THE EFFECTS OF GLYCEROL ON RECRYSTALLIZATION

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THESIS

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Abstract

Freeze-tolerant organisms face several challenges affiliated with ice held in the frozen state, including conditions whereby “recrystallization” occurs. Recrystallization is a phenomenon in which larger ice crystals grow at the expense of smaller ones in a frozen sample or during a melt (Mazur 1984), and it can be very damaging to over-wintering organisms. Currently, no information exists that examines how cryoprotectants such as glycerol may influence recrystallization behavior of ice; therefore, the current study addresses this void. We assessed how a 1% and 4% glycerol solution impacts recrystallization, using a splat-freezing method to examine photographically growth of ice crystals overtime when held at -6°C. Quantification was done via measurement of mean largest grain size (mlgs). Results indicate that glycerol promotes recrystallization events over those seen with pure water. When further tested in saline, both glycerol and saline accelerate recrystallization, with a statistical interactive effect suggesting that while in the presence of AFP, the cryoprotective agent differentially impacts recrystallization depending upon both solvent and amount of glycerol used. Finally, studies were conducted to assess whether glycerol affects antifreeze proteins’ (AFPs) ability to inhibit recrystallization. Interestingly, results indicated that glycerol displays an opposite effect in this case. By enhancing the ability of AFP’s to inhibit ice recrystallization. The conclusions drawn from this study indicate that glycerol’s cryoprotective effects are restrictive in freeze-tolerant organisms. Where it may be protective during the onset of freezing, it may prove more problematic during an extended freezing incident,
actually promoting recrystallization. However, freeze-tolerant species displaying AFP’s appear to have the potential to reverse this effect, and make use of glycerol as an enhancer to promote AFP’s ability to inhibit recrystallization. Thus, the outcome of this series of experiments has strong implications for the preservation of tissues.

Our Study was conducted using the splat-cooling assay and thermal hysteresis measurements. The conclusions drawn from this study indicate that glycerol may work independently to accelerate recrystallization, but synergistically with AFPs to better inhibit recrystallization.
Dedication

This thesis is dedicated to me, my hard work and perseverance.
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Introduction

Many organisms face the challenge of surviving extremely cold temperatures throughout the winter. To overcome this, an organism must have the capacity to survive exposure to low temperatures through specific biochemical and physiological processes. Organisms with this capability are referred to as cold hardy. Depending on the stratagem a cold hardy organism employs for overcoming freezing conditions, it can be classified as either freeze-avoidant or freeze-tolerant. As the name implies, freeze-avoiding organisms must avoid both intra- and extra-cellular ice formation in order to overwinter, because the presence of ice would be lethal. Avoiding ice is done primarily through a phenomenon known as supercooling, the ability of an organism to lower the temperature at which spontaneous ice formation occurs in the body (supercooling point). Consequently, these organisms overwinter in an unfrozen, supercooled state. In contrast, freeze-tolerant organisms are able to tolerate extracellular ice formation, but must still avoid intracellular ice formation. Surviving extracellular ice growth is largely due to an accumulation throughout winter of cryoprotectants such as polyhydric alcohols (polyols) like glycerol and sorbitol, as well as sugars such as fructose throughout winter (Zachariassen 1985). Glycerol is typically the most abundant cryoprotectant produced, and is also produced in freeze avoiding species to enhance supercooling.

Freeze-tolerant organisms must also face the challenge affiliated with ice held in the frozen state. This situation can create conditions whereby “recrystallization” occurs. Recrystallization is a phenomenon in which larger
ice crystals grow at the expense of smaller ones in a frozen sample or during a melt (Mazur 1984). The process of recrystallization involves a dynamic change in ice crystal structure due to the presence of liquid at the interface of the ice grain boundary. This liquid is adsorbed by the larger ice grains, leading to their growth. A common example of this is what happens to ice cream over time when stored in a freezer. With organisms, excessive ice recrystallization could cause freeze damage, since as larger ice crystals grow, a sheering of tissue membranes may occur. Also, as extracellular ice increases, salts will precipitate, and water is then drawn out of the cell to re-dilute the salts thus, accentuating cell dehydration.

I. Glycerol

In the past 50 years many studies have reported the roles that glycerol may play in cell survival of freeze-tolerant organisms. This paper will outline a number of reported roles that glycerol may serve the freeze-tolerant organisms during winter.

The cryoprotective effect of glycerol encompasses a wide range of modalities Zachariassen (1979). Glycerol is a penetrating cryoprotectant. Therefore, it works intracellularly. Its hydrophilic property allows it to bind readily with water or in fact take the place of water as a solvent when water is drawn out of the cell during extracellular freezing. Glycerol leads to increased viscosity of the body fluids, and its solvent effect allows it to serve as an enzyme stabilizer (Hochachka 1973), for even at subzero temperatures cold-tolerant organisms are dynamic systems with many catabolic and anabolic reactions taking place.
Much of the cryoprotective role of glycerol stems primarily from its usefulness in limiting and preventing damage due to cell dehydration. Freezing injury is often not due to the presence of ice per se, rather due to the associated cell dehydration brought on by the presence of ice. Simply, as ice grows extracellularly, salts are precipitated out, increasing the osmolarity of the extracellular fluid. Consequently, water in the cells will be drawn into the extracellular fluids, thus causing dehydration. This mode of cell desiccation will lead to the decrease of the cell volume, precipitation of solutes, protein denaturation, and protein-protein interactions, all of which comprise freeze injury.

Studies of cell desiccation on *Pythos americanus* detail the importance of glycerol in limiting cell injury (Ring and Tesar 1980). The results suggest that as winter acclimation proceeds, glycerol accumulation occurs and it becomes progressively more difficult to remove water by the process of freeze drying. Complete dehydration can only be brought about by increasing the length of time in the freeze drier from 24 to 72 hr. This suggests that glycerol may serve to limit the dehydration that occurs in overwintering insects by “locking in” a certain proportion of water as “bound water”. A similar phenomenon of glycerol increasing bound water has been reported by Storey *et al.* in 1981 from experiments done on the larva of the goldenrod gall fly, *Eurosta solidagensis*. These results provide evidence that there is a direct correlation between low temperature acclimation and an increase in water “bound” by soluble sub-cellular components originating from both low-molecular-weight metabolites, such as glycerol, sugars, and amino acids, and
high-molecular-weight compounds such as proteins and glycogen. Kanwisher (1955), show that significant mortality is associated with 64-80% of the body water frozen. Zachariassen (1976) confirmed that in freeze tolerant species there is a certain maximal fraction of the body water, common to all species, which can freeze without injurious consequences. Thus glycerol limiting cell dehydration during a freezing episode is protective.

Polyols, especially glycerol, also enhance supercooling in freeze susceptible species, enabling them to overwinter in a non-freezing state. These substances increase supercooling on a colligative basis; i.e., colligatively they lower the freezing point of the body based on increased number of particles in solution. These accumulating polyols on a molar basis depress freezing point and consequently, the supercooling point of bodily fluids by increasing the total concentration of solutes in the body fluids, not by virtue of their particular chemical properties interacting with ice.

II. Antifreeze Proteins

One group of cold-hardy agents which do appear to interact directly with ice, are proteinaceous antifreezes known as antifreeze proteins (AFPs) (DeVries, 1971). Earlier studies on AFPs describe these proteins as thermal hysteresis proteins, because they generate a thermal hysteresis effect, which is the lowering of the freezing point of a substance without affecting the melting point (DeVries, 1986). Antifreeze proteins can lower the freezing point of bodily fluids non-colligatively because of specialized chemical properties, which allow for direct protein-ice interaction. A variety of insects and marine fish produce AFP polypeptides and glycopeptides that are believed to act by
binding to burgeoning ice crystals in geometrically specific ways, thereby suppressing growth of ice by preventing water molecules from freely joining any crystals that begin to form (Raymond et al, 1989). These non-colligative antifreeze proteins effectively depress the freezing point hundreds of times more than can be accounted for by simple colligative principles. Thus, these proteins can be very dilute and yet highly effective. Moreover, non-colligative antifreezes do not depress the melting point any more than colligative principles explain. Also, AFPs have been shown to lower the supercooling point and enhance stability of the supercooled state (Zachariassen 1976). Thus, having such a substance in the hemolymph, effectively increases supercooling potential.

In 1964 the first AFP activity was actually discovered by Ramsay, using the mealworm beetle, *Tenebrio molitor* (Ramsay 1964). He described the existence of a substance that lowers the freezing temperature of the insects’ hemolymph, while not impacting the melting temperature, thus generating what is now known as thermal hysteresis. His studies established that the cryptonephridial complex fluid contained a substance that exhibited a difference in melting and freezing temperatures of up to 8° to 9° C. Although the agent causing the thermal hysteresis phenomenon was not isolated, nor has a definitive function of this thermal hysteresis producing substances in the rectal complex been established, this physical behavior with respect to seed ice crystals was thought to be related in some way to the powerful water reabsorbing behavior of the *Tenebrio* rectal complex. It wasn’t until a
substance producing a similar thermal hysteresis affect was found in Antarctic fish that the non-colligative antifreeze activity was shown to be significant.

**III. Fish Antifreeze Protein**

DeVries (1971) using Antarctic Nototheniid fishes, then discovered a similarly acting substance, which allowed these fish to depress their freezing points below that of sea water. Therefore, they remained unfrozen, while swimming in ice-laden water. DeVries isolated this material and identified a glycoprotein that was responsible for generating this non-colligative thermal hysteresis effect. These fish glycoproteins are present in eight distinct molecular weight classes ranging from approximately 2,500 to 34,000. They consist of a peptide backbone made up of repeats of the tripeptide alanyl-alanyl-threonyl, with the disaccharide sugar \( \beta \)-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy- \( \alpha \)-D galactopyranose attached via a glycoside linkage to the hydroxyl side chain of each threonine (DeVries, 1971; DeVries et al., 1971; Shier et al., 1975). In the last two decades, further studies of other cold-water fish species have yielded the discovery of other antifreeze proteins distinct in structure as compared to the antifreeze glycoproteins. These are generally grouped by structural characteristics into four categories: type I, II, III and IV fish antifreeze proteins (Davies and Hew, 1990) and are not glycoproteins. The type I antifreeze protein, commonly found in winter flounders, yellowtail flounders, shorthorn sculpins and grubby sculpins is characterized by its alpha helical structure and its molecular weight of 3kDa. They are long, alanine rich, single alpha-helices. The helices are stabilized by elaborate N and C termini cap structures with complementary charged groups,
and by internal salt bridges (Davies and Hew, 1990). They also generally consist of tandem repeats of the amino acid sequence thr-X2-polar amino acid-X7, where X donates an alanine residue and the polar amino acid is usually represented by an aspirate, asparagine, or lysine residue (DeVries and Lin, 1977).

Types II and III antifreeze proteins are characterized by their difference in amino acid sequences from the type I antifreeze proteins. The type II antifreeze, which is the largest of the non-glycoprotein fish thermal hysteresis protein, is commonly found in the sea raven, smelt and Atlantic herring. The type II antifreeze protein is characterized by its globular shape its abundance of cysteine residues (up to 9.1 molar %) (Davies and Hew, 1990). Its amino acid sequence revealed the presence of 10 cysteine residues out of 129 total residues (Slaughter et al. 1981). Five of these residues are involved in disulfide linkages. The type II antifreeze proteins appear to be homologs of the carbohydrate recognition domain of calcium dependent C-type lectins and have the same three-dimensional fold (Ewert, 1992). The type III antifreeze proteins, having a molecular mass of 7kDa, have, to date, exhibited even fewer distinguishing features than the type II antifreeze proteins, and amino acid sequence data show no obvious bias toward particular residues, nor are there any repeating sequences. The type III antifreeze protein is commonly found in both Northern and Antarctic eelpout and wolf fish. These are globular proteins and their structures are not dominated by any particular amino acid. Unlike the type II antifreeze proteins cysteine residues are not particularly abundant in the fish type III antifreeze proteins (Davies and Hew, 1990).
They consist of short, non-canonical β-strands, with a single turn of alpha-helix in the loop region (Sonnichsen 1996). Finally, the type IV antifreeze protein, the most recently described, is commonly found in the longhorn sculpin and is characterized by its folded α-helical structure, and its low density lipoprotein receptor-binding domain of apolipoprotein E (Deng, et al. 1997)

IV. Insect Antifreeze Proteins

The antifreeze proteins in insects have not been studied as extensively as fish antifreeze proteins have. The best characterized insect AFPs appear to be those of type II class, i.e. possessing high cysteine content. The newly described THP species from Tenebrio molitor, Choristoneura fumiferana, and Dendroides canadensis possess a relatively high complement of cysteine residues (Graham et al. 1997, Tyshenko et al. 1997, Duman et al. 1998). All of the cysteine residues in the most recently isolated isoforms of the D. canadensis THPs are postulated to be involved in disulfide linkages (Duman et al. 1998); thus some high cysteine insect THPs may share structural characteristics with the type II fish AFPs.

In addition, a recently discovered family of type III AFPs has been identified in Tenebrio molitor (Horwath et al, 1996). The antifreeze protein 12.86 being used in our study is a type III-like AFP extracted from the mealworm beetle, Tenebrio molitor. This antifreeze protein is of interest, because for a type III AFP, its shows very high thermal hysteresis activity (2.5°C at 30mg/ml), surpassing the thermal hysteresis maxima of fish.
antifreeze proteins and other insect type III-like antifreeze proteins (1.5°C or less).

V. Enhancers of Thermal Hysteresis

Interestingly, there also appears to be substances that can enhance the thermal hysteresis effect of AFPs. This was first described for the AFPs of *D. canadensis*. A purified *D. canadensis* protein exhibited relatively high maximal activity of 2.7°C at high THP concentrations (>60 mg/ml) (Wu et al. 1991), yet thermal hysteresis of winter acclimated *D. canadensis* hemolymph often reaches 5-6°C (and as high as 8-9°C for some individuals). With the addition of immunoglobulins to purified THP solutions, thermal hysteresis activity was significantly enhanced. For the *D. canadensis* THP, T.H. activity was approximately doubled at THP concentrations equal to or greater than 3 mg/ml THP. However, the immunoglobulins alone did not exhibit thermal hysteresis behavior. This thermal hysteresis enhancing effect was accompanied by the discovery of endogenous “activators” in *D. canadensis* (Wu et al. 1991). The purified *D. canadensis* activator significantly enhanced the thermal hysteresis behavior of a purified *D. canadensis* THP, but did not exhibit thermal hysteresis activity itself (Wu and Duman 1991). More recently, an activator substance, not displaying thermal hysteresis by itself but capable of enhancing purified type 3, AFP from in *T. molitor* has also been detected (Poggioli and Horwath, 1996).

Certain other proteins were likewise shown to be capable of causing some amount of somewhat lesser activation of AFPs. A 0.25% gelatin solution (too low to produce the gel state) activated the *D. canadensis* THP, as
did a protein ice nucleator from *Tipula trivittata*. The mechanism of these activations is unknown at this time, but perhaps the THP aggregates with the activator protein and, in a fashion similar to the THP-antibody complex, the aggregated proteins block a greater surface area of the seed crystal than does the THP alone (Duman et. al.). Some salts, and possibly glycerol have also been suggested to provide enhancement of thermal hysteresis by AFPs. Finally, while antifreeze proteins are known for inducing thermal hysteresis, which is an effective component for freeze susceptible species, other studies have shown that these proteins may also enhance survival of freeze tolerant species by limiting post freezing ice growth in the extracellular regions (Knight and Duman, 1986). This inhibition of ice growth in the frozen state has come to be known as the inhibition of recrystallization. The inhibition of recrystallization characteristic of the antifreeze protein serves as a means to stymie damages that may incur due to ice growth. Furthermore, apart from generating the thermal hysteresis effect, recent studies have shown that AFPs inhibit recrystallization on a non-colligative basis (Knight and Duman, 1986). No research has been done on the effects of glycerol on the AFPs ability to inhibit recrystallization. If information on glycerol in conjunction AFPs is provided, we may be better able to provide a more potent cryoprotectant for preserving tissue cultures for scientific research.

**VI. Mechanism of Recrystallization**

Recrystallization involves the movement of interface boundaries between ice crystal grains (Knight 1995). Some grains grow larger at the expense of smaller neighboring ice crystals, which are gradually absorbed by
the growing crystals. The boundary between an actively growing crystal grain and a neighboring grain is never a straight line but always exhibits curvature. The boundary tends to migrate toward its center of curvature such that the degree of curvature is reduced. Therefore grains with concave boundaries tend to grow while those with convex boundaries tend to decrease in size. The straightening of curved ice grain boundaries decreases interfacial area, thus reducing interfacial free energy.

According to this reasoning, boundary curvature between neighboring crystals should eventually straighten and the associated recrystallization process should cease. However, the tendency of interfacial tensions to maintain equilibrium must also be considered (Knight and Duman, 1986). Neighboring crystal boundaries usually meet in a “Y” shaped configuration separating the three adjacent crystals. The three angles at the junction tend to assume a configuration such that the three tensions achieve equilibrium. For ice crystals, the angles tend to approximate 120° at equilibrium. For most junctions, the process of boundary straightening or migration tends to disrupt the 120° equilibrium angles. Since grain boundary shortening or straightening usually tends to counteract the balance of interfacial tensions at the three-grain junctions, curvature is continually introduced at the junctions to maintain the 120° angles. The two antagonistic processes—boundary shortening followed by curvature adjustments at “Y” junctions—cause the continual propagation of grain boundaries during the course of recrystallization (Burgers 1963).

In 1999, Myers developed a quantitative method for assessing ice recrystallization behavior. This involved photomicroscopy of an annealing ice
wafer, over time, and then the assessment of an average of the largest ice grain sizes for each sample. This observed mean largest grain size (mlgs) provides a statistical approach to evaluate the recrystallization process of ice, and the impact of soluble factors, such as salts, polyols, and AFPs on recrystallization. In so doing, Myers determined the ice wafers held at -6°C over time displayed recrystallization at a rate of 0.0055 mm²/hour and that ice recrystallization was dramatically accelerated in the presence of 0.9% saline to 0.012 mm²/hour, while the presence of AFPs halted or significantly limited ice recrystallization growth, down to 10⁻⁸ m of AFP concentration. The impact of polyols on recrystallization has yet to be determined.

VII. Specific Aims

No information exists that examines how glycerol may influence recrystallization behavior of ice; therefore, the current study will address this void. Specifically, it will assess quantitatively whether glycerol accentuates or retards ice growth during recrystallization and in a concentration dependent fashion, i.e., consistent with seasonal fluctuations in glycerol levels associated with overwintering. This study will also examine whether glycerol serves as an activator of AFP, to enhance antifreeze proteins’ ability to inhibit recrystallization. The outcome of this series of experiments will be useful to apply to studies focusing on the preservation of tissues.
Materials and Methods

I. Stock Solutions

Samples to be subjected to thermal hysteresis measurements and assessment of recrystallization were derived from the following stock solutions: A physiological saline solution (0.9% NaCl), 1% and 4% glycerol solutions, and 1% and 4% glycerol in physiological saline.

II. Collection of Hemolymph Samples Containing Antifreeze Proteins

Hemolymph samples from *T. molitor* were collected by puncturing the cuticle between anterior segments with a needle, then collecting the droplet with a 10 µl capillary pipette. For recrystallization inhibition studies, the hemolymph volume was determined by measuring the length fraction of the capillary occupied by hemolymph compared to the total 10 µl capillary volume. Each hemolymph sample was expelled into an appropriate volume of saline (0.9% NaCl) and glycerol solution (1% and 4%), usually to produce a hemolymph dilution of 1/10,000. The hemolymph dilution was selected because studies done by Myers (1999) show that at this dilution the effects of the antifreeze proteins are apparent, i.e., the ice grain sizes are significantly smaller. However, these crystals are still large enough to be measured using the mean largest size approach. For T.H. measurements, non-diluted hemolymph was sealed in the capillary, as detailed in Myer (1999).

III. Measurement of thermal hysteresis

The micro-capillary method of Ramsay and Brown (1955) as detailed by Horwath and Duman (1982) was used to determine the presence and amount of thermal hysteresis in the various samples. Briefly, sealed
capillaries containing hemolymph are immersed in a refrigerated alcohol bath, and ice seed crystals examined. The freezing point of a thermal hysteresis protein solution is defined as the temperature at which a small seed crystal begins to grow in size; the solution melting point is the temperature at which the seed crystal completely disappears (Myers 1999). The amount of thermal hysteresis is the difference between the freezing and melting points of the solution. This thermal hysteresis phenomenon is antifreeze-protein specific (DeVries, 1971)

IV. Assessment of recrystallization inhibition (R.I.)

The “splat cooling” technique (Knight et al. 1988) was used to produce fine-grained ice samples for recrystallization inhibition studies. For this procedure, 10 µl sample droplets were released approximately 2.6 m above a polished aluminium plate maintained at dry ice temperature (~-78° C). Upon contact with the surface of the aluminium plate, a thin, fine-grained ice wafer about 8mm in diameter was formed. The wafer was immediately transferred to the cold stage holding chamber (preset to -6° C) using a metal weighing spatula maintained at -78° C. The cold stage, consisting of a brass extension piece drilled through to form the ice sample holding area, was cooled via a Peltier device (Laboratory Instruments Services). Heat transferred by the Peltier was absorbed by a second brass head cooled via a circulating alcohol bath at -8° C.

A coverglass was placed at the bottom of the cold stage to form a holding chamber; ice samples were positioned on a small polypropylene ring (cut from the top of a 0.5 ml microfuge tube) at the bottom of the chamber,
which was then sealed with a second coverglass. The temperature within the chamber was monitored using a Type T thermocouple needle microprobe (Physitemp type MT-26/2) with digital thermometer (Physitemp BAT-10RLOP) and a second Type T thermocouple sensor (Physitemp type MT-4) immersed in an ice-water bath for differential temperature measurements.

Ice samples were sealed with the holding chamber and allowed to anneal at a constant temperature for a specified time period. An annealing temperature of -6° C and annealing times of 30 minutes and 1 hour were primarily studied, with the chamber temperature adjusted manually by varying electric current supply to the Peltier unit. The Peltier current was monitored with a Fluke SP-7 digital multimeter.

Following placement of a sample within the cold stage holding chamber and allowing for a specified annealing time, the top coverglass was removed from the chamber and the cold stage placed between crossed polarizing filters for microscopic observation of an ice sample. A stereoscope (Bausch and Lomb Stereozoom 7) with eyepiece mounted camera (Nikon N6006) was used to photograph each ice sample, with crossed polaroids producing contrast between individual ice grains by birefringence.

V. Quantifying Grain Size

Recrystallization, observed in photomicrographs, was quantified using the average of the largest ice grain sizes of each sample as detailed by Myers (1999). In each photograph, the five largest ice grains were each assessed for cross sectional area. Grain cross sectional area was approximated as an elliptical axis, and the largest linear dimension orthogonal to the major axis
serving as the minor elliptical axis. A schematic representation of a typical ice sample photograph at high magnification (30x) and the process of mean largest grain size (mlgs) determination is presented in (Fig 1)

VI. Statistical Analysis

Differences between multiple categories of THP and non-THP solution mean largest grain sizes were tested for statistical significance using analysis of variance (ANOVA) calculated at the a< 0.05 or 0.001 level. Testing for differences in mean largest grain sizes between only two treatment categories was performed using one-way t-test with a critical test statistic calculated at the a<0.05.

Regression analysis was also performed on the time profile of water, in this case to examine mlgs as a function of time. Linear regression was then performed using Microsoft Excel.
Grain area = $0.25AB\pi$

Fig. 1. Scanned photomicrograph of a recrystallized water ice sample wafer held at -6°C for 30 minutes, magnification of 30x. The process by which five largest grains per photograph are chosen and grain sizes approximate as elliptical areas is also shown. A) Grains numbered 1-5 are the largest ice grains of the sample. B) Representation of grain number 1 and how elliptical area is determined.
Results

I. Effects of glycerol on recrystallization of water

Frozen water droplets held at an annealing temperature of -6° C display recrystallization overtime. Photomicrographs of this recrystallization over a two hour period are seen in Figure 2. The ice wafer at time zero is composed of multiple extremely small ice crystal grains (Fig. 2A). After 30 minutes of being held at -6° C, the ice wafer now shows fewer and somewhat larger ice crystals (Fig. 2B). This recrystallization was continuous throughout the two hour interval, and with every additional 30 minutes of annealing time. The ice crystal numbers diminish while the size of the remaining ice grains becomes progressively larger (Figs. 2C-2E). Myers (1999) developed a means to quantify these recrystallization events, through measurement of a composite mean largest grain size (mlgs) detailed in Fig. 1. When this quantification assessment was made of water recrystallization in the present study, the results for the two hour interval are shown in Fig. 3A. At time zero, mlgs was less than 10 mm². Over the two hour interval, the mlgs grew more than 10 fold to greater than 120 mm². Regression analysis defined a highly significant slope [Fig 3B. (R=1.1)], indicating that ice crystal growth is substantial and continuous in this frozen ice wafer, throughout the entire annealing interval. Since recrystallization is evident even after 30 minutes of annealing at -6° C, i.e., the mlgs of each ice wafer showed a significant increase in grain size, we used this time period to assess the impact of glycerol on recrystallization. Glycerol solutions (1% and 4%) were subjected to the splat cooling procedure.
Fig. 2A-E. Photomicrographs illustrating recrystallization of nanopure H$_2$O at -6°C for 0, 30, 60, 90, and 120 minutes. Magnification for each was 30x (A) H$_2$O sample at time 0. (B) H$_2$O sample at 30 minutes. (C) H$_2$O sample at 60 minutes. (D) H$_2$O sample at 90 minutes. (E) H$_2$O sample at 120 minutes. Bars = 0.1 mm
Fig. 3A. Quantitative assessment of recrystallization of H2O annealed for two hours at -6°C. Mean largest grain size (mlgs) assessed from photomicrographs taken of the same sample at 30 minute intervals with a sample size of 5 for each time interval. Differing letters above histogram depict statistical relationships of at least p<0.05 significance (error bars are calculated standard error).
Fig. 3B. Regression analysis of the recrystallization of H$_2$O annealed for two hours at -6°C. The slope of the regression line of mlgs as a function of annealing time was highly significant (R= 1.1) indicating that a significant proportion of the variation in mlgs can be ascribed to annealing time.
These frozen solution droplets were held at an annealing temperature of -6°C for 30 minutes and the extent of recrystallization over time was assessed. Photomicrographs of this recrystallization over 30 minutes are seen in Fig. 4. The ice wafers at 30 minutes show fewer numbers of ice crystals, but those present are significantly larger when compared to the crystals in the ice wafer at time zero, thus recrystallization occurred. In fact, the extent of recrystallization seen in the 1% glycerol samples after 30 minutes was even more pronounced (p <0.001) than the recrystallization seen with water alone, after 30 minutes (compare Fig. 4B and 4D, also see Fig. 5). 4% glycerol solutions (Fig. 4C), also displayed significantly higher mIgs (p <0.001) than at time zero or than the grain sizes of H2O recorded after 30 minutes of annealing (Fig. 4C and Fig. 5). Interestingly, the 1% glycerol solution displayed the most recrystallization, showing statistically higher ice crystal size (p<0.001) than those of 4% glycerol (Fig. 5).

II. Effects of glycerol on recrystallization of 0.9% saline

We next examined the effects of glycerol on recrystallization of a saline solution. Previous studies have revealed that recrystallization is significantly increased in the presence of NaCl (Myers 1999). Our study confirms this as the ice grain size of a 0.9% saline solution (mIgs = 120mm²), splat frozen and annealed at -6°C for 30 minutes (Fig. 6A) shows significantly larger grain size (p<0.05) than pure water after 30 minutes, mIgs = 30mm² (compare Fig. 6A to Fig. 2B). In fact the mIgs of the 0.9% solution observed after 30 minutes correlates with the mIgs of H2O extrapolated at 80 minutes (Fig. 3B).
Fig. 4. Photomicrographs illustrating recrystallization of glycerol in nanopure H$_2$O annealed at -6°C for 30 minutes. Magnification for each sample was 30x. (A) Sample of 1% glycerol in H$_2$O at time zero. (B) Sample of 1% glycerol in H$_2$O at 30 minutes. (C) Sample of 4% glycerol in H$_2$O at 30 minutes. (D) Sample H$_2$O at 30 minutes. Bar = 0.1 mm.
Recrystallization of H$_2$O in the presence of Glycerol after 30 minutes

Fig. 5. Quantitative assessment of recrystallization of H$_2$O in the presence of 1% and 4% glycerol annealed for 30 minutes at -6°C. Mean largest grain size (mlgs) assessed from photomicrographs taken of the same sample at 30 minutes. Statistical analysis reflects a significant difference in grain sizes of each solution (p<0.05) as designated by differing letter above the histograms (error bars are calculated standard error). Sample size is 5 for each of the treatment conditions.
Fig. 6. Photomicrographs illustrating recrystallization of glycerol in 0.9% saline annealed at -6°C for 30 minutes. Magnification for each sample was 30x. (A) Sample of 0.9% saline at 30 minutes. (B) Sample of 1% glycerol in 0.9% saline at 30 minutes. (C) Sample of 4% glycerol in 0.9% saline at 30 minutes. Each treatment reflects a size of 5. Bar = 0.1 mm.
We then tested a 1% and 4% glycerol in 0.9% saline solutions. They too, showed pronounced recrystallization over that seen with water alone. Also, the mlgs of the 1% glycerol solutions were not significantly different from the already elevated ice grains of 0.9% saline after 30 minutes (Fig. 6 & 7). The 4% solution appears to show a somewhat slower rate of recrystallization than in 1% glycerol in saline (Fig 7), and in fact, these differences were statistically significant (p<0.05), so 4% glycerol in saline has less of an accelerating affect on recrystallization, than either 1% glycerol/saline, or saline alone. Regardless, glycerol had no further ice growth accelerating effects over saline. Also, the observed grain sizes of the 4% glycerol/saline solution after 30 minutes were mostly similar to what was observed with water containing 1% (compare Fig. 7 & Fig. 5). Yet the 4% glycerol in saline did enhance recrystallization over that of 4% glycerol/water (compare Fig. 7 & 5). These distinctions perhaps reflect the fact that a 2-way ANOVA indicates that there is a significant interaction of glycerol and solvent solution. Thus, the extent of glycerol’s ability to accelerate recrystallization is dependent upon the underlying solvent solution.

III. Effects of Glycerol on recrystallization in the presence of AFP

We then undertook the task of examining the effects of glycerol on recrystallization in the presence of antifreeze proteins. *Tenebrio molitor* hemolymph containing antifreeze proteins was diluted 1:10,000 in saline and glycerol solutions. It has been shown by Myers (1999) that this dilution will demonstrate the effects of AFPs, i.e., significant inhibition of recrystallization, while allowing ice growth large enough to delineated measurable ice grains.
Recrystallization of 0.9% Saline in the presence of glycerol after 30 minutes

Fig. 7. Quantitative assessment of recrystallization of Saline in the presence of 1% and 4% glycerol annealed for 30 minutes at -6°C. Mean largest grain size (mlgs) assessed from photomicrographs taken of the same sample at 30 minutes. Statistical analysis reflects a significant difference in grain sizes the 4% glycerol solution (p<0.05) as designated by differing letters above the histograms (error bars are calculated standard error). Sample size is 5 for each treatment condition.
Photomicrographs of these ice grains were taken at 30 and 60 minutes. Figures 8A and 8B show effects of 0.9% saline in the presence AFP over a 30 and 60 minutes time period, respectively. After 30 minutes of annealing at -6°C the ice grains are significantly smaller than what was observed under the same conditions in the absence of AFPs (Fig. 8C). Recrystallization was then measured again after 60 minutes of annealing at -6°C. Ice grain measurements reveal a significant increase in grain size when compared to the measurements taken after 30 minutes, but still significantly smaller when compared to the grain sizes of 0.9% saline under the same conditions in the absence of AFP. Quantification assessment of recrystallization in the presence of AFP is shown in Fig. 9. At time zero the mlgs was less than 10 mm². After 30 minutes, the mlgs grew to 44 mm², more than four times the mlgs measured at time zero, but less than half the size of the mlgs measured with saline in the absence of AFP. Therefore while a 1:10,000 dilution of hemolymph containing AFPs displays some inhibition of recrystallization, it is still sufficiently dilute to allow for moderate ice growth during the annealing interval. This makes it possible to now consider the impact of glycerol on AFPs ability to restrict ice growth.

We then looked at 1% glycerol in the presence of AFP. Hemolymph diluted 1:10,000 in 1% glycerol solution containing saline was subjected to the splat cooling assay. Splat wafers were annealed at -6°C over an hour time period. Photomicrographs were taken after 30 and 60 minutes of annealing. Figures 10a and 10b shows recrystallization of these wafers respectively. Quantitative assessment of these composites was done and is shown in Fig. 11.
Fig. 8. Photomicrographs of 0.9% saline containing AFP (A) Photomicrograph showing recrystallization at 30 minutes of annealing at -6°C. (B) Photomicrograph showing recrystallization at 60 minutes. (C) Photomicrograph showing recrystallization of saline without AFP at 30 minutes. Bar = 0.1 mm.
Fig. 9. Quantitative assessment of recrystallization of 0.9% saline compared to 0.9% saline in the presence of 1:10,000 dilute AFP solution annealed for 30 minutes at -6°C. Mean largest gain size (mlgs) assessed from photomicrographs taken of the sample at 0, 30. Statistical analysis reflects a significant difference in grain sizes between the grain sizes of 0.9% saline and 0.9% saline w/AFP after 30 minutes and an hour, as designated by the letters above the histograms (error bars are calculated standard error). Sample size was 5 for each solution.
Fig. 10. Photomicrographs showing recrystallization of 1% and 4% glycerol in saline containing AFP. (A) Photomicrograph showing recrystallization of 1% glycerol in saline containing AFP at 30 minutes of annealing at -6°C. (B) Photomicrograph showing recrystallization of 1% glycerol in saline containing AFP at 60 minutes of annealing at -6°C. (C) Photomicrograph showing recrystallization of 4% glycerol in saline containing AFP at 30 minutes. (D) Photomicrograph showing recrystallization of 4% glycerol in saline containing AFP at 60 minutes. Bar = 0.1 mm.
Fig. 11. Quantitative assessment of recrystallization of 1%, and 4% glycerol in saline with 1:10,000 dilute AFP annealed for 30 minutes at -6°C. Mean largest grain size (mlgs) assessed from photomicrographs taken of the sample at 30 minutes. Statistical analysis reflects a significant difference in grain sizes between the grain sizes of 0.9% saline and 1% glycerol in saline w/ AFP (p< 0.05) after 30 minutes, as designated by letters above the histograms (error bars are calculated standard error). Sample size was 5 for each solution.
At time zero the mlgs was calculated to be less than 10 mm². After 30 minutes of annealing, recrystallization of the glycerol/saline/AFP solution occurred and an increase in ice grain size to 21.6 mm² was measured, albeit, this was still significantly smaller than the mlgs of 0.9% saline control after 30 minutes. While an hour annealing allowed the mlgs of the glycerol/AFP solution to increase further to 39.18 mm², again this remained significantly smaller than the grains of 0.9% saline measure after an hour of annealing (Fig. 12). Interestingly, the glycerol/AFP solutions also displayed significantly smaller mlgs when compared to the saline/AFP solution alone.

Similar measurements were recorded for 4% glycerol in the presence of AFP. Fig. 10c and 10d are photomicrographs of 4% glycerol solutions in saline containing AFP after 30 and 60 minutes respectively. The quantitative assessment of this recrystallization was recorded and presented in Fig. 11 (30 min) Fig. 12 (60 min) respectively. At time zero the mlgs was less than 10 mm². After 30 minutes of recrystallization, the mlgs of the 4% glycerol/AFP solution was measured to be 20.5 mm². After an hour of annealing at -6°C the mlgs doubled to 40.86 mm², measurements similar to what was recorded for 1% glycerol. Thus, both 1% and 4% glycerol/AFP solutions significantly restrict ice recrystallization. Interestingly, it also appears that the glycerol/AFP solution is more potent at restricting ice recrystallization, than is the AFP solution alone (Fig. 11 & 12). This demonstrates that under these conditions, glycerol enhances the AFP’s ability to inhibit recrystallization.
Fig. 12. Quantitative assessment of recrystallization of 1%, and 4% glycerol in saline with 1:10,000 dilute AFP annealed for 30 minutes at -6°C. Mean largest grain size (mlgs) assessed from photomicrographs taken of the sample at 30 minutes and 60 minutes. Statistical analysis reflects a significant difference in grain sizes between the grain sizes of 0.9% saline and 1% glycerol in saline w/AFP (p< 0.05) after 30 minutes, as designated by letters above the histograms (error bars are calculated standard error). Sample size was 5 for each solution.
IV. **Thermal Hysteresis**

We also assessed solutions containing a 1:10,000 dilution of AFP for thermal hysteresis using the Ramsay and Brown (1955) technique. Interestingly the solution containing the dilution of AFP in water shows no thermal hysteresis despite the fact that we had previously observed for this solution significant inhibition of recrystallization (Fig. 11). This underscores Myers’ (1999) observations that detection of the recrystallization inhibition is a more sensitive measurement of AFP activity than that of thermal hysteresis. However with the addition of NaCl and/or glycerol, thermal hysteresis of these solutions was observed. The 0.9% saline solution containing 1:10,000 dilute AFP shows a small thermal hysteresis measurement (0.15°C), while the same solution containing 1% glycerol show a 3-fold increase in thermal hysteresis (0.45°C). The same was observed for the 0.9% saline solution with 1:10,000 dilute AFP containing 4% glycerol. So, saline and glycerol effectively enhances the thermal hysteresis activity of our dilute AFP solutions.
<table>
<thead>
<tr>
<th>% Solution</th>
<th>Melting Point</th>
<th>Freezing Point</th>
<th>Osmolarity (Osm)</th>
<th>Thermal Hysteresis</th>
<th>MLGS (mm²) at 30 Minutes</th>
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</thead>
<tbody>
<tr>
<td>H20</td>
<td>0°C</td>
<td>0°C</td>
<td>0</td>
<td>0</td>
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<td>-0.20 °C</td>
<td>0.12</td>
<td>0</td>
<td>99.11</td>
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<td>-0.75 °C</td>
<td>0.40</td>
<td>0</td>
<td>45.50</td>
</tr>
<tr>
<td>0.9 % Saline (Sal.)</td>
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<td>-0.45°C</td>
<td>0.24</td>
<td>0</td>
<td>120.00</td>
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<tr>
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<td>-0.65°C</td>
<td>0.35</td>
<td>0</td>
<td>113.13</td>
</tr>
<tr>
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<td>-1.15°C</td>
<td>0.62</td>
<td>0</td>
<td>79.29</td>
</tr>
<tr>
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<td>-0.65°C</td>
<td>0.35</td>
<td>0.15°C</td>
<td>44.39</td>
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<tr>
<td>H20 w/AFP</td>
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<td>0°C</td>
<td>0</td>
<td>0</td>
<td>~10.00</td>
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<tr>
<td>1% Gly. in 0.9% Sal. w/AFP*</td>
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<td>-1.20°C</td>
<td>0.65</td>
<td>0.45°C</td>
<td>21.60</td>
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<tr>
<td>4% Gly. in 0.9% Sal. w/AFP*</td>
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<td>-2.65°C</td>
<td>1.42</td>
<td>0.45°C</td>
<td>20.50</td>
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</table>

Table 1. Thermal hysteresis measurements, difference between melting point and freezing point, of our winter hemolymph solutions were subjected to the Ramsay and Brown technique. Sample size, N=3. Gly = glycerol, Sal. = saline, AFP* = antifreeze protein (1:10,000 dilution of winter *Tenebrio molitor* hemolymph containing AFP)
Discussion

I. Water, glycerol and recrystallization

Our study first examined the role of glycerol on recrystallization of frozen water. We found that the ice grains in frozen ice wafers held at -6°C over a two-hour time period displayed significant recrystallization and grew at a rate of 55mm²/hour. The presence of 1% glycerol further increased this rate more than 2-fold to 120 mm²/hour. The rates of recrystallization observed for our glycerol solutions clearly demonstrate that glycerol acts to accentuate recrystallization. One explanation for this is that the glycerol solution may provide an increase of liquid water at the interface between grain boundaries, resulting in an acceleration based on colligative effects. Our melting point measurements reveal that 1% glycerol in water depresses the melting point of the solution (Table 1). An increase in glycerol concentration, particularly up to 4%, further depresses the melting point. Both solutions also show different osmolarity values, with 4% having a higher osmolarity as expected due to colligative principles. The lowering of the melting point would result in more mobile liquid water between grain boundaries. The migration of water molecules between adjacent ice grains probably requires that each water molecule leaving one grain must reorient itself to match the lattice orientation of the second grain. This would occur more readily with the availability of more liquid water at the ice crystal interfaces in the glycerol solutions due to their lower melting points. Thus, recrystallization would be promoted. This explanation, however, does not account for the significantly smaller mIGs observed for the 4% glycerol solution when compared to the 1% glycerol
solution. The fact that there is a significant decrease in mlgs, in spite of a
decrease in melting point and an increase in osmolarity, may indicate that
beyond the colligative impact of glycerol on recrystallization, there is also an
impact due to the chemical behavior of the substance. In both glycerol
solutions, recrystallization was significantly accentuated, but the degree by
which recrystallization was enhanced may lie in the viscosity of the different
concentrations of glycerol. A 4% solution of glycerol is more viscous than
1%. As a result of this elevated viscosity, the solute particles, at the grain
interfaces may act as an obstacle or barrier against the moving water
molecules, thus dampening somewhat the rate of recrystallization. So,
although in the 4% glycerol solution there is a further depression of the
melting point and an increase in osmolarity, the viscosity may be an additional
characteristic of the solutions that tamper how fast recrystallization can occur.

II. Saline and recrystallization

Next we determined the effects of saline on recrystallization and the
impact of glycerol on recrystallization in saline solutions. These conditions
more clearly reflect endogenous physiological conditions found in
overwintering insects. A quantitative assessment of the mean largest grain
size shows convincingly that recrystallization of nanopure water was
significantly enhanced in the presence of a 0.9% physiological NaCl solution
when annealed for 30 minutes at -6°C. The physical explanation for how
NaCl may enhance recrystallization of nanopure water, under particular
circumstances, was articulated by Knight (1995). Presumably, the addition of
sodium chloride increases the volume of liquid between the ice grain
boundaries due to a colligative melting point depression effect, thus allowing
greater ice growth at the boundary layer, and hence, a more pronounced
recrystallization effect.

We then determined the impact of glycerol on recrystallization in these
saline solutions. Glycerol showed a similar effect to that of NaCl. At the
concentrations used in our studies, the glycerol/saline solutions enhanced
recrystallization. Colligatively, glycerol may again accelerate recrystallization
in a similar fashion to that seen with NaCl. If so, then we might expect to see
similarities in the colligative properties of our glycerol and saline solutions
(Table 1). However, this was not the case. Melting point measurements
show, that the 1% glycerol solution further depressed the melting point when
compared to the saline solution alone, and even further with the 4% glycerol in
saline solution. Each solution also showed an increase in osmolarity as
concentrations increased. While it may be difficult to explain the accelerating
effect of both NaCl and glycerol at certain concentrations with respect to
recrystallization, it may, nonetheless, be related to the presence of liquid water
at the interfaces between ice grains caused by the colligative melting point
depression of NaCl and glycerol in solution. The movement of H$_2$O
molecules between adjacent ice grains is promoted with liquid at ice boundary
interfaces. So the presence of liquid H$_2$O would, in effect, decrease the
“activation energy of reorientation” of migrating molecules (Knight, 1995).
For frozen H$_2$O samples lacking NaCl or glycerol, liquid water would be less,
consequently, recrystallization would proceed at a slower pace of that seen
with glycerol/saline solutions.
Furthermore, our results again suggest that glycerol impacts recrystallization in ways that reflect its chemical nature. If glycerol were functioning solely on a colligative basis, we might have observed an enhancement of recrystallization in the 1% glycerol in saline solution. However what was observed was a very similar degree of recrystallization when compared to 0.9% saline alone. This may mean that the addition of glycerol to the 0.9% saline did not have an additional affect on the amount of liquid inclusions between the ice grains. The ice wafer generated via the splat-cooling technique would have excluded all salts and glycerol from the ice lattice. This may have concentrated the glycerol between ice grains, and while this effectively lowers the melting point and may promote liquid water at those interfaces, it would likely also increase viscosity at these boundaries, and therefore this may dampen the more accelerated ice growth derived from colligative means.

Recrystallization behavior of the 4% glycerol solution further supports this explanation. In this case, the 4% glycerol solution has much lower melting point than the 0.9% saline solution, and a corresponding greater osmolarity (Table 1). However, the ice grain sizes of the 4% glycerol solution were significantly smaller than those of both the 0.9% saline and 1% glycerol/saline solutions. Although recrystallization was still accelerated, it was not accelerated as much as the 0.9% saline and 1% glycerol solutions. Again, an increase in viscosity associated with an increase in glycerol concentration may explain this effect. As glycerol becomes more concentrated, its viscosity increases. This increase in viscosity may mean that
there will be not only an increase in liquid volume at the interface, but also solute particles. These solute particles may slow or partially block the migration of water molecules, thus slowing recrystallization as observed with the 4% glycerol/saline solution, when compared to the 1% glycerol/saline solution.

III. Glycerol in the presence of dilute AFP and recrystallization

Lastly, we determined the role of glycerol on recrystallization in the presence of antifreeze proteins (AFPs). Antifreeze proteins have the ability to directly interact with ice, and block further ice growth (Duman 1986). Also, AFPs have been shown to directly inhibit recrystallization (Knight and Duman 1986, Myers 1999). This is an AFP-specific response seen under conditions that eliminate non-THP recrystallization inhibition (Knight 1995, Myers 1999). As stated previously, particular concentrations of NaCl will accelerate the rate of recrystallization in pure H₂O, due to an increase in liquid volume between the boundaries of bordering ice grains. On the other hand, AFPs function to counter this increase in rate of recrystallization presumably by adsorbing directly to the surface of burgeoning ice crystals, thus preventing recrystallization. Our studies provide further support for this. With a very dilute concentration of AFP (1:10,000 winter hemolymph), recrystallization was significantly inhibited.

Since the present study has indicated that glycerol promotes recrystallization, we were especially interested in examining whether the addition of AFPs to these glycerol solutions would counter glycerol’s enhancement of recrystallization. In fact, this was shown to be the case. The
1% glycerol/saline solution with AFP displayed nearly a 6-fold decrease in recrystallization, and the 4% glycerol/saline solution with AFP showed a nearly 4-fold decrease in recrystallization. Both 1% and 4% glycerol/saline/AFP solutions showed a near equal level of recrystallization after 30 minutes of annealing. Melting point measurements indicate that even at a 1:10,000 dilution, the AFPs were still potent at inhibiting recrystallization. Presumably, this is because the AFPs inhibit recrystallization by specifically binding to the ice lattice.

Furthermore, our data indicate that glycerol actually has an enhancement or activator effect on the ability of AFPs to inhibit recrystallization. We observed that recrystallization inhibition was further enhanced in the presence of glycerol, regardless of concentration, when compared to the saline/AFP solution. The idea of a colligative effect may be ruled out, as the melting point measurement decreased as the percentage of glycerol increased, but the grain sizes were not affected by the corresponding osmolarity values. Therefore glycerol may have a chemical interaction with the AFPs, allowing for the AFPs to generate a greater inhibition of recrystallization.

There are now several instances where the thermal hysteresis activity of antifreeze proteins has been shown to be enhanced by certain solutes, including salts, generic proteins like gelatin, immunoglobulins, and natural endogenous activator proteins (Duman and Wu, 1992). Glycerol has also been found to enhance thermal hysteresis activity of purified insect AFPs (Duman et al 1991). The mechanism by which this is done is not fully understood,
however it may be similar to the way antibodies appear to activate THPs. Presumably the THP aggregates with the activator protein and the aggregated proteins block a greater surface area of the seed crystal then does the THP alone.

Our studies are the first to document an enhancement of AFPs’ ability to inhibit recrystallization, in this case, by glycerol. This enhanced AFP/glycerol inhibition of recrystallization, may be a consequence of two different modes of actions of glycerol. The first again relates to increased viscosity. In terms of recrystallization a high concentration of glycerol may be very viscous and gelatinous similar to a 25% gelatin solution, another well known activator. A very viscous glycerol solution would provide additional solute particles at the grain interfaces to slow down recrystallization, when compared to a less viscous glycerol solution. This slowing of the time of recrystallization might be giving THPs additional time to adsorb to the seed crystal before recrystallization would occur under normal circumstances. The second mode of action by which glycerol might promote AFP’s ability to inhibit recrystallization involves direct AFP-glycerol interaction. The AFP-glycerol complex may have altered binding characteristics, which would allow the complex to adsorb to a larger surface of the growing ice crystal effectively limiting the sites by which additional water molecules may bind, thus dampening or restricting recrystallization. These two pathways by which glycerol may contribute to AFP’s inhibition of recrystallization may account for the substantial retardation of recrystallization seen with our AFP/glycerol/saline solutions.
Finally, glycerol is a well known cryoprotectant (Duman 1986). In these cases, during freezing, glycerol works to compensate for the intracellular water loss that a cell undergoes as extracellular ice and solute accumulate. However, our studies clearly show that glycerol works to enhance recrystallization, a phenomenon that is deleterious to an overwintering freeze tolerant organism. This paradox may suggest that glycerol is cryoprotective during the initial bout of freezing, but may prove problematic during an extended freeze, and/or during the melt; these are times when recrystallization tends to occur. To overcome this challenge some organisms may have evolved AFPs, whose potency to inhibit recrystallization is enhanced in the presence of glycerol and possibly other colligative antifreezes like sorbitol, manitol, etc., thus limiting this type of damage in natural conditions. Therefore, our study may be consequential to clinical studies, which would improve the cryopreservation of tissues.
References


