for RI activity using the splat assay (Figure 5.9). The SM had ice crystals about 40 µm in diameter and these grew to almost 60 µm when the SM was diluted 2-fold. In all cases the fractions collected from the ion exchange run had crystals that were larger than the diluted SM. Both the cation exchange fractions (Q bound and Q FT) had ice crystals between 80 and 90 µm. The anion exchange bound (SP bound) fraction had ice crystals that were 100 µm and the flow-through (SP FT) ice crystals were larger again at over 120 µm.
Figure 5.8: Ion exchange chromatograms. One millilitre of *P. davidi* homogenate (Method 2.3.3.1) was dialysed overnight against ion exchange Buffer A. After dialysis the sample was run through the ion exchange chromatography protocol (Method 2.3.15.2) with either a Q Sepharose (A) or SP Sepharose (B) column in place. One millilitre fractions were collected throughout the run. Both conductivity (left hand axis) and the $A_{280}$ (right hand axis) were tracked throughout the run. Regions pooled to create flow-through (FT) and bound (B) fractions are indicated.
Figure 5.9: Ice crystal sizes of samples collected from ion exchange chromatography. One millilitre of *P. davidi* homogenate (SM) was fractionated by ion exchange chromatography (Methods 2.3.15.2) with either an anion (SP) or cation (Q) exchange column. The $A_{280}$ was measured and the peaks collected and combined to give a flow-through (FT) and a bound fraction from each column. The fractions were dialysed overnight against 25 mM Tris-HCl pH 8.0 and then tested for RI activity by splat assay (Methods 2.3.4). Half SM is a 2-fold dilution of SM. The average diameter of the ten most prominent ice crystals is shown.
5.2.2 Hydrophobic interaction chromatography

Three different HIC columns were chosen of different hydrophobicity. The three columns were butyl (weakly hydrophobic), phenyl (medium) and octyl (strongly hydrophobic). It was anticipated that using a wider range of column types would give a greater chance of finding one suitable for the separation of RI activity.

The three HIC columns tried all produced very similar chromatograms (Figure 5.10). Very large absorbance peaks appear between 1 and 5 min and much smaller, more diffuse peaks emerged between 15 and 25 min. These second peaks are very small and have a maximum absorbance of approximately 6 mAU at their highest point. In all three cases there is also a slight deviation in the conductivity that coincides with the first large absorbance peak. The second smaller absorbance peak coincides with the decrease in conductivity and ends as the conductivity starts increasing again. There is a small kink in the conductivity trace halfway through its decrease. All fractions that showed some absorbance were collected and those from the same peak were pooled to create a flow-through and bound fraction from each hydrophobic interaction column. These fractions were then dialysed overnight against 25 mM Tris-HCl, pH 8.0 and tested for RI activity using the splat assay. In Figure 5.11 we see that the SM had small ice crystals to start with (30 µm) and these grew at both the 2-fold (58 µm) and 4-fold (75 µm) dilutions. All of the bound and flow-through fractions from the HIC had crystals that were larger (95-115 µm) than the 4-fold dilution of SM. This could mean that more than one component is required for RI activity and the HIC is splitting these. Pooling the samples did not bring the activity back, meaning either the components were split into the flow-through and bound fractions, or they require more than simply being mixed in the sample solution to regain activity.
**Figure 5.10: HIC chromatograms.** One millilitre of *P. davidi* homogenate (Method 2.3.3.1) was dialysed overnight into HIC buffer. The sample was then run through the HIC protocol (Method 2.3.16.1) with either a butyl (A), phenyl (B) or octyl (C) hydrophobic column in place. One millilitre fractions were collected throughout the run. Regions pooled to create flow-through (FT) and bound (B) fractions are indicated.

![HIC chromatograms](image)

**Figure 5.11: Ice crystal sizes of HIC fractions.** One millilitre of *P. davidi* homogenate (SM) was run through the HIC method (Methods 2.3.15.1) with either a butyl, phenyl or octyl hydrophobic interaction column in place. The absorbance at 280 nm was followed and fractions from the same peak were pooled to create either a flow-through or bound fraction for each column type. These pooled samples were then dialysed overnight into 25 mM Tris-HCl pH 8.0. Half SM and Quarter SM are 2-fold and 4-fold dilutions of SM, respectively. Each fraction was then tested for RI activity using the splat assay (Methods 2.3.4) and the average size of the 10 most prominent crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 128 µm ± 16 µm.

**5.2.3 Gel permeation chromatography**

Gel permeation chromatography was carried out using the AKTA prime chromatography system, a Superdex 75 column (GE Healthcare) using a buffer containing 25 mM Tris-HCl, and 100 mM NaCl pH 8.0. The chromatography was carried out with isocratic flow (0.5 mL.min⁻¹) and fractions were collected throughout the run.
Given the previous results i.e. the apparent loss of RI activity during the chromatography, I thought that the RI activity may be due to a complex of more than one molecule and that chromatography was separating these. For this reason I choose to use gel filtration chromatography to try and purify the RI activity, which, due to its different separating conditions, would leave intact any complex associated with the RI activity. The gel filtration chromatogram (Figure 5.12) shows absorbance ($A_{280}$) from the 14th until the 44th min of the run, this equates to fractions 7-22. There are significant peaks at the 18th min and from the 26th to the 31st min and a large peak at the 41st min.

![Figure 5.12: Gel permeation chromatogram](image)

The fractions that showed some absorbance (fractions 7-22) were then tested using the OR method (Chapter 4 - Methods 2.3.13). The OR readings of the gel permeation fractions displayed a wide range of values. The SM had a low reading of approximately 20. Three regions had readings similar to the SM - fractions 8-9, fractions 12-15 and fractions 18-19. Fractions 16-17, 20 and 22 had OR values between 40 and 70 and the remaining fractions (7, 10, 11 and 21) had OR values > 85 (Figure 5.13A). The fractions that had low OR values (8, 9, 12-15, 18 and 19) were then tested with the splat assay to confirm the presence of RI activity (Figure 5.13B). The SM had an average ice crystal size of just under 40 µm and
this increased to 60 µm when it was diluted 2-fold. All of the fractions that had low OR readings and were tested using the splat assay had large ice crystals with the average size being between 140 and 160 µm.
Figure 5.13: RI analysis of gel permeation fractions. A 0.5 mL aliquot of *P. davidi* homogenate (SM) was fractionated on a Superdex 75 gel permeation column (Methods 2.3.15.3) and fractions with significant $A_{280}$ were collected. These fractions were then tested for RI activity using the OR method (Methods 2.3.13) and transmittance was recorded after 24 h at -6 °C (A). Those fractions that had low OR values were then tested for RI activity using the splat assay (B) (Methods 2.3.4). The half SM is a 2-fold dilution of the SM. The average diameter of the ten most prominent crystals is shown. Error bars are ± standard deviation. Buffer control crystal size was 181 µm ± 28 µm.

There is a clear discrepancy between the OR and splat results. Several of the fractions appear to have RI activity using the OR; however, when the splat assay was used to confirm this activity they did not.
5.3 Summary

Due to the failure of CFIB to isolate any molecules responsible for RI activity it was necessary to resort to more classical techniques to find these. In this chapter we investigated some of the basic properties of RIP and tried to isolate them using chromatographic methods.

Initially, we looked at some of the fundamental properties of the RI activity. These included proteinase K sensitivity, effects of EDTA, heating, and ammonium sulfate concentration. Proteinase K digestion showed that there was a time-dependent loss of RI activity in *P. davidi* homogenates, grass extract and *Tenebrio* homogenates and 1D SDS-PAGE showed this loss of activity was matched by protein degradation. Both of these results indicate that the source of the activity is a protein, or at least has a protein component. The addition of EDTA did not affect the RI activity implying that metal ions are not important for RI activity. The heating results were interesting, with heating at 95 °C for 30 minutes not affecting RI activity, and both heated and unheated samples showing the same levels of RI activity. The ammonium sulfate precipitation experiments gave some very interesting results. Partition of the proteins based in their solubility in 50 % ammonium sulfate removed activity, but upon recombination some of the activity was recovered. This suggests that more than one component is necessary for RI activity.

After investigating some of the fundamental properties of the RI activity we moved onto attempting to isolate it using classic chromatographic techniques (ion exchange, hydrophobic and gel permeation). Ion exchange chromatography was tried first, with both anion (Q sepharose) and cation (SP sepharose) used. After running the chromatography neither the bound or flow-through fraction of either of these methods had any RI activity i.e. no activity was recoverable after the separation. There was an interesting result, with very little protein binding to the cation column, despite optimisation of the binding conditions (dialysing into a low pH solution prior to chromatography). This indicates that proteins of *P. davidi* may have an unusual isoelectric point, although further work would be needed to confirm this. Next three different hydrophobic interaction columns (butyl, phenyl and octyl) were investigated. As with the ion exchange chromatography, no RI activity was recovered in either the bound or flow-through fractions. Also of note was that very little of the protein bound to any of the columns (very small peaks in the bound fraction). This was
unexpected and may mean the proteins of *P. davidi* are quite hydrophilic; more testing will be required to confirm this. Based on previous experiments that indicated that RI activity may be due to more than one component, we reasoned that the lack of RI activity from any of the ion exchange or hydrophobic interaction chromatography was due to separation of these components on the column. Using this reasoning we thought that a gentler separation method may keep the complex intact and because of this we chose to try gel permeation chromatography. The chromatography ran smoothly and using the OR method developed in Chapter 4 eight fractions were identified for splat assay to confirm RI activity. When tested for RI activity using splat assay none of these samples showed RI activity. This was very disappointing as we believed the gentler conditions of gel permeation would help retain the RI activity, but this was clearly not the case.

Overall this series of experiments showed that RI activity is sensitive to proteinase K digestion, does not require metal ions for activity, is very heat stable (95 °C for 30 minutes) and may require more than one component for activity. We also tried a variety of classic chromatography techniques to isolate RIPs and in all methods tried we were unable to recover RI activity off the column, which prevented further purification using these methods.
Chapter Six

Proteomic Analysis

6.0 Introduction

Due to the inability to purify the RI protein via either CFIB (Chapter 3) or chromatography methods (Chapter 5) we chose to look at the proteome of *P. davidi* to identify potential recrystallisation inhibition proteins. It is known that acclimation at 5 °C increases the ability of *P. davidi* to survive intracellular freezing (Smith *et al.*, 2008). We planned to investigate whether acclimation lead to an increase in RI activity. If this was the case we would then compare the proteome of acclimated and non-acclimated samples using 2D-PAGE to identify candidate RIPs.

6.1 Recrystallisation Inhibition Activity of Acclimated and Non-Acclimated *P. davidi*

Previous work in our laboratory showed that when *P. davidi* is acclimated at 5 °C for 7 days they are better able to surviving freezing when compared to unacclimated *P. davidi* (Smith *et al.*, 2008). I investigated whether this difference in survival was matched by a difference in RI activity. To do this, a batch of *P. davidi* was grown in liquid culture (Method 2.3.1.2) and when the culture had reached a suitable density, it was split into two. One half of the culture was kept at 20 °C as per the normal procedure (non-acclimated) and the other half was kept at 4 °C (acclimated). After 7 days both samples were harvested (Method 2.3.1.3) at their holding temperature i.e. 20 °C or 4 °C and homogenised (Method 2.3.3.1). These homogenates were tested for protein concentration (Method 2.3.6). This testing showed that acclimated homogenate had a protein concentration of 0.427 mg.mL⁻¹ and the non-acclimated homogenate had a protein concentration of 0.536 mg.mL⁻¹. A sample of each was
taken and adjusted using 25 mM Tris-HCl, pH 8.0 so that both homogenates had the same protein concentration and then tested for RI activity using the splat assay (method 2.3.4).

**Figure 6.1:** Crystal size of acclimated and non-acclimated *P. davidi* homogenates. A liquid culture of *P. davidi* was split and half were grown at 5 °C (acclimated) and the other half kept at 20 °C (non-acclimated). After 7 days the cultures were harvested, homogenised and the protein content of each homogenate standardised. A dilution series of each homogenate was then tested for RI activity using the splat freezing method (Method 2.3.4). The average diameter of the 10 most prominent ice crystals is shown.

From Figure 6.1 it is clear that there is a difference in the RI activity of the acclimated and non-acclimated homogenates. At each dilution tested the crystal size of the acclimated sample is approximately half that of the non-acclimated sample. This clearly shows that acclimation process increases the RI activity of *P. davidi*. 
6.2 2D Gel Electrophoresis

As there is a difference in RI activity between acclimated and non-acclimated samples I could move onto looking at the proteome of both samples using 2D SDS PAGE. To do this each homogenised sample (acclimated and non-acclimated) were analysed on separate 2D-gels. The first dimension separation was based on net charge (isoelectric point) between pH 3 and pH 10. The second dimension separated size in the second dimension using SDS-PAGE. Each sample was run concurrently to make further comparison of the gels as easy as possible i.e. both samples would have been subjected to identical running conditions. After both samples had been separated both gels would be analysed side by side and protein spots with differential expression (higher or lower) would be further analysed.

The protein level of each homogenate sample was adjusted (as when testing RI activity) so that each sample had the same protein loading. This ensures any differences observed after staining were due to differential expression rather than an artefact of the testing.
**Figure 6.2:** Large format 2D-PAGE display of proteins extracted from an homogenate of *P. davidi* acclimated at 20 °C. The homogenate was prepared using the standard production method (see Method 2.3.3.1), followed by a commercial “clean up” procedure. The protein was solubilised in a total volume of 330 μL rehydration buffer that contained 3 μL tributyl phosphine and 2 μL of protease inhibitor. The protein solution was then used to rehydrate a pH 3-10 non linear 18 cm IPG IEF strip. Proteins were then reduced and then alkylated prior to second dimension SDS-PAGE, to separate proteins based on molecular weight. After electrophoresis, gels were soaked twice for 30 min each in 10 % methanol/7 % acetic acid, and then stained overnight with colloidal Coomassie stain. Gels were destained in MQ water over 48 h to remove background. The outlined spots are more prominent than in the 5 °C gel (Figure 6.3).
Figure 6.3: Large format 2D-PAGE display of proteins extracted from an homogenate of *P. davidi* acclimated at 5 °C. The homogenate was prepared using the standard production method (see Method 2.3.3.1), followed by a commercial “clean up” procedure. The protein was solubilised in a total volume of 330 μL rehydration buffer that contained 3 μL tributyl phosphine and 2 μL of protease inhibitor. The protein solution was then used to rehydrate a pH 3-10 non linear 18 cm IPG IEF strip. Proteins were then reduced and then alkylated prior to second dimension SDS-PAGE, to separate proteins based on molecular weight. After electrophoresis, gels were soaked twice for 30 min each in 10 % methanol/7 % acetic acid, and then stained overnight with colloidal Coomassie stain. Gels were destained in MQ water over 48 h to remove background. The outlined spots are more prominent than in the 20 °C gel (Figure 6.2).
The destained 2D-PAGE gels for 20 °C and 5 °C acclimated samples are shown in Figure 6.2 and 6.3, respectively. Both gels are similar, with proteins spread throughout the gel. Using a visual comparison proteins that were expressed different between the two gels and have circled those that were present at a higher level (darker spots) in that gel. This resulted in 15 spots being identified in the 20 °C gel and 46 spots being identified in the 5 °C gel. These spots were excised from the gels in the hope that we would be able to identify them using mass spectrometry methods. Unfortunately due to timing problems and equipment unavailability we were not able to analyse the spots this way and they have been frozen for future analysis.

6.3 Summary

After the unsuccessful attempts to isolate the protein(s) responsible for RI activity using classical chromatography techniques the next logical step was to use an ‘-omics’ method, in this case proteomics.

To do this we used previous knowledge from our lab that *P. davidi* that had been exposed to 5 °C for 7 days (acclimated) are better able to survive freezing (Smith *et al.*, 2008). We reasoned that this may be due to an increase in RI activity of the *P. davidi*. To test this we grew a culture at 20 °C (standard protocol) and then split this culture in two; half were kept at 20 °C (non-acclimated) and the other half were put into 5 °C (acclimated) for 7 days. After this time a homogenate was made from both growth conditions, the protein concentration was then measured and standardised and the RI activity determined using the splat assay. The protein concentration was standardised to ensure any differences seen were due to differences in protein expression, rather than different growth rates, feed levels etc.

The splat assays (Figure 6.1) clearly show that there was a difference in the RI activity between the acclimated and non-acclimated samples. The activity of the 16-fold dilution in the acclimated samples was greater than (smaller crystals) than the 4-fold dilution in the non-acclimated samples. This shows that the RI activity in the acclimated samples is at least 4 × that of the non-acclimated samples.

After showing a difference in RI activity between the acclimated and non-acclimated samples the next step was to carry out 2D SDS-PAGE to separate the proteins of each
sample. Prior to loading the protein concentration was standardised to ensure that the same amount of protein was loaded on each gel. The results of non-acclimated and acclimated 2D gels are show in Figure 6.2 and Figure 6.3, respectively. A side-by-side visual comparison was able to identify a total of 61 spots that were differentially expressed between the samples – 15 were more abundant (darker) in the non-acclimated sample and 46 were more abundant in the acclimated sample. All differentially expressed proteins were excised from the gel and stored for further analysis. The next obvious step was to analyse these spots using mass spectrometry and a protein database search to find matches to known proteins. Unfortunately, the equipment required to do this analysis was not available (damaged) at the time of this work and the time constraints would not permit this part to be done before submission.
Chapter Seven
Discussion

7.1 Cold Finger Ice Binding

The CFIB method involves growing an ice hemisphere very slowly over a relatively long time. Due to the slow rate of growth of the ice hemisphere, all solutes that do not interact with the ice (protein, salts etc.) are excluded from the growing ice hemisphere, whereas those solutes that are compatible with the ice lattice (e.g. AFPs) are incorporated into the ice hemisphere (Kuiper et al., 2003). This produces an ice hemisphere that contains only substances (e.g. proteins) that interact with ice as all others have been excluded. This results in the ice binding proteins being purified due to the exclusion of other substances rather than any active enrichment in the ice hemisphere. It has been shown that ice binding proteins can be purified to homogeneity using two rounds of ice binding (Kuiper et al., 2003). The second round of ice binding is necessary due to a small amount of protein that is incorporated into the ice due to non-specific interactions or that are loosely bound to the outside of the hemisphere at the end of the freezing period. Because of the effect of the P. davidi homogenate on ice crystal growth i.e. recrystallisation inhibition, it was hypothesised that the protein or proteins responsible would interact with ice and prevent ice crystal growth. If this was the case it seems reasonable to assume that the protein(s) responsible would be able to be purified using the CFIB technique.

7.1.1 Panagrolaimus davidi Cold Finger Ice Binding

The P. davidi homogenate was subjected to CFIB. The liquid fraction from the first CFIB run was also subjected to another CFIB run (Method 2.3.5/Figure 2.4), in an attempt to recover as much of the RI activity as possible from the sample. If only a single run had been used approximately 50% of the activity would have remained in the liquid fraction, LF1 assuming 50% of the sample froze and that the amount of activity recovered is equal to the amount of
sample frozen. However, by adding a second run of CFIB it was expected that half of the remaining 50% of activity could be recovered. When these two fractions were combined for a second round of ice binding, we should have approximately 75% of the total RI activity of the SM from which to obtain our pure sample.

After the second round of CFIB there were four fractions of interest, SM, LF1, LF2 and IF2. The volume of each fraction was measured and each fraction was tested for both protein concentration and RI activity. The protein concentrations behaved as expected with the LF1 having a slightly higher concentration than SM due to the concentrating effect of removing water (as the ice hemisphere). Both the LF2 and IF2 had very low protein concentrations (0.013 mg·mL⁻¹ and < 0.01 mg·mL⁻¹) due to the low starting protein concentration of the ice fraction generated from the first round of CIFB (Table 3.1). There was also a marked difference between the ice hemispheres obtained with the hemisphere from the second round of CFIB as they were much clearer than the first (not shown).

The results of the splat assays were also unexpected. The SM behaved as expected with high levels of RI activity (small crystals) in the undiluted sample and the activity was removed (larger crystals) as the sample was diluted. The first unusual result was that the LF1 followed the exact same pattern as SM and had almost identical crystal sizes at each dilution suggesting that most of the RI activity was still in LF1. This was further supported by the fact that both LF2 and IF2 had almost no RI activity with only the undiluted samples showing any RI activity (Figure 3.1). Both of these fractions also illustrate the importance of doing a dilution series on the samples to determine RI activity. If only the undiluted sample had been measured then the SM, LF1 and IF2 would all appear to have very similar RI activities. However, the remaining dilutions clearly show that SM and LF1 have much higher RI activity than the IF2 fraction. This phenomenon had been noticed previously in our laboratory and because of this the dilution series had been introduced to confirm RI activity. The reason for the small crystal sizes in the undiluted samples may be due to several different factors e.g. very low levels of RI protein may be able to still have an effect, or different salt mixtures between the different samples. Again this was unexpected because IF2 should have had more RI activity than either LF1 or LF2 (Table 3.4) and this was not the case. These results suggest that the protein(s) responsible for the RI activity are not enriched in the ice fraction i.e. it does not bind to ice. However, the results could also be due to a number of other
factors e.g. the CFIB cooling too fast, the program not long enough or not cooling to a low enough temperature. If the CFIB was cooling too fast it may not allow the RIP enough time to interact with the growing ice crystal and form a strong interaction that would allow incorporation into the ice hemisphere. Similarly if the program was not long enough or not cooling enough a suitable ice surface may not be forming and therefore the RIP could not bind to this either.

To check whether this result was an anomaly or something unique to RI proteins the CFIB protocol was applied to a winter grass extract. This grass extract also has a RI protein i.e. RI activity but negligible thermal hysteresis, just as the P. davidi homogenate does (Kumble et al., 2008).

### 7.1.2 Grass Cold Finger Ice Binding

The protein concentrations (Table 3.2) of the different fractions from the grass CFIB showed that the SM had the highest protein concentration and that the LF1 had a slightly lower protein concentration. This does not seem to make sense as it was expected that the LF1 would be concentrated in this step. However, the higher protein concentration in the SM (0.62 mg·mL⁻¹ in grass against 0.162 mg·mL⁻¹ in P. davidi) may have led to more of the protein being incorporated into the ice fraction through non-specific interactions. Also the smaller amount of sample frozen during each run (about 50:50 for the grass CFIB against 70:30 in the P. davidi CFIB) would mean that the LF1 has not concentrated as much as the P. davidi sample i.e. the total protein is in 50 mL for the grass LF1 against 30 mL for the P. davidi LF1. It may be that a combination of these two effects resulted in the slightly lower protein concentration in LF1 compared to SM. LF2 has a higher concentration of protein than would be expected, but again this may be due to the high concentration in SM leading to more carry over to the LF2. The grass LF2 had a protein level <0.01 mg·mL⁻¹ just as the P. davidi LF2 did.

All samples were tested for RI activity using the splat assay (Figure 3.2). The pattern was very similar to the P. davidi samples. Both SM and LF1 have small crystals in the undiluted sample and these grew slightly larger as the dilution increased. As with the P. davidi SM and
LF1 they are both very similar at each dilution suggesting that most of the RI activity has been retained in the liquid fraction and has not been incorporated into the ice. This is further supported by the fact that IF2 has almost no RI activity even in the undiluted sample. The grass LF2 had a level of RI activity between the LF1 and IF2. This small level of RI activity may be due to the higher protein concentration in the LF2 suggesting a higher carry over of proteins (and therefore RIPs) during the first round of CFIB. As with the P. davidi fractions, how much activity each fraction should have relative to the others can be predicted (Table 3.4) and these indicated that both the LF2 and IF2 should have very similar levels of RI activity (32 and 35 units, respectively) and that LF1 should have the lowest activity of the samples tested (21 units). The splat assay results do not show this. Both SM and LF1 have very high activity followed by LF2 and finally IF2 with the lowest activity (SM = LF1 > LF2 > IF2). Again this is the opposite of what was expected if the activity was being incorporated into the ice as hypothesised.

While these results very closely match the results obtained with the P. davidi sample they still do not help explain why there is no RI activity in either of the IF2s. There may still be a problem with the CFIB equipment or method or there is something unique about the mechanism of RI proteins. To determine if the CFIB equipment and method were capable of purifying ice binding proteins the procedure was used on a Tenebrio homogenate, which contains an AFP that has both RI activity and thermal hysteresis and has been shown to bind to ice previously (Graham et al., 1997; Marshall et al., 2004; Scotter et al., 2006).

### 7.1.3 Tenebrio Cold Finger Ice Binding

The Tenebrio sample behaved in much the same way as both P. davidi and grass samples throughout the CFIB procedure. All three samples have slightly cloudy ice hemispheres after the first round of ice binding but after the second round all hemispheres were very clear (as in Figure 2.5). As with the grass samples, approximately 50% of the sample was frozen during each ice binding run, although the protein concentration followed the same pattern as that from the P. davidi sample with the LF1 having a slightly higher concentration than the SM (Table 3.3). This suggests that the high protein concentration in the grass SM had a
greater effect on the relatively lower LF1 protein concentration than did the amount of sample frozen. The LF2 fraction had a protein concentration approximately half that of the SM, which suggests that more of the protein is being carried over in the ice hemisphere during the first round of ice binding. As with the previous two samples the protein concentration in the IF2 was extremely low (<0.01 mg·mL⁻¹).

The pattern observed in the splat assays for the *Tenebrio* sample is completely different to both the *P. davidi* and grass samples (Figure 3.3). Both LF1 and LF2 have very little RI activity and dilution does not increase the crystal size to any great degree. Both SM and IF2 had small crystal sizes in the undiluted sample (approximately 40 µm and 30 µm, respectively). The crystal size of both SM and IF2 grew as the fraction was diluted until the 8-fold dilution. This suggests that both SM and IF2 have a lot of RI activity and that most of the RI activity from the SM has been incorporated into IF2. Given the expected relative RI activities of each fraction (Table 3.4) it is surprising that both SM and IF2 were so close together in their RI activity (expected activity units of 100 and 40, respectively). Overall these results indicated that the AFP from *Tenebrio* had been incorporated into IF2 as expected. This showed that the CFIB equipment and procedure were functioning properly and are capable of incorporating ice binding proteins (AFPs) into an ice hemisphere.

### 7.1.4 Protein Analysis

To determine if any protein had been incorporated into the ice fractions (IF2) of any samples (*P. davidi*, grass or *Tenebrio*) 1D SDS-PAGE was carried out on all fractions (Figure 3.4, 3.5 and 3.6). These samples behaved as expected; based on both the protein concentrations and RI activities from the previous experiments. All the SM and LF1 fractions have many different bands over a wide range of sizes as would be expected for crude extracts. Both the grass and *Tenebrio* LF2 have some faint bands as expected from the protein concentration. These proteins are likely to be from proteins that were carried over from the first round of ice binding due to non-specific interactions with the ice. The LF2 lane of *P. davidi* has no visible bands suggesting that no proteins were carried over from the first round of ice binding. Both *P. davidi* and grass IF2 have no visible bands in them. This is not a
surprising result given both the extremely low protein concentration and lack of RI activity in both samples. It also supports the idea that there is something unique about the mechanism of RI proteins and that they exert their effect by means other than binding to ice. The *Tenebrio* IF2 lane has a band at about 45 kDa. This is much larger than the reported size for the *Tenebrio* AFP which is approx. 8.4 kDa. The band observed in the *Tenebrio* lane may be due to contamination of the sample and showed up due to a longer developing time used when staining the *Tenebrio* gels. There is also evidence that the native *Tenebrio* AFP is present in a variety of different isoforms and that some of these may aggregate to form the active AFP. Possibly the 45 kDa band is due to the aggregation of the 8.4 kDa monomers and this aggregation is stable in the presence of SDS (M. Sharma, personal communication). The *Tenebrio* AFP has an unusual amino acid composition and is known to be difficult to stain using standard staining techniques e.g. Coomassie or Silver staining (Graham et al., 1997). It is also possible that the 45 kDa band is a previously unreported AFP from *Tenebrio*. To determine the size of any proteins in the *Tenebrio* IF2 fraction it was submitted for intact mass-spectrometry analysis. The IF2 from *P. davidi* and grass were also submitted to the same mass-spectrometry analysis to determine if any proteins were in the sample but were present below the detection limits of the silver stain.

The mass-spectrometry analysis (Figure 3.7) for *Tenebrio* IF2 shows three peaks (m/z of 8258.1, 8346.4 and 8394.0). These peaks are most likely different AFP isoforms expressed by *Tenebrio*. *Tenebrio* is known to have a family of at least 11 genes encoding the AFP. However, it is not known which of these or how many are expressed (Qin and Walker, 2006). Work is currently underway in our laboratory to determine which isoforms are expressed in *Tenebrio* and to try and determine the relative biological importance of these. It is also possible that the different sizes are due to different post-translational modifications, although the published sequences and structures make no mention of any such modifications.

Both the *P. davidi* and grass IF2 samples show no peaks anywhere within the spectrum tested. It is possible that the lack of any detectable protein is because the mass-spectrometry protocol used was not suitable to induce the peptides to fly. However, the same conditions as used for the *Tenebrio* IF2 were used for these samples and so this seems
unlikely. It is likely that there is no protein in the *P. davidi* and grass IF2 samples; this conclusion is supported by the protein concentration, RI activity and 1D SDS-PAGE analysis.

### 7.1.5 Implications

These results taken together suggest that there is something special and unusual about the mechanism of the RI proteins from both *P. davidi* and grass whereby they can exert their effect on ice crystal growth without binding to ice? This is counterintuitive. Surely a protein that affects the growth of ice crystals must interact or bind to ice in some way and so should be able to be isolated through a CFIB method. AFPs, defined as having thermal hysteresis, are thought to act through an absorption/inhibition mechanism where they must bind to the growing ice crystal to increase the curvature of growth (Raymond and DeVries, 1977; Barrett, 2001; Pertaya *et al.*, 2008). This leads to a decrease in the freezing temperature. All AFPs also have RI activity and the two properties have always been assumed to be linked (Smallwood *et al.*, 1999; Gilbert *et al.*, 2004). However, recent experiments have been able to remove the thermal hysteresis activity while retaining the RI activity. Gibson *et al.* (2004) created a library of small synthetic polymers that were screened for RI activity and found that the –OH group is essential for RI activity. This suggests that the RI activity of AFPs may be a side effect of having a large proportion of –OH (mainly from threonine) on the ice binding surface rather than the RI activity being an important part of the AFP function (Gibson *et al.*, 2009; Balcerzak *et al.*, 2014). Liu and Ben showed that the thermal hysteresis and ice shaping activity of AFGP analogues could be removed while retaining the RI activity (Liu and Ben, 2005). John *et al.* developed a structural model of a RI protein from an Antarctic grass *Deschampsia antarctica* based on the gene sequence (John *et al.*, 2009). This model was very similar to the AFP from perennial rye grass (*Lolium perenne*) (Kuiper *et al.*, 2001). The *D. antarctica* RI protein has a putative ice binding face, the same as *L. perenne*, but with fewer threonines (about 30-40%), which is hypothesised to be related to the different functions of the proteins (AFP vs. RIP) (John *et al.*, 2009). *Deschampsia antarctica* is freeze tolerant and so the protein only needs enough threonines (-OH groups) to enable it to prevent recrystallisation once frozen. It is even suggested that some thermal hysteresis may be detrimental to the survival of *D. antarctica*. If the water supercools (as caused by thermal hysteresis) when it does freeze it may happen too quickly meaning the protein
cannot control the ice crystals (RI). The resulting dendritic growth may damage the cell membranes and structures (John et al., 2009). This may mean that thermal hysteresis and RI are caused by two different mechanisms, both of which are present in the AFPs, but only the RI function is inherent in RIPs.

The mechanism of RI proteins activity remains unknown. However, these results suggest that they work without binding to the ice. This would imply that they are able to control the movement of liquid water between the ice crystals. To do this they would have to immobilise or bind all of the liquid water around the ice crystal. This seems like a difficult way of controlling ice growth. However, after freezing, very little water remains in the liquid state. For a 0.1 M KCl solution > 80% of the water is frozen at -2°C and this increases to >95% at -5°C (Williams and Meryman, 1965). If each RI protein is able to bind a large number of water molecules it may require fewer RI proteins to trap all the water molecules than to cover the ice surface (as AFPs do) and prevent RI. This idea is supported by the presence of RI activity of AFPs at concentrations below which they are able to produce thermal hysteresis (Feeney and Yeh, 1998). A mechanism has been proposed where the RI agent (small carbohydrates in these examples) can sit in the liquid layer between ice crystals (Tam et al., 2008; Abraham et al., 2015). This leads to a disordering of the water molecules and this in turns prevents the water molecules joining the quasi-liquid layer (the slightly ordered layer just outside the actual ice). Without the quasi-liquid layer being ordered the water molecules cannot arrange themselves into an ice structure. This mechanism explains how RI proteins could prevent the recrystallisation of ice without binding to the ice, which is supported by our results (Tam et al., 2008). Although this group only used mono- and disaccharides, there is no reason why this mechanism could not also work with proteins as long as they are able to interact with the water molecules in a similar way and were able to fit in the quasi-liquid layer.

**7.1.6 Cold Finger Ice Binding Conclusion**

I have shown that both *P. davidi* and grass homogenates do not have any proteins that are able to be incorporated into a growing ice hemisphere and therefore no proteins that are able to bind to ice. The absence of protein from the ice fractions was not due to equipment or methodological failures, as demonstrated by the ability to isolate a protein consistent
with the Tenebrio AFP from a crude homogenate of Tenebrio. Therefore ice binding is not a property of RI proteins. This means that the RI activity of these proteins must be due to some mechanism that does not require ice binding. This mechanism is currently unknown but may require the RI protein to be able to sit in the still liquid water between the ice crystals and prevent the water molecules migrating between different ice crystals therefore preventing recrystallization, or disrupting the water molecules and inhibiting the formation of the quasi-liquid layer which in turns disrupts further formation or change of ice crystals.

7.2 Screen Development Discussion

Due to the time-consuming nature of splat assays it was desirable to develop a screening technique that would allow the rapid identification of chromatography fractions that have RI activity. To do this I have tried three different methods based on various properties of RI activity i.e. protection of LDH activity during freezing or the appearance of the sample after freezing. Overall this was relatively successful, with two of the methods showing promise.

7.2.1 LDH Method

Initial experiments were aimed at determining the dilution of LDH required to obtain a change of absorbance in the range of 0.02 – 0.05 ΔAU·min⁻¹. A reaction rate in this range prevents the substrate being used up too quickly, which in turn prevents the reverse reaction occurring, thus maintaining 1st order kinetics. To determine the dilution of LDH necessary to obtain this rate, a series of dilutions were made and tested for LDH activity (Figure 4.1). The 1 in 10 dilution has a very flat line with a low level of absorbance suggesting that all the substrate was used up very quickly before the assay could be measured. The 1 in 100 dilution had a slope of approx. 0.25 AU·min⁻¹. This rate is much higher than what is required and the slope is also not linear. Both the high rate and non-linear slope mean that the 1 in 100 dilution is also too high to use in future experiments. The 1 in 500 dilution produces a linear line with a slope of approximately 0.0451 ΔAU·min⁻¹. This is within the range that was aimed for in terms of a rate of reaction; just to ensure that the 1 in 500 dilution was optimal, a 1 in 1000 dilution was also checked. This also produced a linear rate but the slope was only 0.0132 ΔAU·min⁻¹, too low to detect small changes in LDH
activity. These results show that the 1 in 500 dilution is the optimum dilution of LDH to use in the LDH protection assay.

Once the optimum dilution of LDH had been determined some preliminary work on the method could be done to see if it may be suitable for a screening method. The first thing to be established was whether or not LDH could be protected from freeze-induced loss of activity. To do this both fresh homogenates of both *P. davidi* and *Tenebrio* were made (Method 2.3.3.1 and 2.3.3.2) and homogenates that had been frozen at -18 °C were also tested. A fresh 1 in 250 dilution of LDH was made and diluted 1:1 with Type I water (final dilution of LDH 1 in 500) and this was tested for LDH activity and defined as 100 % activity. The 1 in 250 dilution of LDH was also diluted 1:1 with either the fresh or previously frozen homogenates and all samples were frozen. After freezing all samples were tested for LDH activity and compared to the original fresh LDH activity (Figure 4.2). It is clear that Type I water does not protect the LDH from freeze damage as this sample has almost no activity after freezing. Conversely all of the *P. davidi* and *Tenebrio* homogenates were able to protect most or all of the LDH activity, with the previously frozen *P. davidi* homogenate having the lowest activity after freezing although this was still > 80 % of the original activity. Because both *P. davidi* and *Tenebrio* homogenates protect the LDH it suggests that the protection may be due to RI activity rather than AFP activity (*P. davidi* samples have no AFP activity). It also appears that there is some difference between the fresh and previously frozen samples and that this difference is greater in the *P. davidi* homogenate than in the *Tenebrio*. These differences may be due to freeze/thaw damage or, more likely, due to different RI activities of the original samples. To minimize the differences from this point on only the homogenates that had been made up fresh were used. This meant that the homogenates were exposed to fewer freeze-thaw cycles and so any damage due to this was minimized. Overall these results are very promising and show both *P. davidi* and *Tenebrio* homogenates may be able to protect LDH from freeze-induced damage and that this method may be suitable for use as a way of rapidly screening for RI activity.

Before I can be sure that the LDH activity after freezing is from RI activity protecting the LDH, the possibility that the high LDH activities in the *P. davidi* and *Tenebrio* samples are due to endogenous LDH activity in the homogenates must be ruled out. To do this I repeated the same experiment, but added Type I water instead of LDH to the homogenates. There was
also a control sample of LDH plus Type I water. The samples were chilled (4 °C) to check that it was the freezing of the sample that caused loss of activity and not an effect of being left at a low concentration for a period of time at which LDH is known to lose activity (King and Weber, 1986). After freezing or chilling the sample for 4 h, the LDH activity of each sample was measured. The fresh LDH (control) had high activity that is only decreased slightly by chilling and was almost completely destroyed by freezing, as seen previously. There is almost no activity in either the fresh, chilled or frozen *P. davidi* or *Tenebrio* samples (Figure 4.3). These results clearly demonstrate that there is very little endogenous LDH activity from either of the homogenates.

Another test of whether the LDH was actually being protected by the homogenate or if the homogenate is somehow enhancing the activity after freezing was to check the effect of removing the substrate (pyruvate) from the LDH assay cocktail. I did this because even though there is very little native LDH activity in the homogenates it is possible that the homogenate is contributing some pyruvate (or another substrate) to the reaction conditions. This may have the effect of increasing the activity of the still active LDH and making it appear that the homogenates are protecting the LDH from freeze damage when they are only enhancing the activity of the remaining active LDH after freezing. Prior to freezing, and in the presence of both NADH and pyruvate, all three samples have high activity with both the *P. davidi* and *Tenebrio* samples having slightly higher activity than the LDH alone (Figure 4.4). When pyruvate is not included in the reaction, samples show no activity prior to freezing, as expected. This shows that the *P. davidi* and *Tenebrio* homogenates are not providing any pyruvate (or other substrate) for the LDH to act on. Again the results after freezing were checked to ensure that the freezing process was not somehow activating or releasing some substrates for the LDH. The result is as expected with both the *P. davidi* and *Tenebrio* samples providing some protection to the LDH during freezing but the LDH without either homogenate (Type I water) showed no activity after freezing. These results again support the assumption that the *P. davidi* and *Tenebrio* homogenates are protecting the LDH during freezing rather than enhancing the activity that remains after freezing.

The final test was to confirm that the apparent freeze protection by the *P. davidi* and *Tenebrio* homogenates was due to something specific to them (i.e. RI activity) rather than some general protection that is conferred due to non-specific protein interactions. To
determine this LDH protection assays were carried out using different concentrations of BSA. The BSA concentration did not affect the LDH activity with all fresh samples having LDH activity close to 100 % of the LDH with Type I water sample (Figure 4.5). The BSA concentration had very little effect on the LDH activity after chilling also. All the samples have an LDH activity of close to 100 % of the fresh LDH activity. The frozen samples, however, show a concentration dependent pattern. The LDH with Type I water (0 mg·mL⁻¹ BSA) has lost nearly all of its activity after freezing, as would be expected from previous experiments. By adding 0.05 mg·mL⁻¹ of BSA to the sample resulted in approximately 50 % of the LDH activity being retained after freezing. As the concentration of BSA added to the sample was increased the percentage of LDH activity after freezing increased, with 90 % of the activity still present after the freezing when 0.2 mg·mL⁻¹ of BSA was added (Figure 4.5). This result shows that the LDH activity can be protected by adding relatively low concentrations of BSA to the sample. Given that BSA has not been reported to have either antifreeze or RI activity (at these concentrations) this is a surprising result. Whereas a very high concentration of BSA may have been expected to provide some protection to the LDH due to non-specific interactions e.g. colligative effects, to have this protection appear at such low concentrations was unexpected. It suggests that any protein, even at relatively low concentrations, may give positive results in this assay. The concentration of both the P. davidi and Tenebrio homogenates are in the 0.3-0.5 mg·mL⁻¹ range (not shown) which is around the concentration of BSA required to retain approximately 100 % of LDH activity after freezing and this is exactly the level of activity observed (Figure 4.2 and Figure 4.4). This means that the LDH protection observed in the P. davidi and Tenebrio homogenates may be due solely to the protein concentration of the sample rather than any special properties of the homogenate e.g. RI activity. This in turn suggests that this version of the LDH protection assay is not suitable as a method to screen for RI activity (Wisniewski et al., 1999). This does not mean that other screening methods using LDH activity are not valid, but extra controls may need to be included to ensure that any positive results are due to RI (or AFP) and not non-specific protection (Griffith et al., 2005; Pruitt et al., 2007; Jarzabek et al., 2009).
7.2.2 Nanolitre Osmometer Method

After determining that the LDH method was not going to be suitable as a screen for RI activity it was necessary to attempt another method. The next method tested used a nanolitre osmometer (Otago Osmometers) that is more commonly used to determine either thermal hysteresis or ice crystal shape. This instrument has a large surface that is temperature controlled (to set up a large number of samples) and can freeze the samples quickly resulting in a frozen disc with a large number of small crystals that could grow over time if there was no RI activity. The aim was to develop a method similar to that described by Tomczak et al. (2003) using available equipment.

Grass extract was used as an RI-positive sample and diluted both 10-fold and 100-fold and then assayed. The result of this initial experiment was very promising and showed that there were clear differences as the grass extract was diluted (Figure 4.6). As the extract was diluted the appearance of the sample after the holding period got less uniform and more ‘patchy’ or ‘starry’. The reason for the increase in the ‘patchy’ appearance is probably due to the change in ice crystal size. As the crystals grow they will lose uniformity of size and there will be crystals of many different sizes, which will show up as ‘patchy’. The ability to detect RI activity in a typical set of chromatographic fractions was then tested.

A simple cation exchange chromatography method was used and five fractions collected throughout the run (from injection until after the gradient was complete). These fractions were then tested using both the nanolitre osmometer method and splat assay (not shown). The splat assay showed that both fractions 2 and 3 had more RI activity than any of the other fractions. When the fractions were tested using the nanolitre osmometer (Figure 4.7A-E) all looked very ‘patchy’ and similar to the buffer (Figure 4.7F), suggesting that all had very low RI activity. This was not useful as a screen as it means that unless the sample has extremely high RI activity, it may not show up as positive and is instead a false negative. The nature of chromatography means that the amount of activity in any single fraction will be somewhat less than that of the injected sample because of dilution. Because of this, it is necessary for any screening methods to be able to detect low levels of activity. The findings of this experiment suggest that this method will not be sensitive enough to detect the level of RI activity that could be expected in chromatography fractions.
To try and increase the sensitivity of the test, powdered graphite was added to emphasise the crystal boundaries. It was hoped that the addition of graphite would make the crystal boundaries easier to see because it would be excluded from the ice crystal i.e. it does not bind to ice and it would therefore concentrate in the boundaries between the crystals. It was anticipated that concentration of the graphite in the crystal boundaries would help differentiate between samples with intermediate crystal sizes. The addition of graphite was successful in that it made the crystal boundaries easier to see (Figure 4.8). However, it did not make it significantly easier to determine the crystal size, or differentiate between samples with intermediate RI activity and so the presence of graphite was not helpful in improving the sensitivity of this assay.

Overall this method showed some promise: it would have been easy to do up to 20 samples at a time (and possibly more), there were obvious differences between samples with high RI activity and no RI activity, and very little sample was used (2 µL). However, due to the difficulty in identifying samples with intermediate levels of RI activity, even with the addition of graphite to emphasise the boundaries, and the high level of subjectivity in identifying samples with RI activity, I decided not to continue developing this method. Nonetheless, it is probable that this approach could be developed into a successful screening method for RI activity.

### 7.2.3 Optical Recrystallometer Method

The third method used an optical recrystallometer. This measures the transmittance of light through a sample and is calibrated so that 100 units corresponds to all light transmitted, and 0 units to no light transmitted. Previous work had shown that if a frozen sample was held at a high sub-zero temperature there would be a change in the transmittance of light over time depending on the level of RI of the sample i.e. samples with high RI did not change transmittance much over time whereas samples with no RI had a large change in transmittance over time (Wharton et al., 2007).

Samples with a range of RI activities were tested to ensure that the OR method would be able to identify samples with and without RI activity. To do this five different samples (P.
*davidi* homogenate, *Tenebrio* homogenate, grass extract, BSA and beef heart extract (F1)) were tested with varying protein compositions. To make sure that the protein concentration was not having an effect on the RI activity all samples were standardised to 1 mg·mL$^{-1}$ and serial dilutions made (1:1) to a lowest concentration of 0.0625 mg·mL$^{-1}$. To ensure that there was a range of RI activities across the samples they were analysed using the splat assay.

The splat assays show that there is a range of activities (Figure 4.9). Both the grass and *Tenebrio* samples have high RI activity, shown by their small crystal size in the higher concentration samples that is slowly diluted out as the concentration decreases. In fact the *Tenebrio* sample still has more RI activity at its lowest concentration than either F1 or BSA at 1 mg·mL$^{-1}$. The *P. davidi* sample has an intermediate level of RI activity with slightly larger ice crystals at 1 mg·mL$^{-1}$ (about 50 µm) that increase in size as concentration decreases before losing RI activity at 0.125 mg·mL$^{-1}$. The F1 sample has almost no RI activity with relatively large crystals at 1 mg·mL$^{-1}$ (about 70 µm) and then crystals >100 µm from the 0.5 mg·mL$^{-1}$ sample onwards. The BSA sample appears to have no RI activity as the most concentrated sample (1 mg·mL$^{-1}$) has large ice crystals (about 90 µm) and these sizes change very little across the concentrations tested. These results were as expected with grass, *Tenebrio* and *P. davidi* extracts all having been reported as having thermal hysteresis and RI activity. Conversely neither F1 (beef heart extract) nor BSA was expected to have RI activity.

All samples were tested in the OR at three different holding temperatures (-4 °C, -6 °C and -8 °C) and the OR readings were taken at 0, 1, 4 and 24 h to examine the effect of both holding temperature and holding time. Both the holding temperature and holding time have significant effects on the reading obtained from the OR (Figure 4.10, 4.11 and 4.12).

When the holding temperature was -4 °C (Figure 4.10A and B) the change in OR readings was very quick with most of the samples reaching a maximum reading (> 90) after only 4 h. It was expected that the -4 °C samples would change very quickly because as the holding temperature gets closer to the melting point of the sample the recrystallisation rate increases. The rapid change in OR reading may suggest that -4 °C would be a good temperature for a screening method due to its speed. However, it also means that small changes in the initial (0 h) reading may have greater effects. Furthermore some of the
samples have very similar readings in both the grass (high RI activity) and F1 (low RI activity) which suggests that it may be hard to separate samples with RI activity and those that do not (especially in the 1 h readings). When the holding temperature was decreased to -6 °C, the difference between the samples (grass and F1) was much more noticeable, especially at the 24 h reading. The grass reading stayed very low (about 20) throughout the entire time, whereas the F1 sample changed significantly during the 24 h. The 24 h readings were especially encouraging because of the wide spread of values, that also increased with decreasing concentration (therefore decreasing RI activity (Figure 4.11)). The -6 °C results suggested that by decreasing the holding temperature, both high and low RI activity could be identified. When the holding temperature was decreased further to -8 °C there was almost no change in the OR readings over 24 h for either sample (Figure 4.12). This may be due to -8 °C being too cold and not allowing enough recrystallisation to occur in the sample to produce a noticeable change in the OR reading within 24 h. These results taken in combination suggest that -6 °C for 24 h is the optimal holding temperature and time to identify samples with or without RI activity. Samples held at higher temperatures (-4 °C) change too quickly and those held at lower temperatures (-8 °C) do not change enough. Also, samples held for less than 24 h did not change enough (except in the case of -4 °C) to confidently and consistently identify samples that have RI activity. From these results it was decided that 24 h was the best holding time for three reasons, the first is that it gives the most time for recrystallisation to occur, making changes easier to observe. The second was that at 24 h nearly all samples had increased in value, which is not always the case at earlier times. In a previous paper using the OR to detect RI activity, it was concluded that any change in OR reading (increase or decrease) was a sign of recrystallisation occurring (Wharton et al., 2007). The third reason is that small changes in the OR reading at 0 h (e.g. 30 instead of 15-20) can have a large impact on the apparent rate of recrystallisation i.e. the OR readings will reach higher values much quicker (data not shown). By using the 24 h OR readings it minimizes the effect of any small differences in the initial readings that could have an effect on the final OR reading. These small changes may represent differences in RI activity that show up during the short warming time, but by waiting for 24 h these changes would also become more obvious and prevent any random small differences in the initial values affecting the final interpretation of the results.
After deciding that 24 h was the optimum time for holding the samples, the change in transmittance of samples with high and low RI activity (as determined by the splat assay) were tested. All samples were measured at -4 °C, -6 °C and -8 °C and the 24 h OR readings plotted, together with ice crystal size as measured by the splat assay (Figure 4.13). In every case the -4 °C reading indicated a high level of transmittance (>90), suggesting that it is too high for a holding temperature since recrystallisation occurs too quickly. With the -8 °C samples the OR readings started low (about 10-20) and remained low (maximum of 30), indicating that the changes are too small to determine if a sample has RI activity or not. The -6 °C samples appear to have a good range of readings that match the changes expected based on RI activity. Both grass and Tenebrio have high RI activity and both have very small changes in OR reading at 24 h as the concentration decreases (Figure 4.13A and B). This is as expected since as the concentration decreases so does the RI, leading to more recrystallisation and a larger increase in transmittance. The P. davidii sample has a moderate amount of RI activity, which is lost at the lowest concentration (0.0625 mg·mL$^{-1}$); this suggests that there is some RI activity but it is diluted out (Figure 4.13C). The F1 sample has a small amount of RI activity, which is diluted out very quickly (Figure 4.13D). This is also seen in the splat assay (Figure 4.9). The BSA sample also has very high readings (>90) for all concentrations except for 1 mg·mL$^{-1}$, but this reading is still higher than any other 1 mg·mL$^{-1}$ samples (Figure 4.13E). When these results are taken in together it is very clear that the 24 h OR readings for samples held at -6 °C can be used to test for RI activity and samples with low OR readings have more RI activity than those with high OR readings.

This method has several advantages over other RI tests. The sample can be recovered, which may be important, and it is quick and easy to perform on a large number of samples. It gives an absolute number, making the test less subjective e.g. any samples with a value <90 can then be tested using the splat assay to confirm RI activity. Also there appears to be a lag between the ice crystals reaching a large size (>90 µm) and the OR reading reaching its maximum value (Figure 4.13). This greatly reduces the chances of samples with intermediate to low levels of RI being missed (false negatives). While this suggests that this method may be a more sensitive method to detect RI activity than the splat assay, it is still possible that what is being followed is not recrystallisation but some other closely linked phenomenon. Because of this it is necessary to confirm RI activity of samples using the splat
assay as this allows individual crystals to be measured and confirm RI activity in the sample as opposed to some other phenomenon that results in a change in the OR readings. To ensure consistent results when using this method it is important to have a low 0 h reading (ideally <15). To ensure this it is important to maintain the temperature of the ethanol/dry ice slurry by adding dry ice regularly and to minimize the time the samples spend out of the Al block i.e. transfer the frozen sample to the Al block as quickly as possible. Another problem with this method is that it is sensitive to elevated salt concentrations (Figure 4.14). If the method was not sensitive to salt, all readings taken at the same time would be expected to be very similar; this is clearly not the case. There appears to be a critical point at which the amount of salt becomes problematic (approximately 0.4 M NaCl). Once the salt is above this level there is a very rapid increase in the transmittance value. The reason for this increase is probably due to the salt decreasing the freezing point of the solution and this means that there is more liquid water that is able to migrate between the crystals and results in faster recrystallisation. One possible way of preventing the problem is by dialysing the sample after the chromatography run but before the OR analysis. This is not unexpected as the splat assay can also be affected by salt levels and some samples required dialysis before they can be accurately assessed in this assay (Knight et al., 1988, Knight et al., 1995). However, dialysis of a large number of samples is time-consuming and cumbersome.

7.2.5 Screen Development Conclusion

In Chapter 4 I have attempted to develop a method that would allow RI activity to be screened but would remove the need to test all fractions and samples with the time-consuming splat assay. To do this three different methods were tested, each focussing on a different effect RI may have on the sample. The first method was based on the protection of LDH activity during freezing and was shown to be ineffective as a screen because the protein level of the sample was also important i.e. even low levels of BSA (no RI activity) were able to induce an effect similar to samples with RI activity. The next method used a nanolitre osmometer to observe many samples at the same time under a microscope. This method showed promise but was too subjective, had difficulty detecting intermediate levels of RI and there was a high chance of missing samples with RI (false negatives). The final method used an optical recrystallometer, which measures the change in light transmittance through
a sample. This is known to be related to the amount of RI in the sample (Wharton et al., 2007). Several different temperature and time combinations were tested and it was determined that 24 h at -6°C was the optimum combination for this method. This method was used for further work because it gave a value (OR reading) and this would remove any subjectivity from selecting samples. It was also unlikely to give false negatives, meaning it would be unlikely any samples with RI activity would be missed.

7.3 Protein and Chromatography Discussion

In Chapter 5, I aimed to characterise some of the basic properties of the causative agent of RI activity from *P. davidi* including proteinase K sensitivity, ammonium sulfate solubility, heat sensitivity and salt dependence. I also attempted to purify the RIP using classical chromatography techniques.

7.3.1 Proteinase K

Although it had been assumed that a protein was responsible for the RI activity of the *P. davidi* and grass homogenates, I was unable to find any report of this in the literature. This was important to determine as recently there has been several reports of small, non-protein molecules e.g. carbohydrates, that have been shown to have RI activity (Wu et al., 2011; Capicciotti et al., 2012). To determine if the activity was due to a protein a series of proteinase K digestions were carried out on *P. davidi* grass and *Tenebrio* homogenates. If a protein was responsible for RI activity the activity would be lost after proteinase K digestion. The samples were incubated with proteinase K for 1, 6 or 24 h (at 37 °C) to see if there were any time-dependent effects. *Tenebrio* was included as a control because in this case it is known that a protein is responsible for RI activity (*TmAFP*) (Graham et al., 1997; Liou et al., 2000). To ensure that any loss of RI activity was due to the action of proteinase K, rather than the activity being heat sensitive i.e. activity was lost due to 24 h at 37 °C, a control where the proteinase K was replaced by Type I water was included. The final control was to ensure that the proteinase K itself did not have any RI activity. To confirm this, 5 µL of proteinase K was added to 1 mL of Type I water and then treated it the same as the homogenate samples.
In all cases both SM and SM + H$_2$O (negative control) samples had very similar crystal sizes (Figure 5.1 A-C). This suggests that whatever causes the RI activity is not heat sensitive, at least not at 37 °C for 24 h. This means that any loss of activity can be attributed to the effect of proteinase K. In nearly every case the 24 h digestion had larger crystals than the 6 h digestion, which had larger crystals than the 1 h digestion. The only exception was the 8-fold dilution for *P. davidi* homogenate and the difference in this is not large. This time-dependent loss of RI activity was expected because as the protein is exposed to PK for longer, more digestion will occur and more activity will be destroyed.

The 1D SDS-PAGE results also support the assumption that the loss of RI activity is due to protein degradation. In both the *P. davidi* and grass gels (Figure 5.2 and 5.3) both the SM and SM + H$_2$O lanes are very similar, which suggests that the proteins are all very stable. They are not affected by exposure to relatively high temperatures for prolonged periods of time (37 °C for 24 h). This was expected given that both the SM and SM + H$_2$O samples had very similar RI activity (Figure 5.1A-C). Interestingly the SM and SM + H$_2$O lanes for the *Tenebrio* sample are different (Figure 5.4). The SM lane is very dark/intense and no individual bands can be identified; however, after exposure to 37 °C for 24 h much of this intensity disappeared and three distinct bands are left exposed. This suggests that the *Tenebrio* homogenate is more heat sensitive than either the *P. davidi* or grass homogenate. However, given that the RI activity of both the SM and SM + H$_2$O are nearly identical it seems that the *Tm*AFP must be relatively heat stable even if many of the other proteins in the homogenate are not (Figure 5.1C and Figure 5.4). The incubated lanes all have bands that line up with the band in the H$_2$O + PK lane. The size of this band (approximately 30 kDa) is consistent with the reported size of PK and is most likely the PK added to the homogenates (Jany and Mayer, 1985). The *P. davidi* gel (Figure 5.2) also has many low molecular weight bands at the bottom of the gel. These are fragments of the proteins that have been digested by the proteinase K that have not run off the gel. This is supported by the fact that the 24 h lane has much fainter bands than either the 1 or 6 h incubations and is presumably due to the longer incubation allowing for more complete protein digestion. The grass gel (Figure 5.3) only has the proteinase K band (30 kDa) in each of the incubated lanes, suggesting that the proteins were broken down to very small fragments in a short time (< 1 h). The *Tenebrio* gel (Figure 5.4) is very similar to the grass gel, with only faint bands that
match to the proteinase K (30 kDa) and some very faint digestion fragments at the bottom of the gel. The gel results suggest that the protein is destroyed very quickly by the proteinase K (< 1 h). However, splat assays suggest that it takes much longer than this to destroy the RI activity (at least 6 h). This may be because small fragments of the protein are enough to maintain RI activity and it may take longer than 1 h to destroy these small fragments and render them inactive. Many AFPs are known to come in different isoforms that have different activity and often the difference in the activity is due to a different number of the repeating unit of the AFP e.g. more repeats of the loop leads to a greater TH gap (Graether et al., 2000; Leinala et al., 2002b; Marshall et al., 2004). It is possible that only 1-2 (20-30 amino acids) of these loops may be enough to maintain RI activity but not TH activity. The initial digestion may break down the AFP into the individual loops (therefore lower molecular weight bands) and then more time is required to digest the loops. This may be due to the peptide bonds being difficult to access or the amino acid sequence being less favourable for the proteinase K digestion (Hilz et al., 1975; Betzel et al., 1986). Disulfide bonds may play an important role in holding the protein together and enable the protein to be more resistant to proteinase K digestion (Liou et al., 2000, Marshall et al., 2004). Regardless of the exact reason for the lag between protein breakdown and loss of RI activity, it is very clear that whatever is responsible for the RI activity of both P. davidi and grass homogenates is sensitive to proteinase K digestion and is therefore most likely a protein.

**7.3.2 Other properties**

Because it was determined that a protein was responsible for RI activity some other properties of the protein were investigated to see if any of these may be useful for initial purification steps.

**7.3.2.1 Metal Ion Dependence**

Some AFPs (type II) are homologous to the carbohydrate binding domain of C-type lectins and some of these require Ca\(^{2+}\) for activity. It was important to check whether the RIP had a similar requirement (Gilbert et al., 2005). To test whether the RIP was dependent on metal ions a metal ion chelator was added (EDTA to a final concentration of 5 mM). From the crystal sizes it is clear that the RI activity was not affected by EDTA with the crystal size of
both the samples almost identical at every dilution tested (Figure 5.5). This meant that the addition of 5mM EDTA had no effect on the RI activity and therefore the RIP does not require any metal ions for activity or they are so tightly bound that they cannot be stripped away by EDTA. Based on other work it seems unlikely that the Ca$^{2+}$ could not be removed using EDTA, as the Ca$^{2+}$ is routinely removed using either dialysis or chelating agents (Gilbert et al., 2005; Guo et al., 2013)

### 7.3.2.2 Heat Sensitivity

Heating the *P. davidi* homogenate had very little effect on RI activity. After 30 min at 95 °C the activity of both the heated and unheated supernatant was very similar. This suggests that the heating did not affect RI activity. Both heated and unheated pellets also had very similar crystal sizes, although these were much larger than those for the supernatants. This means that there was very little RI activity in the pellet and that heating the sample did not increase activity in the pellet. It is possible that heating the sample resulted in irreversible damage to the RIP and this would also result in the pellet having large crystals. If this was the case a loss of activity in the supernatant would be expected i.e. larger crystals, but this is not the case. The RIP is not heat sensitive and remains in solution after heating to 95 °C for 30 min (Figure 5.6). This may be useful as a first step in purification as many of the other proteins will denature after 30 min at 95 °C, therefore removing many interfering proteins.

### 7.3.2.3 Ammonium Sulfate Precipitation

The ammonium sulfate precipitation produced some very interesting results. Despite starting with a homogenate that had a high level of RI activity (small crystals) neither the 50 % supernatant nor pellet had any RI activity (large crystals) (Figure 5.7). Somehow the RI activity was lost during the precipitation. This is unusual as proteins usually redissolve when the ammonium sulfate is removed. However, it is clear that the activity is affected by ammonium sulfate precipitation (O’Conner et al., 1973; Madhusudhan et al., 2008; Liu et al., 2010). When the original sample was reconstituted (by mixing the supernatant and pellet in equal amounts) a large proportion of the RI activity was recovered. This suggests that RI activity was not destroyed by precipitation but different components ended up in different fractions. It may be that it requires more than one component to be active and that these components were separated during the precipitation and recombining them allows them to
regain activity. This is an interesting finding as no AFP have been reported as requiring aggregation to have TH activity (Yamatodani et al., 1985; Krenkova et al., 2009). Many INPs do require aggregation to have activity because it is thought many subunits are required to form a complex large enough to act as nucleation site for water (Kajava and Lindow, 1993; Zachariassen and Kristiansen; Duman, 2001). In addition to this experiments using combinations of small molecules (sugars) have suggested that an element of cooperativity may be involved in the mechanism by which they inhibit recrystallisation (Abraham et al., 2015). Since the mechanism of RI activity is not known, but does not require ice binding (Chapter 3), it is possible that a large complex must be formed to be able to control the movement of the still liquid water between the ice crystal boundaries or to cover the surface of the ice crystals and prevent the binding of new water molecules. If this is the case, it is possible that during the precipitation this complex is dissociated and that some components go to the supernatant and some go to the pellet, and combining these two parts allows the restoration of RI activity.

7.3.3 Chromatography

Due to the results of the ammonium sulfate precipitation i.e. activity lost by precipitation, I chose to load crude P. davidi homogenates (Method 2.3.3.1) onto the chromatography columns. Initially, step gradients for both ion exchange and HIC were used to determine if the activity would bind to the column and could be recovered. If this was the case a gradient could be utilised to purify the protein further.

7.3.3.1 Ion Exchange Chromatography

In the anion exchange (Q Sepharose) chromatography (Figure 5.8A) there was a small absorbance peak near the start of the run and a large peak between 15-20 min. These peaks correspond to the flow through (unbound) and bound proteins. The cation exchange (SP Sepharose) chromatography (Figure 5.8B) has an inverted peak pattern with a large flow through peak near the start of the run, followed by a smaller bound peak at 15-17 min. This was an unusual finding as the pH of the solution had been lowered to promote binding to the cation exchange resin and therefore it was expected that most of the proteins would bind to the column and a chromatogram similar in shape to that from the anion exchanger.
(Figure 5.8A) would result. One explanation of this could be that the sample had not equilibrated properly in the cation exchange (pH 6.0) buffer. This seems unlikely as the samples were dialysed overnight and this is a standard procedure of exchanging buffer. The result was also the same each time the procedure was carried out (> 3 times) (Meyer et al., 1991; Bassé et al., 1996; Porter et al., 1998). Another explanation of these results may be that many of the P. davidi proteins have a low pl e.g. <6.0. If this is the case, at pH 8.0 (anion exchange buffer), most of the proteins would have a negative charge and bind to the positively charged column. Because the pH of the cation exchange buffer was only lowered to 6.0 many of the proteins would have maintained a negative or no charge and therefore would not bind to the negatively charged column. The IEF step of the 2D gel electrophoresis (see Chapter 6) did not indicate an abnormally large proportion of low pl proteins i.e. the spread of proteins in the first dimension was relatively even. Whether this is the case or not and if or how this would give an evolutionary advantage to P. davidi requires further investigation.

Both the flow through and bound peaks from each chromatography run were collected and pooled to give a total of four fractions; Q flow through, Q bound, SP flow through and SP bound. Each of these fractions were dialysed overnight into 25 mM Tris, pH 8.0 and tested for RI activity. In all cases the fractions had less RI activity (larger crystal sizes) than the diluted SM (Figure 5.9). This suggests that the fractions have very low or no RI activity. There are several possible reasons for this loss of RI activity. Based on the findings of the ammonium sulfate precipitation, it may be that a potentiating protein or complex is being separated and the individual and separated components are not active (Shi et al., 1995; Banci et al., 1998; Fernandez-Lafuente, 2009). This seems unlikely, especially in the anion exchange, as most of the proteins (> 95 %) appear to have bound to the column. I would expect that the potentiating protein or the parts of the active complex would be in the bound fraction, unless a) the potentiating protein/active complex were split up during the chromatography or b) the complex is large and made up of many individual proteins and so at least one of these would end up separated. Even if the complex was split up during the column binding, all of the bound fractions were pooled together and so, based on the ammonium sulfate results (Section 5.3.2.3), it would be expected that at least some of the activity would be recovered in this fraction. Another possibility is that the protein is
somewhat heat labile and that the chromatography process leads to some heat induced loss of activity. Based on the results of the PK and heat stability experiments this would seem unlikely as neither 24 h at 37 °C or 30 min at 95 °C had any effect on the RI activity of the homogenate (Figure 5.1A and Figure 5.6).

7.3.3.2 Hydrophobic Interaction Chromatography

All of the HIC chromatograms look very similar (Figure 5.10 A-C). All exhibit a large absorbance peak near the start of the run (approximately 1-6 min) and a very small diffuse peak at approximately 15-25 min. The conductivity is also very similar in all the chromatograms. There is a small change in the absorbance that corresponds to the large absorbance peak and then from the 15th min until the 25th min the absorbance decreases, with a small absorbance increase at the 20th min. From here a steady rise is seen until the original level at the end of the run (30 min).

These chromatograms are not as expected. This method was set up as a step method, to determine if and where RI activity would bind to the HIC columns. This would be useful information for development of a gradient method if it was successful. However, given the running conditions used it appears that an effective step gradient was not produced, in fact it was nearly a linear gradient with only a small kink where the step should have been. This may have been overcome by altering the method so that each part of the step was given longer to equilibrate e.g. 10 min at 50 % buffer A instead of the 5 min used (Method 2.3.16.1). Given the apparent lack of protein binding to the columns, as indicated by the large flow through peaks and small elution peaks it seems that the binding conditions were not optimal. This is strange as the sample was dialysed against a high salt buffer (HIC buffer A) which should have promoted binding to the columns (Queiroz et al., 2001; Lienqueo et al., 2003). This was clearly not the case with any of the columns used. There are several possible explanations for this. The first is that *P. davidi* has an extremely hydrophobic set of proteins and that it requires an even higher level of salt and/or a more hydrophobic column to allow the proteins to bind. In the dry environment of Antarctica or during periods of being frozen, a more hydrophobic set of proteins may allow *P. davidi* to survive with less liquid water (Block, 2003; Wharton, 2003). As with the possibility that *P. davidi* has evolved to have unusually low pI proteins, if and why this is the case requires further investigation. Another
explanation is that something may have been wrong with the use of the columns or the columns themselves. Given that three different columns were used it would be unlikely that all three were misused or faulty in the same way.

Despite the unusual chromatograms, all fractions that had absorbance were collected and fractions from the same peak were pooled to create flow-through and bound samples for each column. Each of these fractions and the starting material were dialysed overnight against 25 mM Tris pH 8.0 and then tested for RI activity using the splat assay. The results of the splat assays for the HIC fractions are shown in Figure 5.11 and it is clear that all of the fractions have very little or no RI activity. In all cases the HIC fractions had larger crystals than the 4-fold dilution of the SM. This means running the SM through the HIC columns resulted in loss of >75% of the RI activity. This is very surprising given that almost all of the protein comes out in the flow-through peak and should have been altered little. As with the ion exchange chromatography results, this suggests that whatever caused the RI activity in the *P. davidi* requires two or more components for activity and that it is very sensitive to being run through any kind of separation conditions or media. Because both ion exchange chromatography and HIC lead to the loss of RI activity it is likely that some protein(s) essential for RI activity are being separated, rather than the loss of a charged particle. HIC would not be expected to affect the presence of charged molecules (ions) within the protein (Wu et al., 2011). The EDTA result also supports this as it has no effect on activity either (Figure 5.5) making it unlikely that a metal ion is important for the RI activity.

### 7.3.3.3 Gel Permeation Chromatography

Because both the ion exchange and HIC resulted in the loss of RI activity, gel permeation chromatography was tested. It was hoped that the isocratic flow and low flow rate of gel permeation chromatography would preserve activity and allow detection of RI activity in some fraction(s) after separation. A Superdex 75 column (GE Healthcare) was chosen as appropriate for the separation of proteins between 3 and 75 kDa, which should cover most of the globular proteins in the sample (Andrews, 1964).
The chromatogram (Figure 5.12) showed $A_{280}$ (protein) between 15 and 43 min. In this range there are distinct peaks at 18 min and 41 min. The 18 min peak is near the start of the run and is likely to be all the proteins that are too large to enter the matrix i.e. >70 kDa. The 41 min peak is near the end of the run and corresponds to the void volume of the column and may have contained small proteins and peptides (<5 kDa) and other small molecules. There is also a broad peak at 25-33 min. There appears to be an even spread of proteins throughout the run (fractions 7-22). Because of this I chose not to pool fractions and instead tested each fraction individually using the OR method developed in Chapter 4 (see 2.3.13) to screen for RI activity.

The OR showed three different areas that had readings similar to the SM (Figure 5.13A). Two of these areas matched regions of the chromatogram that had high absorbance – fractions 8 and 9 (min 16-19) and fractions 12-15 (min 24-30). The other area that had a low OR reading was fraction 18 and 19 and this corresponded to an area on the chromatogram that had very low absorbance (min 36-39). This suggests that the gentler conditions of the gel permeation chromatography had preserved the protein(s) or complex responsible for the RI activity. It also suggests that there may be more than one protein or complex that has RI activity. It is also possible that whatever is responsible for the RI activity is active in several different states and each of these states has a different molecular size. There are many cases of each of these scenarios already known, many AFPs are known to have a large number of different isoforms and often more than one of these isoforms can be found in an organisms at a time (Barrett, 2001; Qin and Walker, 2006). Furthermore, many large complexes do not need all of the associated proteins to have activity and can be found in several different sizes that still have activity (Coux et al., 1996; Kawahara, 2002; Thornton et al., 2004).

As discussed in above, the OR method may not be measuring RI activity so the apparent RI activity of fractions was confirmed using the splat assay (Yu et al., 2010). When the fractions that had low OR readings were tested using the splat assay they all had very large crystals (Figure 5.13B). In all cases the fractions had crystals that were >140 µm, much larger than the SM (40 µm) and the 2-fold dilution of the SM (60 µm). This meant that none of the fractions had RI activity and the all the presumptive RI containing fractions identified by the OR method are false positives, at least judged by the splat freezing method. As with ion
exchange and hydrophobic interaction chromatography most or all of the RI activity was lost during the chromatography. This means that even the very gentle separating conditions used in gel permeation chromatography led to the dissociation of the protein or complex responsible for RI activity. This was unexpected because the buffer was not altered during the run and I know from other experiments that the RI activity was stable in this buffer e.g. PK experiments (Chapter 5.3.1). It was therefore concluded that running the sample through the column must have affected the RI activity in some way, perhaps by separating different components into different fractions.

7.3.4 Protein and Chromatography Conclusion

In Chapter 5 I was able to show that RI activity is sensitive to proteinase K. Therefore RI activity is caused by a protein or at the very least has a protein component that is vital for activity. This was clearly shown by the decrease in RI activity of *P. davidi*, grass and *Tenebrio* homogenates in a time-dependent manner when exposed to proteinase K. I was also able to show that EDTA had no effect on the RI activity of *P. davidi* homogenates and so it is unlikely that any metal ions are required for RI activity. The RI activity of a *P. davidi* homogenate was not affected by 30 min at 95 °C.

When the *P. davidi* homogenate was precipitated with ammonium sulfate (50 % saturation) the RI activity was not in either the precipitate or the supernatant i.e. the RI activity was lost. However, by recombining the precipitate and supernatant I was able to recover most of the RI activity. This suggests that the RI activity does not result from a single protein but requires more than one component. These were separated, but not destroyed, during ammonium sulfate precipitation and were able to recombine to recover activity once back in favourable conditions and in contact with each other.

Running *P. davidi* homogenate through different chromatography columns (ion exchange, HIC and gel filtration) resulted in the loss of the RI activity. This also suggests that whatever is responsible for RI activity is very sensitive and can be separated or destroyed very easily resulting in a loss of RI activity.
Overall Chapter 5 did not produce many positive results. I was able to show some basic properties of RI activity. It is caused by a protein (Figure 5.1) and EDTA (Figure 5.5) or heating (Figure 5.6) have little or no effect on RI. I also showed that RI activity could be lost by precipitating \textit{P. davidi} homogenate with 50% saturation of ammonium sulfate and that this loss of activity was reversible and could be recovered by recombining the precipitate and supernatant. This suggests that RI activity requires more than one component. Attempts to purify the RI protein using chromatography were unsuccessful. Regardless of the column type used, RI activity was destroyed by running the sample through the column. This meant that fractions could not be selected for further rounds of purification. From these results it was concluded that these classical chromatography techniques were not suitable for purifying RI proteins from \textit{P. davidi}.

### 7.4 Proteomic Analysis

From previous work in our lab we know that when \textit{P. davidi} is acclimated at 5 °C for 7 days it is better able to survive freezing than when kept at 20 °C (Smith \textit{et al.}, 2008). In this chapter we wanted to see if there was any difference between the RI activity of acclimated and non-acclimated \textit{P. davidi} homogenate samples and then also investigate if there was also a difference in the protein profile of samples using 2D PAGE.

#### 7.4.1 Recrystallisation Inhibition Activity of Acclimated and Non-Acclimated Homogenates

The first thing to do was determine if there was a difference in the RI activity of the homogenates from cultures from different holding temperatures. As part of this I needed to ensure that the protein level of each sample was the same. This was to make sure that any differences observed were due to differences between the RI activity of the samples and not an artefact of the homogenates having a different protein amount (Chapter 4). There was a small difference in the protein concentration of each sample, with the acclimated homogenate having a lower concentration than the non-acclimated. This may be due to any of several reasons. There may have been more nematodes in the non-acclimated sample, although there was a similar volume of nematodes in each sample before homogenisation and so it would be unlikely that this is the reason. Another possibility is that nematodes in
the acclimated sample were somehow less healthy than those in the non-acclimated sample (D. Wharton, personal communication). This would be possible as the liquid cultures fed on *E. coli* that grow in the culture with them, and at 5 °C the *E. coli* may not grow as fast and so these nematodes could be partially starved. Again this seems unlikely due to the appearance of the cultures i.e. they were still cloudy, which is indicative of a good supply of *E. coli* for the nematodes to fed on (Broeze *et al.*, 1978; Kovarova *et al.*, 1996). Alternatively, protein expression may simply be greater in 5 °C acclimated samples. Support for this possibly comes from the greater number of apparently up-regulated proteins in the 5 °C gel. Whatever the cause of the difference in the protein concentration, it was standardised by diluting the non-acclimated sample.

The results of the splat assay clearly show that there is difference between the two samples, with acclimation decreasing the size of the ice crystals (Figure 6.1). At every dilution the average size of the ice crystals in the non-acclimated homogenate is at least double the size of the acclimated homogenate. Even in the 16-fold dilution the acclimated sample crystal sizes have not reached the size of the buffer crystals, suggesting that acclimation is a very potent method for increasing the RI of a sample. This result is not surprising given the assumed link between RI activity and freeze survival (Ramløv *et al.*, 1996; Wharton *et al.*, 2005b). Due to only have a small volume of culture freeze survival was not tested as well; however, previous work in our lab had shown a link between acclimation and freeze tolerance (Smith *et al.*, 2008). If an acclimated sample is able to better inhibit recrystallisation i.e. smaller ice crystals, it will be more likely to limit any potential damage caused by those crystals and therefore survive the freezing process better. It is well known that many AFP expression levels are increased with exposure to low temperature and so it is not surprising that it is also the case with RI activity (Xu and Duman, 1991; Graham *et al.*, 2000; Jarzabek *et al.*, 2009). While it would have been nice to show a definite link between freeze survival and RI activity of these homogenates, for the purposes of this research it is not vital. This research is focused on the RI activity of the sample and this experiment has a difference in the RI activity between the two samples meaning that we can proceed to see if there are any differences at the proteomic level.
7.4.2 2D Gel Electrophoresis

The next step was to find out if the differences in RI activity of the homogenates could be caused by a change in the proteome of the nematodes. This was done by 2D gel electrophoresis. This is a very powerful tool for separating a complex mixture e.g. whole cell homogenates, due to the two different methods of separation (Gygi et al., 2000. Vanrobaeys et al., 2005) The first separation relies on the charge of the protein, with protein moving along a strip under an electric current until they reach a point neutral charge, at which point they move no further. The second dimension then separates the proteins based on size, with the smaller proteins moving further through the gel (down in the orientation shown in Figure 6.2 and 6.3). By combining these two techniques nearly every protein will be separated and have its own unique place (spot) on the gel (Gygi et al., 2000).

As with the splat assays the amount of sample loaded onto each gel was adjusted to have the same amount of total protein. This would ensure that any differences in spot intensities were due to differences in expression rather than just a different amount being loaded i.e. if more protein was loaded onto one gel we would expect all the spots on that gel to be darker. The gels worked very well and presented a wide range of proteins, as is typical with this type of analysis (Gygi et al., 2000). This indicates that *P. davidi* does not have an unusually large amount of low pI proteins, which was one possibility for the unusual results found during the ion exchange chromatography in Chapter 5.

The next task was to identify those protein spots that were differentially expressed between gels. This was done by comparing the scanned gels side by side using visual evaluation and marking those spots that appeared darker in one gel compared to the other. We chose to select the spot from the gel on which it was darkest as this indicates a higher amount of protein making any downstream work more likely to give good results. This analysis resulted in 15 spots being identified on the 20 °C gel and 46 spots being identified on the 5 °C gel. *Panagrolaimus davidi* is known to grow best at 20 °C and so it is to be expected that the 5 °C treatment would have more increased intensity spots as this is the ‘stressed’ state and usually stress induces protein production (Wharton and Brown, 1989). There is however, no way of knowing which level of each protein is normal i.e. it is possible that spots that appear darker in one gel have been down regulated in the other gel, rather than up regulated in
that gel. The differentially expressed proteins were spread all over the gels, both high and low molecular weight (top and bottom) and high and low pl (left and right). This indicates that a wide variety of proteins are required to handle the acclimation process. In addition to the proteins visualised on the gel there is also likely to be other components that are important to the freeze tolerance or RI of *P. davidi* e.g. membrane proteins, lipids and carbohydrates that were not able to be investigated using this technique (Schmid, 1982; Storey and Storey, 1988; Wharton 1995).

The final part of this work was to try and identify the proteins that had been differentially expressed. This requires removing the spots from the gel, carrying out an in gel tryptic digest followed by mass spectrometry and a protein database search of the resulting protein fragments to find matches to known proteins (Shevchenko *et al*., 2007). Unfortunately, the equipment required to do this analysis was not available (damaged) at the time of this work and the time constraints would not permit this part to be done before submission. The spots were still removed from the gels, numbered (not shown) and stored at −80 °C so that they could then be analysed when the equipment was available. This may provide some clue as to which protein/proteins are responsible for the RI activity in *P. davidi*.

### 7.4.3 Proteomic Analysis Conclusion

The final part of this work was to determine if there were differences in the proteomic profile of acclimated and non-acclimated *P. davidi* homogenate samples. First we showed that there was a difference in the RI activity of the two homogenate samples, with the acclimated sample having higher RI activity. After determining this was the case we compared the proteomes of both homogenates using 2D gel electrophoresis. From this we were able to select 61 proteins (15 in the non-acclimated gel and 46 in the acclimated gel) that were differentially expressed between the samples. These spots have been excised from the gel and are awaiting identification.
Chapter Eight

Conclusions and Future Directions

8.1 Project Overview

The Antarctic nematode *P. davidi* is the only animal currently known to survive intracellular freezing. Many years of research have gone into understanding the processes that enable it to do this e.g. effect of freezing rates, acclimation etc. and it has been shown that RI plays an important role in this unique ability. Despite all this work very little is known about the biochemical nature of this animal and its ability to survive freezing. The first aim of this project was to identify any ice-binding molecules in a homogenate of *P. davidi*, the second aim was to develop an accurate screen for RI activity which could be used in the third part, which was to determine some basic properties of the protein(s) responsible for RI activity and attempt to purify them using chromatographic methods. The final aim was to compare the RI activity and protein expression profile of acclimated and non-acclimated *P. davidi* homogenates.

8.2 Cold Finger Ice Binding

In this part we attempted to isolate ice binding molecules using the cold finger ice binding technique. After two rounds of ice binding nothing from the *P. davidi* homogenate had bound to the ice; this lead to also trying a grass extract (known to have RI activity) and a *Tenebrio* homogenate (known to have thermal hysteresis activity). Of these three samples only the *Tenebrio* homogenate had any molecules bind (a protein consistent with the *Tenebrio* AFP). This indicates that there is something special about both the *P. davidi* and grass extracts that do not require ice binding for RI activity and that the mechanism of RI is very different to that of AFP. This lead to the conclusion that RI activity does not require ice binding and a new method of activity was proposed. This mechanism proposes that the recrystallisation is inhibited by either preventing the movement of the still liquid water between ice crystals by binding the free water molecules and preventing their movement, or by disrupting the structure of the quasi-liquid layer just above the ice crystal surface. By
preventing this initial structuring of the water molecules in the quasi-liquid layer it prevents the water molecules moving into the ice crystal and therefore prevents the ice crystal growing.

8.3 Screen Development

While the discovery that RI activity does not require ice binding was novel and interesting it did not help with identifying the protein(s) responsible for the activity. This would likely now require more traditional chromatography techniques, which generate a large number of fractions for screening. The current test for RI activity is the time consuming splat assay, which is impractical for testing a large number of samples.

This part of the project involved testing three novel methods for their suitability as a screen for RI activity; the LDH method, nanolitre osmometer method and optical recrystallometer method. Each of these methods allowed the rapid (relatively) screening of a large number of samples. Despite this, one method stood out above the others as most suitable – the optical recrystallometer method. The LDH method was determined to be susceptible to false positive due to non-RI protein interference i.e. a high enough protein concentration would result in a positive result. The nanolitre osmometer method, while promising, was deemed too subjective at this stage, but with more time and work may prove feasible. The optical recrystallometer method had several advantages over both the other methods; the number of samples that can be tested at once is limited only by the size of the Al block, it gives a numerical value to remove any subjectivity, the risk of false negatives is very low and it is easy to set up and monitor.

8.4 Protein and Chromatography

As already mentioned very little was previously known about many of the basic properties of the protein(s) RI activity of *P. davidi*. Chapter 5 aimed to go back to first principles and uncover some of these basic properties, with the aim of being able to use the information gathered to isolate the protein using a combination of chromatography and the optical recrystallometer screen developed in the previous chapter.
The most significant property demonstrated was to show that the RI activity of the homogenate was susceptible to proteinase K digestion. This is a strong indication the RI activity is dependent upon a protein or proteins; this had never previously been shown conclusively. Once it was shown to be a protein we tested for several other properties and found that RI activity was not affected by EDTA i.e. no metal ions were required for activity, it appeared to be very heat stable, being able to withstand 95 °C for 30 min with no loss of activity. Another interesting discovery was the ability to destroy the RI activity by ammonium sulfate (50 % saturation) precipitation, but it was then possible to restore the activity by recombining the two parts. This suggests more that two or more components are required for the activity.

With this new knowledge and the nanolitre osmometer screening method an attempt to purify the RI activity using traditional chromatography techniques was made. These included ion exchange (both anion and cation), hydrophobic interaction (butyl, phenyl and octyl) and gel filtration. Regardless of the type of chromatography none of the fractions had any RI activity on the splat assay (despite some positives on the screen).

8.5 Proteomic Analysis

The final part of this project was to investigate the proteomic profile of *P. davidi* homogenates from acclimated and non-acclimated cultures. Previous work had shown that acclimation lead to an increase in freeze survival and it was hypothesised that this was due to an increase in RI activity. A liquid culture of *P. davidi* was split into two parts and one stored at 20 °C for 7 days (non-acclimated) and the other stored at 5 °C for 7 days. After the 7 days both cultures were harvested and homogenised, the protein concentrations of both were standardised and then tested for RI activity. It was very clear that the homogenate from the acclimated sample had higher RI activity i.e. smaller crystals than the non-acclimated sample.

The next step was to see if this difference in RI activity was matched by a difference in the protein profile. This was tested using 2D gel electrophoresis. Both homogenates were again standardised and then run on concurrent 2D gels and then compared. A total of 61 proteins (15 on non-acclimated and 46 on the acclimated), that were differentially expressed were
identified in the gels. These spots were excised from the gel and are now awaiting further analysis.

8.6 Future Directions

Like all good projects, this research has added to the knowledge of RI in the Antarctic nematode *P. davidii*; however, it has also indicated several new avenues for future work.

The cold finger ice binding work indicated a new mechanism for control of ice growth that did not require any compounds to bind to the ice. There are many possible paths this work could take, of which the most interesting will be to determine if the proposed mechanism is correct. This could involve chemically labelled compounds that can be visualised in real time and their location in the ice-water matrix tracked. There could also be some work on whether the RI activity and thermal hysteresis activity could be separated in AFPs. This could provide information about whether thermal hysteresis can exist without RI or give clues about the evolution of either RIP or AFP.

The optical recrystallometer screen has already been used successfully in at least one other project that came from this work investigating *Tenebrio* AFP expression. Also the nanolitre osmometer assay could be further developed into a screening technique; the main part of this would be to remove the subjectivity from the test.

There are also still lots of untried chromatography techniques that could yet be attempted. With the wide range of separation techniques now available it is possible that one of these could prove useful in isolating the RI activity. Further work could also be carried out on the ammonium sulfate precipitation to see if other saturation levels produce the same results.

Finally the excised spots from the 2D gel electrophoresis can be analysed and identified. It may also be possible to do some genetic work on these samples and see if there is any increased expression of genes (mRNA) in the acclimated samples. Either of these techniques may lead to new targets of investigation for RI activity.

Based on the recent discovery of many small molecules that have RI activity it would be interesting to test if there are any in the *P. davidii* homogenate that may have RI activity or
affect the RI activity. This could be tested by destroying the carbohydrate in the homogenate and testing for RI activity – similar to the Proteinase K experiments.
References


Xu L. and Duman J. G. (1991) Involvement of juvenile hormone in the induction of antifreeze protein production by the fat body of larvae of the beetle *Dendroides Canadensis*. *Comparative Physiology and Biochemistry*. 258, 288-293


