Investigation of Antifreeze Protein Activity in Blue Mussels and Amyloid-Like
Transition in a Predominant Winter Flounder Serum Antifreeze Protein

by

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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

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ABSTRACT

The study of marine antifreeze proteins has provided new findings. The blue mussel (*Mytilus edulis*) was known to have antifreeze activity; however, the antifreeze protein or other molecule responsible has never been characterized. Activity was evident in mussels from each of the Maritime provinces, Canada. The antifreeze molecule was shown to alter ice crystal morphology. It functioned over a wide range of pH values and it showed protease resistance. Nonetheless, its purification was not achieved. A winter flounder (*Pseudopleuronectes americanus*) α-helical antifreeze protein, wflAFP6, has been shown to form amyloid-like fibrils during freezing. Separation of different aspects of the freezing process demonstrated that equilibrium freezing with an ice template is necessary for conversion of the wflAFP6 to the amyloid-like conformation. Amyloid-like conformation was determined by dye binding and electron microscopy. The effects of wflAFP6 concentration and solution properties were determined in order to better understand the process of conversion.

LIST OF ABBREVIATIONS AND SYMBOLS USED

A	alanine
A	amp
Aβ	amyloid β
AU	absorbance unit
AFGP	antifreeze glycoprotein
AFP	antifreeze protein
Ala	alanine
Arg	arginine
Asp	aspartic acid
Asn	asparagine
Asx	aspartic acid or asparigine
C	celsius
CAPs	N-cyclohexyl-3-aminopropanesulfonic acid
Cys	cystine
Da	dalton
DEAE	diethylaminoethyl
EDTA	Ethylenediaminetetraacetic acid
FPLC	fast protein liquid chromatography
g	gram
g	gravity
Gln	glutamine
Glu	glutamic acid
HPLC	high performance liquid chromatography
HR	high resolution
INP	ice nucleating protein
k	kilo
L	litre
Lys	lysine

m	metre
m	milli
μ	micro
μ	micron
M	mega
M	molar
min	minute
n	nano
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PIN	protein ice nucleator
RFU	relative fluorescent unit
RT	room temperature
SDS	sodium dodecyl sulphate
Ser	serine
TEM	transmission electron microscopy
Thr	threonine
ThT	Thioflavin T
UV	ultraviolet
Val	valine
wflAFP	winter flounder liver antifreeze protein

ACKNOWLEDGMENTS

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CHAPTER 1: INTRODUCTION

1.1 COLD AS A SELECTIVE PRESSURE

Species constantly encounter challenges and undergo selective pressures, one of the most fundamental being the temperatures of their habitats. Extreme temperatures pose a great risk to many organisms (Storey & Storey, 2004; Hancock *et al.,* 2008; Hancock *et al.,* 2011). Oceans around the globe are generally cold, with approximately 90% of ocean water below 5°C and surface temperatures reaching -1.9°C in polar and temperate regions (Storey & Storey, 2004). The present time is considered to be an interglacial, or warm period, yet this was certainly not always the case. In the period from approximately 118,000 to 9,000 years ago, the earth was significantly cooler. In fact, warm periods comprise only approximately 10% of the last 2.5 million years (Adkins *et al.,* 1997). Furthermore, in warm periods such as the present time, there can still be substantial cold regions on earth. Low temperature is therefore a far-reaching and important selective pressure for diverse organisms.

1.2 ADAPTATION OF SPECIES TO COLD

Low temperatures affect structural integrity, metabolism and other processes. Temperatures below the freezing point of water present an even greater risk because the bodily fluids of an organism may freeze below their colligative freezing points. Freezing presents many risks. An organism that freezes can experience rupturing of the vasculature, halting of blood/fluid flow, dehydration and the arresting of nervous functions, all of which can prove fatal (Storey & Storey, 2004; Pearce, 2001). Survival

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mechanisms to either prevent or modulate ice formation within the body allow an organism to survive these selective pressures. One such mechanism, and the focus of this thesis, is antifreeze proteins (AFPs) (Storey & Storey, 2004).

1.3 ANTIFREEZE PROTEINS

1.3.1 General Antifreeze Protein Information

Species that avoid freezing and cannot survive freezing, are termed freeze-avoidant while those that can survive the freezing process are referred to as freeze-tolerant or freeze-resistant (Storey & Storey, 2004). AFPs have important protective roles in many of these species. The AFPs bind to ice crystals, which can have two selective effects in different contexts. In freeze-avoidant species, AFPs lower the freezing point and thereby prevent the freezing of bodily fluids, whereas in freeze-resistant species, AFPs can modulate ice growth when freezing and thawing occur such that crystals remain small (Davies *et al.*, 2002; Ewart *et al.,* 1999) . The AFPs that have these ice-binding roles in various species are highly varied, in size, structure and properties (Griffith & Ewart*,* 1995; Davies & Sykes, 1997; Ewart *et al.,* 1999).

Despite the diversity of AFPs, which will be reviewed later in this chapter, they all function by binding to ice crystals, thereby preventing freezing damage. The ice growth-inhibiting feature of AFPs was first discovered due to the ability of AFPs to depress the freezing point of fish blood serum to a much greater extent then colligative properties would suggest (Devries, 1970). This means that AFPs are effective at preventing ice formation at much lower concentrations than would be necessary for many types of

chemical antifreeze that act only colligatively, such as ethylene glycol (Harding *et al.,* 1999). AFPs can therefore impart substantial freezing point depression while having a negligible impact on the osmotic pressure of an organism and they potentially require less of a metabolic investment in synthesis.

The currently accepted mode of action for the prevention of ice crystal growth by AFPs has been a matter of intense investigation for several decades. Overall an inhibition-absorption mechanism involving the binding of the AFP to a specific plane on the surface of a growing ice crystal surface and sterically hindering the thermodynamically favored growth of ice by separating the liquid water phase and the solid crystal phase(Raymond & Devries, 1977; Burcham *et al.,* 1986). As an ice crystal begins to grow, AFPs will bind specific ice surface planes and prevent further growth of the crystal structure outward from those planes. This results in distinctive morphologies for ice crystals growing in the presence of AFPs. They are often sharp and angular with distinct faces (Figure 1). The exact morphology of the ice crystal depends on the planes to which the AFP binds; however, a hexagonal spicule shape is quite common (Scholander & Maggert, 1971; Knight *et al.* 1984). Binding of AFPs to ice seems largely to rely on van der Waals forces and hydrogen bonding; however, the mechanism is still not completely understood for many AFPs, due in part to the difficulty of studying protein-ice surface interaction (Jorov, 2003). For example, it has not been directly feasible to co-crystallize an AFP with its ligand ice, as it would so readily be for other proteins with their interacting ligands.
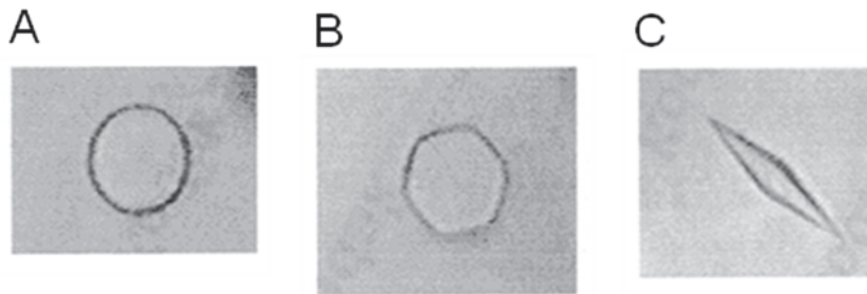
Figure 1. Ice crystals demonstrating the characteristic morphologies observed in the absence and presence of AFPs. The panels show a solution containing no AFP (panel A), trace amounts of AFP (panel B) and high levels of AFP (panel C). (Modified from Ewart *et al.*, 1998)

Recent findings do reveal that AFPs may bind water in ice-like configurations through hydrogen bonding. This arrangement of water molecules simulates the primary prism and basal planes of ice, providing a form of AFP-ice interaction that can be readily viewed by x-ray crystallography. Both hydrogen bonding and hydrophobic interactions are important in the AFP-ice interaction, and the ordered water molecules may play in role in absorption to ice. This binding method has been termed the "anchored clathrate" mechanism of action (Garnham *et al.,* 2011).

The binding of AFP to ice stabilizes the ice to an extent, thereby lowering the actual freezing temperature. The crystal is normally stable within the resulting temperature gap between the melting and freezing temperatures, named a thermal hysteresis. As temperatures decline through the hysteresis interval, the growth of ice will become increasingly favoured energetically, until a point is reached at which the ice surface will rapidly expand over the AFP and resume normal growth. The difference between the temperature at which the ice crystal melts (melting point) and the temperature at which it grows rapidly (freezing point) when it overcomes the inhibition of the AFP is the thermal hysteresis (Davies & Sykes, 1997). The magnitude of thermal hysteresis generated on a molar basis can vary wildly among the different AFPs. Some of these differences in AFP efficacy may have evolved in response to differing selection pressures in the environments of different species and in their evolutionary histories.

Freeze-avoidant organism AFPs are capable of preventing freezing across the range of temperatures encountered in their environment through the year, otherwise death would

be the result. A freeze-avoidant individual that experiences a wide range of temperatures would have sufficient AFP to lower the freezing temperature to the lowest environmental temperature encountered. Those in more stable environments may have AFP capable of protecting against ice growth in a narrow band of temperatures (Duman & Devries, 1974). This may explain why insects, as an example, have antifreeze molecules with very high thermal hysteresis (Scotter *et al.,* 2006), while many fish have AFPs which are only effective for a couple of degrees below zero. The spruce budworm (*Choristoneura fumiferana*) AFPs demonstrate very high thermal hysteresis level on a mass basis, 10-100 times greater than most fish AFPs (Doucet *et al.,* 2000; Doucet *et al.,* 2009).

Freeze-tolerant organisms present an interesting question, as they are capable of macroscopic freezing and thawing, after which they are able to resume normal activity. Here, thermal hysteresis would have little purpose. Instead, it has been observed that AFPs may play a role in stabilizing cellular membranes (Storey & Storey, 2004) and, during freezing and thawing in these animals, preventing ice recrystallization. Ice crystals can undergo localized freezing and melting, even when appearing macroscopically to be "frozen solid". This recrystallization phenomenon can result in several smaller crystals coalescing into a single larger ice crystal with a lower surface area (Knight *et al.,* 1995). It is these large crystals that are believed to be harmful and disruptive to cellular membranes and to cause the mechanical damage experienced during freezing. Binding of AFPs to the smaller crystals surface prevents this recrystalliaztion effect. Combined with protein ice nucleators (PINs or INPs) who act nucleate ice crystal formation, ensuring ice formation occurs at higher sub-zero temperatures with many small ice crystals, an

organism can avoid the tissue damage associated with freezing and simply resume

metabolic activities once thawed (Clark & Worland, 2008; Storey & Storey, 2004;

Valliere *et al.,* 1990).

1.3.2 AFP Types

While AFPs appear to have ice-binding functions in all species in which they are found,

there is large variation in structure among these proteins. AFPs are widespread, having

been found in bacteria, plants, fungi, invertebrates, insects and fish, and they are loosely

placed into 5 types for fish, 2 types for insects and 3 types for plants, with work ongoing

to characterize AFPs observed in other organisms (Ewart *et al.*, 1999). A single AFP type

may include proteins that have a variety of sequences, but these proteins are normally

homologous or share remarkable compositional similarities. Moreover, most AFPs

appear to be recently evolved variants within existing protein families with other major

ligand binding functions. For these reasons, sequence database searches are of little use in

finding new AFPs; instead, directly assaying proteins for AFP activity is the only

practical way to identify AFP presence in an organism (Griffith & Yaish, 2004).

Fish type I antifreeze proteins from the winter flounder (*Pseudopleuronectes americanus*)

are the best known, and include the first to have a 3-dimensional structure determined.

They consist of amphipathic α- helical proteins, approximately 3.5-4.5 kDa, with three

faces to their structure, a hydrophilic, hydrophobic and an intermediate Thr-Asx face

(Patel & Graether, 2010). A subset of type I exist, known as hyp-type I, in a dimeric

form, approximately 32 kDa or two 17 kDa subunits, and are far more efficient at

freezing point depression than most fish antifreezes (Patel & Graether, 2010; Gauthier *et al.,* 2005; Marshall *et al.*, 2005). These proteins can be found in several righteye flounders and sculpins. Fish type II antifreezes are cysteine-rich globular proteins containing 5 disulfide bonds that are homologous to the C-type lectins. They are found in sea raven, rainbow smelt and Atlantic herring (Ewart & Fletcher, 1993; Yamashita *et al.,* 2003). Fish type III antifreeze proteins, which are found in eel pouts (family Zoarcidae), are approximately 6 kDa in size and they appear to have evolved from sialic acid synthase (Baardsnes & Davies, 2001). The types III AFP differ from the type I in that they are globular proteins with a single flat side. Residues on the flat side of this AFP have been shown to have roles in ice binding (Howard *et al.,* 2011). The fish type IV AFPs found in longhorn sculpin (*Myoxocephalus octodecimspinosis*) are α-helical, like the type I, but they are rich in Gln and Glu and their sequence predicts a 4 helix-bundle homologous to serum apolipoproteins (Deng & Larsen, 1998). Cod and Antarctic notootheniids produce antifreeze glycoproteins, often referred to as AFGPs because of their glycosylation, although the discovery of other AFP glycoproteins makes this name less relevant and they are considered as a type among the AFPs. These AFPs are polymers of the tripeptide Ala-Ala-Thr with a few minor sequence variants, and there is an O-linked dissacharide on each Thr (Davies & Sykes, 1997; Griffith & Ewart 1995; Ewart *et al.* 1999).

AFPs are also found in beetles, such as the mealworm (*Tenebrio molitor*), and generally consists of 12-mer or 13-mer repeats approximately 8.3 to 12.5 kDa long (Graham *et al.*, 2007; Doucet *et al.*, 2009). These proteins are considered highly active, and are

commonly referred to as hyperactive, as they generate a very high freezing point depression on a mass basis. They are highly enriched in Thr and Cys, containing a cysteine at least every sixth residue (Graham et al., 2007; Doucet *et al.*, 2009). Another type of AFP found in spruce budworm is also enriched in Thr and Cys, but does not contain repeats (Graham *et al.,* 2007; Doucet *et al.*, 2009). There exists an interesting third anti-freeze molecule from the Alaskan beetle (*Upis ceramboides*), a xylomannan glycolipid that contains no protein. Yet the molecule displays remarkable thermal hysteresis properties (Walters *et al*., 2009).

Many species of overwintering plants also appear to contain several types of AFPs. For example, the winter rye (*Secale cereale*) produces AFPs homologous to the pathogenesis-related proteins chitinase, glucanase and thaumatin (Griffith & Yaish, 2004; Ewart *et al.*, 1999; Hon *et al.,* 1995). Plants generally have low thermal hysteresis levels and their AFPs likely play a larger role in the prevention of recrystallization than in lowering the freezing point (Knight *et al.,* 1995; Hon *et al.,* 1995).

Bacterial AFPs are also being characterized, showing again the great variety in AFP structures. The Antarctic bacterium *Marinomonas primoryensis* produces a Ca^{2+}-dependent AFP greater than 1 MDa in size, which also has hyperactive activity. The ice binding site has been localized to a 322 amino acid region containing a β-helical fold. The ice binding site appears to consist of parallel repetitive arrays of Thr and Asp/Asn (Garnham *et al*., 2008).

These examples only represent a portion of AFPs now discovered. More are being observed, through their activity, in bacteria, fungi, marine microorganisms and invertebrates. For example, a multigene family of AFPs was recently found and characterized in the polar diatom *Fragilariopis cylindrus* (Bayer-Giraldi *et al.*, 2011). However, as there is no true consensus in sequence for AFPs, the isolation and characterization of these proteins can be a very labour-intensive initiative. Nonetheless, a great deal of exciting progress is being made.

1.3.3 Novel Uses for AFPs

The ability of AFPs to halt ice growth and limit ice crystal size has proven to be a highly desirable feature for many applications. Many industries in the frozen food sector could make products more tolerant to the freezing process and limit their recrystallization, which causes hardness in foods like ice cream and contributes to freeze dehydration known as "freezer burn". This could have the added benefit of maintaining nutrients for longer periods in frozen products (Griffith & Ewart, 1995). Strains of frost-resistant crop species could be produced for agriculture in colder climates by introducing into them appropriate genes for AFPs, particularly those from other freeze-tolerant plants. Fish farming may also benefit from freeze-resistant, as has been done with Atlantic salmon (*Salmon salar*). This species does not produce its own AFP, but the flounder type I AFP gene has been introduced into this salmon, where it is appropriately expressed (Hew et al., 1999). This may eventually allow Atlantic salmon aquaculture in more northerly locations at lower temperatures. The use of AFPs in medical applications such as cryosurgery could also prove useful, where the AFP actually plays the interesting role of

generating spicular ice crystals that enhance the destruction of undesirable tissue (Pham *et al.,* 1999).

Use of AFPs for the above applications requires a strong understanding of AFP biology. Structure, mode of action, sequence and the ability to isolate the proteins, amongst other potential properties, all play a role in their potential use for future applications. This requires that extensive research be done on candidate AFPs. A winter flounder AFP may also have a novel use in medical research due to its protein misfolding and amyloid-like fibril formation properties (Graether *et al.,* 2003; Graether & Sykes, 2009).

The current study focuses on two antifreeze protein-synthesizing species, blue mussels (*Mytilus edulis)* and winter flounder. The focuses of the two projects, while similar in their focus on AFPs, are quite different in scope. One, and the first that will be discussed, is the attempted isolation of an AFP from blue mussels. Evidence has previously been found, in the form of measurable thermal hysteresis values, indicating this organism contains AFP (Guderley *et al.,* 1985; Valliere *et al.,* 1990); however it has never been isolated or examined in any great detail. The second project examines an unusual feature of an AFP found in winter flounder. This AFP is well studied with a known structure and sequence; however, it undergoes a conformational change to an amyloid-like fibril formation upon freezing that is not directly linked to its AFP activity (Graether *et al.,* 2001; Graether *et al.* 2003).

1.4 THE BLUE MUSSEL (*M. EDULIS*)

1.4.1 Biology and Environment

Blue mussels (*M. edulis*) are marine invertebrate bivalve molluscs, found on the north Atlantic and Pacific coasts and other temperate or polar waters. Living in intertidal zones on coastal rocks and beaches, the mussel is alternatively submerged in the ocean and exposed to the open air with the tidal cycle. Locations such as the coasts of Canada, in winter, regularly have air temperatures of approximately -20° C. Despite these extreme temperatures, mussels survive well, freezing solid in the open air and resume metabolism upon re-submerging once thawed (Williams, 1970).

Blue mussels are freeze-tolerant species and they are likely to accomplish this with both AFPs, which play a key role in survival, and PINs. These two protein types act in concert to promote gradual (gentle) freezing at high subzero temperatures and to limit tissue damage that occurs during freezing (Guderley *et al.,* 1985; Valliere *et al.,* 1990; Theede *et al.,* 1976). When temperatures fall to a point where a mussel would freeze, PINs act to ensure that ice crystals have many nucleation points (Lundheim, 2002). This limits the size to which any one ice crystal will grow by creating many small crystals (Storey & Storey, 2004). AFPs would then ensure that recrystallization is inhibited, preventing coalescence into larger crystals, which are more damaging (Knight *et al.,* 1995). Once maximally frozen and with small non-damaging crystals maintained, the mussel is able to survive until thawing to resume normal activities.

1.4.2 AFP Information

The exact nature of the blue mussel AFP is unknown; however, evidence of its existence was obtained by observing the ice crystal morphology of growing crystals in the presence of mussel protein extract as well as characteristic thermal hysteresis (Guderley *et al.,* 1985; Valliere *et al.,* 1990). The angular surfaces and spicule shapes of grown ice crystals are characteristic of AFPs, strongly suggesting the presence of an AFP or agent that acts much like an AFP. Work done by Guderley *et al.* 1985 suggests that the AFP is a glycoprotein, as indicated by its presence in the bound fraction when passed through a concanavalin A (Con A) column. Con A binds to mannose groups that may be found on the surface of many glycoproteins (Goldstein & Hayes, 1978; Brewer & Bhattacharyya, 1985). The size, sequence, conformation, and possible subunit structure of this AFP are currently unknown and would require further study.

1.5 THE WINTER FLOUNDER (*P. AMERICANUS*)

1.5.1 Biology and Environment

Winter flounder (*P. americanus*) is a right-eye flounder found in the northerly coastal waters of Canada and the United States. Winter flounder can often be found in shallow estuaries, rivers and bays during the winter months where waters can be several degrees below freezing and filled with ice. The temperature is low enough that without a protective adaptation the flounder's bodily fluids would freeze. Freezing of a winter flounder results in its death, and as such it is a freeze-avoidant organism (Pearcy, 1961).

1.5.2 Adaptations

Winter flounder possess several AFPs that have been well studied (Duman & Devries, 1976; Gong *et al.,* 1996; Tong *et al.,* 2000). These proteins behave as typical AFPs, binding to a specific plane of growing ice crystals and inhibiting further growth. The major AFPs of winter flounder have weak thermal hysteresis on a mass basis, but they are abundant in serum and since the flounder only requires freezing avoidance to the freezing point of sea water, which is approximately -1.9°C, depending upon salinity, they contribute a large portion of this freezing point depression.

1.5.3 AFP Information

There are two different forms of AFP in the winter flounder. The short AFPs are less than 40 residues in length. They are expressed throughout the fish and encoded by many gene copies (Gong *et al*., 1995).When isolated from winter flounder serum by HPLC, two distinct AFP isoforms are observed, HPLC6 (also referred to as wflAFP6) and HPLC8 (wflAFP8) (Figure 2). Scrapings from skin also show several similar AFPs, including sAFP1, sAFP2 and sAFP3 (Gong *et al*., 1995). These proteins are α-helical, like all type I AFPs (Patel & Graether, 2010). Winter flounder also produce a much longer helical hyperactive type I AFP (hyp-type I) that have far greater activity on a mass basis and that are comparable to the insect AFPs in that regard (Marshall *et al.,* 2005; Gauthier *et al.*, 2005). This additional AFP provides the additional freezing point depression of winter flounder serum that brings its freezing point to that of the surrounding seawater, together with the shorter and less efficient AFPs (Marshall *et al*., 2005).

Figure 2. Sequence alignment of the type 1 AFPs wflAFP6 and -8. Only three amino acid differences distinguish between the two products.

1.6 WꜰʟAFP6

The winter flounder liver antifreeze protein 6, or wflAFP6, is a relatively small protein, at 37 amino acids, and it is highly soluble. The protein sequence includes three repeats 11 amino acids in length, Thr-X$_2$-Asx-X$_7$, where X usually indicates Ala (Sicheri & Yang, 1995). X-ray crystallography studies have shown that the protein is α-helical (Figure 3) with an amino-terminal cap, Asp 1, Thr 2, Ser 4 and Asp 5, and a carboxy-terminal formed by the amidated Arg 37. A salt bridge also exists between Lys 18-Glu 22 stabilizng the helix. The α-helix has three apparent faces, a hydrophobic face composed of Ala and Thr methyl groups, a hydrophilic face formed by Arg, Glu, Ser and Asn residues and Thr-Asx face containing the hydrophilic groups of the Thr and Asx (Sicheri & Yang, 1995).

WflAFP6 behaves typically for AFPs, binding to ice surfaces in the intermediate "slushy" layer between ice and water, separating the crystalline ice from liquid water and preventing further ice growth (Burcham *et al.*, 1986). Initially, it was thought that the binding of this AFP to ice surfaces was based on hydrogen bonding between the Thr residues in the Thr-Asx face and the ice crystals surface (DeVries 1974; Raymond & DeVries 1977; Brooke-Taylor *et al*. 1996; Haymet *et al*. 1999), however, substitution of the Thr for Val did little to change the AFPs activity (Haymet et al. 1999). Substitution of Thr to Ser eliminated AFP activity (Zang & Larsen, 1998) and mutations of Ala 19 and Ala 21 on the hydrophobic face reduced AFP activity whereas mutations of Ala 17 and Ala 20 did not (Baardness *et al*., 1999). These findings would suggest that van der Waals and hydrophobic interactions play the larger role in wflAFP6-ice interaction (Haymet *et*

al. 1999). The results noted above coincide with the most recent work indicating that a combination of hydrogen bonding and hydrophobic interactions may act to coordinate liquid waters into ice like configurations bound to an AFPs ice binding surface (as noted above) (Garnham *et al.,* 2011).

In addition to ice crystal binding, wflAFP6 has shown another interesting property. Following the freezing of high concentrations of wflAFP6, characteristic signs of amyloid-like structure were observed, including dye binding and structural changes (Graether *et al*., 2003; Graether & Sykes, 2009). Amyloid can be defined briefly as self-associated, stable, insoluble aggregates that contain cross-β sheet structure (Eichner & Radford, 2011). A single freeze-thaw event is capable of inducing a conformational change in wflAFP6 (Graether *et al*., 2009) from soluble α-helical protein to a β-sheet conformation and that the protein will then assemble into amyloid-like fibrils, as indicated by ThT assay, mass spectrometry and transmission electron microscopy (TEM) (Figure 4). Amyloid forming proteins play important roles in several human diseases, such as Alzheimer's disease and Parkinson's disease (Haass & Selkoe, 2007; Laferla *et al.,* 2007).

The exact conditions that favour the conversion of wflAFP6 to an amyloid state are not understood, with observations performed at only two concentrations, 23 mM and 15 mM, and with one kind of freezing method, leaving a great deal to be learned. Understanding the effects of various conditions, such as concentration, pH, and features of the freezing conditions, on wflAFP6 amyloid-like fibril formation are important to optimizing assays

that could eventually use wflAFP6 and also as clues concerning the exact mechanism by which wflAFP6 forms amyloid.
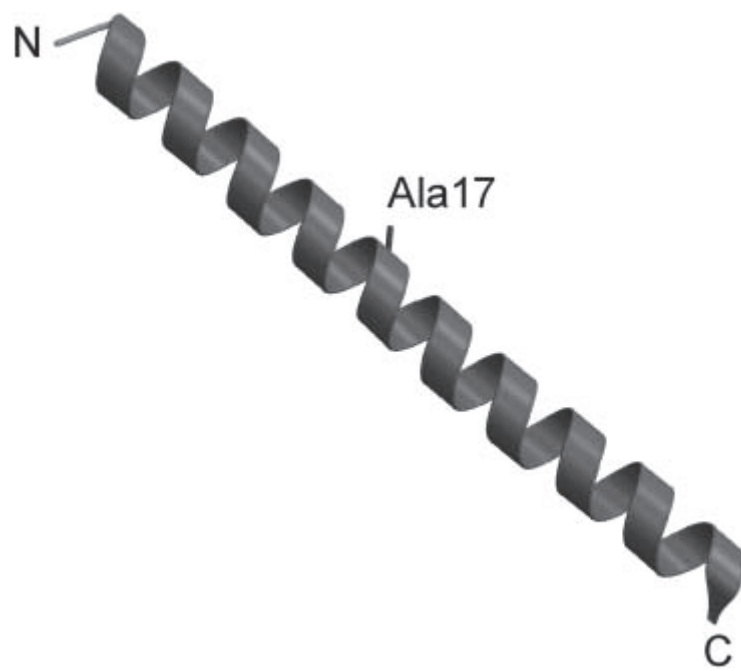
Figure 3. Basic structure of type I AFP shown as a ribbon diagram. (Modified from Graether *et al*., 2003- Open source)

Fibrils
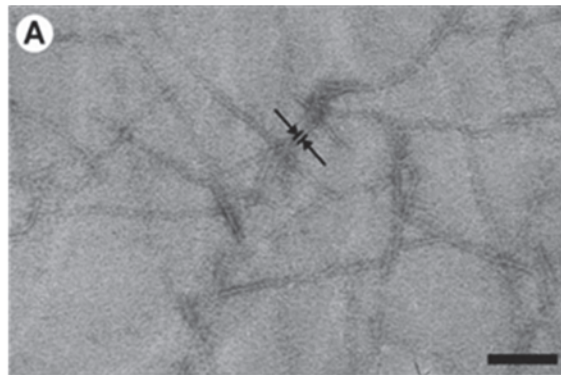
Proto-
filament
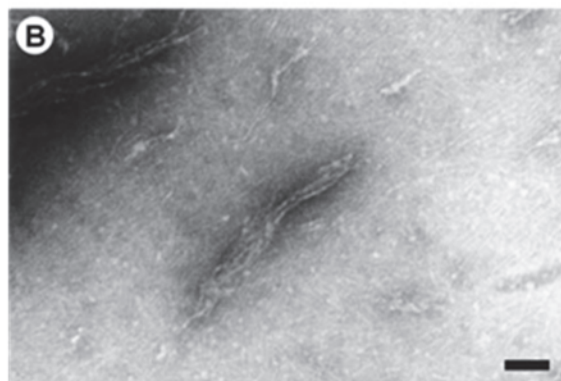
Bar is 100 nm.

Figure 4. Images of the amyloid-like fibrils formed by wflAFP6, following a freezing event, taken by transmission electron microscopy. The first panel shows mature amyloid-like fibrils, while the second focuses on a proto-filament. (Modified from Graether *et al.*, 2003- Open source)

1.7 AMYLOID

1.7.1 Protein Misfolding and Role in Disease

Maturation of a functional protein involves the folding of newly translated protein into its functional form. The process of folding is complex, as proteins have a near limitless variety of possible spatial conformations (Levinthal, 1969). While several models are quite successful in predicting protein folding, the process still holds many questions and no model so far can account for the folding of all proteins.

Currently it is believed that a protein moves through a series of conformations settling upon the lowest possible energy state, which often coincides with the protein's native form (Dobson, 2003; Dobson & Karplus, 1999). This involves interactions between sites within the protein as well as the solvent and solute components in the surrounding environment. The progression to this lowest energy state is not a linear path, but a series of conformations, and the path can vary between two individual molecules of the same protein.

The complex nature and crowded cellular environment (Ellis & Minton, 2003) in which proteins must fold can result in proteins misfolding, i.e. adopting non-native conformations. Some of these conformations can be prone to aggregation, and this can be disruptive to a cell's normal operations/ functions and ultimately damaging. Cells contain several safeguards against potential misfolding, such as chaperone proteins and degradation pathways (Luo & Le, 2010; Ding & Yin, 2008). Chaperones act to protect a nascent protein against detrimental interactions, maintaining the protein in a fold-

competent conformation and allow the protein to fold in isolation. They do not increase the rate of folding themselves, but instead increase the efficiency. A folding protein may go through several rounds of chaperone interactions to attempt folding to a stable state; however, this process is not always successful. Enzymes may then recognize proteins that have failed to reach native conformation and tag them with several ubiquitin molecules. This targets the misfolded protein for degradation by the proteasome (Luo & Le, 2010; Ding & Yin, 2008).

1.7.2 Amyloid-Forming Proteins

While cells contain many features to prevent and eliminate misfolded proteins, a number of diseases have been associated with misfolded proteins (Chiti & Dobson, 2006). Many of these diseases revolve around proteins that undergo a conformational change to amyloid fibrils, producing insoluble aggregates that contain cross-β sheet structures (Eichner & Radford, 2011). These aggregates are often protease-resistant and cannot normally be destroyed *in vivo*. All amyloid fibrils share a similar conformation, β-sheets with associated hydrogen bond strands, parallel or anti-parallel, running perpendicular to the axis of the fibril which is why they are given the name "cross-β" (Eanes & Glenner, 1968). These fibrils also tend to share similar physical characteristics once assembled, fibrils being composed of proto-filaments lightly twisted together into unbranched fibrils 7-12 nm in diameter (Makin & Serpell, 2005).

Many amyloid-forming proteins are well known due to their role in mammalian disease (Benson *et al.,* 2008; Westermark *et al.,* 2007). These proteins, such as the amyloid β-

peptide in Alzheimer's disease, α-synuclein in Parkinson's disease or prion proteins in

the many different prion diseases, seem to share little in common in terms of sequence.

Nonetheless, all produce similar amyloid fibrils. In fact, many proteins produce similar

amyloid-like fibrils under certain conditions, suggesting that amyloid may be a universal

conformation that proteins can be induced to take (Toyama & Weissman, 2011; Stefani &

Dobson, 2003). However, with recent advances in biophysical techniques it has begun to

become apparent that on a molecular level amyloids are quite diverse. The same

polypeptides have in fact been found to produce different amyloid forms, a notable fact in

the prion field, for example, where different conformations of the infectious prion protein

may manifest as a different disease phenotypes. These variations in amyloid structure

could in part be why the study of amyloid formation has proven difficult, as determining

baselines and relevant statistical data has been perturbed by the seemingly varied nature

of amyloid (Toyama & Weissman, 2011).

1.7.3 Amyloid Formation Process

Amyloid fibril assembly is a multi-stage process that is only partially understood. The

current hypothesis is that there is an initial lag phase in which native proteins reversibly

shift between the amyloidogenic β-sheet conformation and an alternative "denatured"

state. Interactions between several protein molecules in the amyloidogenic conformation

can result in the assembly of small amyloidogenic oligomers that are able to further

assemble into an amyloid "seed". These seeds then act as a core for elongation into

filaments which can then assemble into pre-fibrils and fibrils. Seeds can also assemble

into the core of a fibril and then elongate into a full length amyloid fibril (Chiti & Dobson, 2006).

The most common method for detecting and quantifying amyloid fibril formation is through the use of fluorescent dyes, such as Congo red and thioflavin T (ThT) (Levine, 1999). ThT dye is an effective method of detecting amyloid, as a result of its ability to bind most amyloid and amyloid-like fibrils while showing no appreciable interaction with non-amyloid folds. The binding of ThT dye to amyloid fibrils induces a spectral emission shift from the dye that can be detected by fluorescence. The exact mechanism of ThT binding to amyloid fibrils is unknown. Various other dyes, such as Congo Red are also used. Amyloid can also be examined by transmission electron microscopy (TEM) in which filaments and fibrils are evident, providing distinct evidence of amyloid presence.

X-ray diffraction is also extensively employed in verifying the presence and characteristics of amyloid, as the cross-β structure of amyloid presents a characteristic pattern. X-ray diffraction also allows a potentially clearer picture on how specific protein molecules may stack to form amyloid structures (Eichner & Radford, 2011). However, the technique can be quite challenging in implementation.

1.8 RESEARCH OBJECTIVES

The discovery of new AFPs can advance our knowledge in distinct areas. Identifying an AFP in a species allows a better understanding of the cold adaptation and habitat range of the species, particularly as AFP gene dosage and expression can be easily monitored. A

distinct AFP can offer new opportunities to examine protein-ice interaction at the molecular level. Furthermore, the properties of some AFPs make them informative models for studies on protein structural elements and versatile products for biotechnology applications. Therefore, the research objective here was to conduct studies on novel AFPs and AFP properties.

1.8.1 Activity-Guided Mussel AFP Isolation

Evidence exists suggesting that the blue mussel contains an AFP or similarly functioning molecule; however, this protein has not been purified nor studied in detail. The goal of this project was to isolate the blue mussel AFP and to characterize it. To achieve this goal a series of protein isolation strategies were used, guided by qualitative analysis of AFP activity within fractions by observing ice crystal morphology. Various details concerning the activity and stability of the AFP were also determined using this same qualitative method.

1.8.2 Characterization of WflAFP6 Amyloid-Like Fibril Formation

Although the amyloid forming ability of wflAFP6 when frozen has been previously observed, the process is not understood. Therefore, it presents an interesting opportunity to examine the transition of a soluble protein to amyloid-like fibrils and the specific conditions that lead to this transition, which have not been identified. This study involved the manipulation of several parameters, such as cooling and freezing events, concentration and pH, and observing the effect on amyloid-like fibril formation by wflAFP6. This will be achieved by quantifying amyloid-like structure formation under

various conditions using ThT binding assays and TEM. With a baseline set of conditions

optimized to produce amyloid-like structure, use of the process for amyloid structure-

function studies or amyloid formation assays can be explored.

CHAPTER 2: AN AFP IN BLUE MUSSEL

2.1 INTRODUCTION

The occurrence of an AFP in the blue mussel, *M. edulis,* has been entertained for some time because of the ability of this species to freeze solid and then resume normal activity upon thawing (Williams, 1970); however, the first evidence of antifreeze activity was reported by Guderley *et al.,* 1985. Quantifiable thermal hysteresis was obtained, which was found to coincide with the glycoprotein fraction when mussel homogenate was passed through a Con A lectin column, suggesting the possibility of a glycoprotein AFP. Nonetheless, isolation and identification of the protein responsible for antifreeze activity in the blue mussel has not yet been achieved.

The isolation of AFPs presents many interesting challenges and opportunities. Due to the recent evolution of AFPs from a wide range of non-homologous progenitors, these proteins cannot easily be identified by elements such as conserved sequences in a database (Griffith & Ewart*.,* 1995; Davies & Sykes, 1997; Ewart *et al.,* 1999). There is no single sequence and structure in a database that would predict an AFP. An effective algorithm has been developed based upon patterns of ice interaction surface carbons in protein 3-D structures that was reported to identify AFPs (Doxey *et al.,* 2006), but it has not yet been widely used. It would also require a library of 3-D structures, which is not a resource available for mussels. For these reasons, the most practical approach to isolation of an AFP from mussels is antifreeze activity-guided fractionation of extracts. Ice crystal morphologies generated by AFP presence are a convenient marker for AFP

presence (Scholander & Maggert, 1971; Knight *et al.* 1984; Griffith & Yaish, 2004).

Most proteins are excluded from ice during freezing. Therefore, the ice binding activity

of AFPs also presents the possibility that the AFP could be isolated using a method that

exploits this for affinity purification. Kuiper *et al.* (2003) have purified AFP using a cold

finger device (Figure 5) to repeatedly freeze the AFP into the ice and then thaw it,

thereby enriching for this protein.

With these considerations in mind, the goal of this project was the isolation and

characterization of the blue mussel AFP.
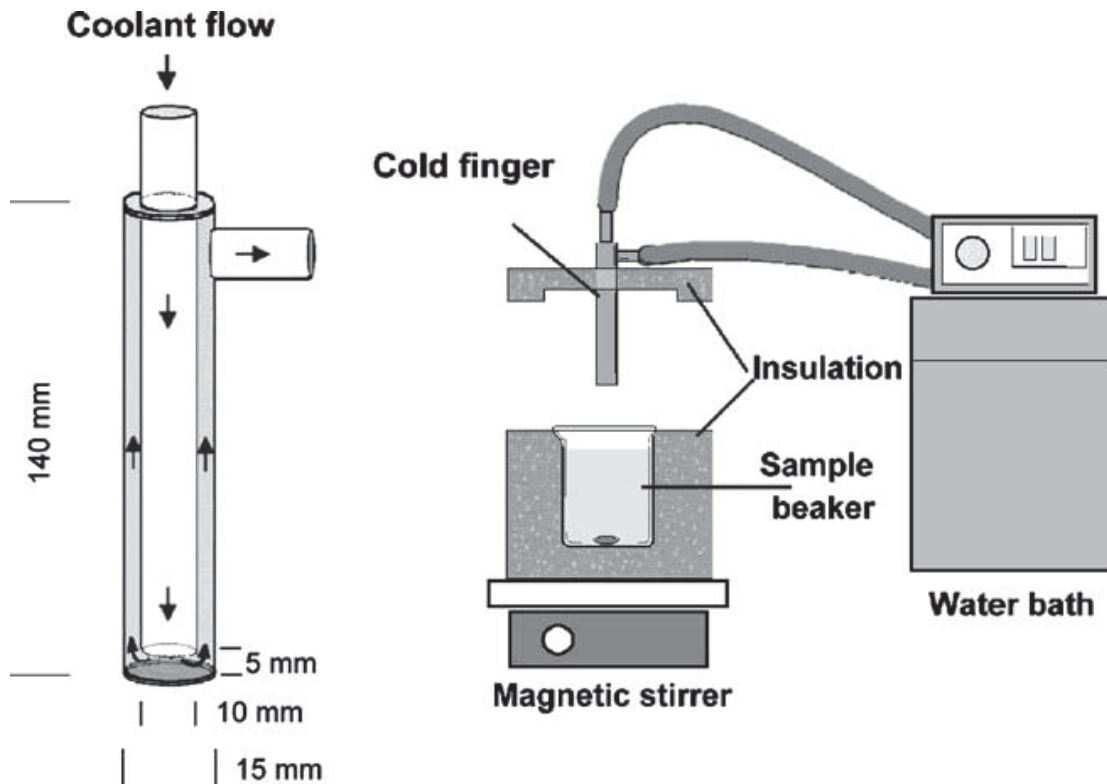
Figure 5. The cold finger apparatus for isolating AFPs using their ice binding activity, or ice affinity purification. The apparatus is constructed of thermally conductive materials such as aluminium or brass for effective heat transfer and best paired with a refrigeration unit/circulator with a high degree of temperature control. (Image adapted from Kuiper *et al*, 2003- with permission from Elsevier)

2.2 MATERIALS AND METHODS

2.2.1 Blue Mussel Sample Collection

Three geographically separate populations of mussels were sampled over the course of the study for the purpose of identifying a mussel population with sufficiently high AFP concentrations to allow effective isolation of the protein.

Mussels from a mussel and salmon farm source on the New Brunswick (NB) side of the Bay of Fundy were provided by Dr. Thierry Chopin (University of New Brunswick). The mussels were collected during the winter (January 2009), in order to increase the likelihood of maximal AFP concentrations. Samples were shipped packed in wet ice, in shell, and then transferred to freezer bags at -80°C upon arrival at the NRC building in Halifax until use. These mussel samples will be referred to as NB mussels.

Wild mussels in an intertidal habitat were sampled on the lower beaches at Sambro, Nova Scotia (NS). The mussels were collected during the winter (February 2010), and placed on ice immediately upon collection. Upon arrival at the NRC building in Halifax, the mussel meat was removed from the shells by cracking the shells and using a clean knife and scissors to remove any remaining connections between shell and tissue. Mussels were then placed in individual freezer bags, frozen on dry ice and stored at -80°C until use. These mussel samples will be referred to as NS mussels.

Farmed mussels from long line culture were provided by a mussel farm along the Montague-Brudenell river watershed in Prince Edward Island (PE). The mussels were

grown submerged and they were collected with sea ice present on the water surface (Jan 2010), to maximise the likelihood of AFPs being present. The mussels were immediately packed in wet ice for shipment, and they were washed in an ice water bath upon receipt. Shells were removed as above and the mussel samples were placed in freezer bags on dry ice and stored at -80 °C until use. These mussel samples will be referred to as PE mussels.

2.2.2 Isolation of Total Protein from Blue Mussels

Verification of AFP activity in the NB, NS and PE mussels required the preparation of homogenates (Cutler, 2004; Valliere *et al.,* 1990; Rosenberg, 2005). Sample mussels were thawed on wet ice for approximately 10 minutes and then, for those requiring shell removal, the rear shell hinge opened with a clean knife. An ultra-clean knife and scissors were used to remove the mussel tissue from the shell and to cut the tissue into approximately 2.5 mm pieces. Care was taken to ensure that the mussel samples remained as cool as possible, using float trays on ice to perform the work. The mussel pieces were then placed in a 50 mL Falcon tube containing approximately 10 mL of 100 mM NH_4HCO_3 (pH ~ 8), and held within in an ice water bath. For large mussels the pieces may have been divided between 2 tubes, while small mussels were occasionally combined, in order to maintain a consistent volume of tissue equivalent to 5-10 mL per tube.

For each preparation, a polytron homogenizer was then used to homogenize the mussel pieces with the sample tubes contained in an ice water bath during the entire process. The

homogenate was then spun at approximately 4000 x g for 30 minutes in a chilled centrifuge (4°C). The supernatant was transferred from the pellet into a new 50 mL polypropylene tube (Falcon) and this centrifugation step repeated up to 3 times to eliminate insoluble material. The final supernatant was decanted to a clean 50 mL Falcon tube. This set of preparation conditions will be referred to as standard mussel homogenate protocol.

Further steps were also taken in some instances to increase homogenate clarity and eliminate further contaminants. Samples from the standard protocol were then placed into a 55°C water bath for 25 minutes to remove heat-labile proteins as described in the procedure of Guderley *et al*. (1985). It was then spun at 4000 x g in a chilled centrifuge (4°C). This preparation shall be referred to as the heated mussel homogenate protocol.

For some analyses, protein extracts were prepared from either the standard or heated protocol mussel homogenates by acetone precipitation. The new supernatants were transferred to clean acetone-resistant polypropylene tubes (Oakridge).In a fume hood, ice-chilled acetone was added to the above mussel supernatants at a 4:1 acetone to mussel homogenate ratio, mixed briefly with a vortex mixer, and allowed to incubate for 1 hour on ice. The preparations were then spun at 10 000 x g, in a chilled centrifuge (4°C) for 10 minutes. The spin step was repeated if the supernatant was not clear or if the pellet was not discrete.

Supernatants were then decanted and the acetone pellets allowed to dry for approximately 30 minutes in a fume hood at room temperature (RT). The dried pellets were placed in 2 mL microfuge tubes and either stored dry at -20°C or dissolved in minimal 100 mM NH_4HCO_3 and frozen at -80°C. Once dissolved, care was taken to keep the samples cool during any procedure. Sample mussel protein precipitates from this procedure will be referred to as either acetone-precipitated protein or heated acetone-precipitated protein, dependent on the original homogenate protocol.

Samples were rarely quantified by Bradford analysis at this stage, as the solutions were pigmented. Quantification of total protein would be pursued following further isolation steps, where the desired AFP protein may constitute an appreciable quantity of the total protein present. It was also quite apparent that total protein quantity was significant based on sample colour/clarity and protein extract sizes. It is important to note that samples were not the same mussel preparation for all experiments and apparent AFP activity could change a great deal between one mussel homogenate preparation and another.

2.2.3 Photomicroscopy of Ice Crystals

Mussel protein solutions derived from the standard or heated homogenate protocols and, acetone-precipitated protein dissolved in 100 mM NH_4HCO_3 were qualitatively analysed for AFP activity by examining growing ice crystal morphology. Mussel samples were maintained on wet ice, for thawing and when prepared for viewing by miscroscopy. If required, the samples were passed through a 45 µm syringe filter to eliminate particulates; however this step was avoided whenever possible due to the potential risk of

AFP loss. Samples could also be concentrated prior to the procedure using 3K Amicon

Ultrafree®-MC centrifugation filtration units (Millipore). This required 400 µL of the

desired sample, which would be concentrated to 120 µL per the manufacturer`s

instructions in a chilled (4°C) microcentrifuge. The samples shall be referred to as

concentrated "sample type".

 A Clifton nanolitre osmometer was set up with a cooling stage upon the sample stage of

an Olympus BX51 compound microscope fitted with a long-working distance objective,

which allowed microscopic crystals to be viewed in small (sub-µL) convex discs of

protein solutions. Clifton osmometer sample platforms, specifically created for the

purpose of viewing growing ice crystals, allowed a small volume of sample to be placed

into a sample well, a hole through the platform, using glass capillary tubing needles. The

hole was small enough that water tension keeps the droplet in place. The temperature of

the cooling stage was rapidly lowered to -40°C, using the Clifton device, in order to

allow snap freezing of mussel samples, then raised until thawing began. The sample was

allowed to thaw until only one ice crystal remained, and then lowered to a point where

the crystal grew slowly. Then, digital images of growing ice crystals were taken using a

digital camera (Motic) integrated into the microscope. Growth of an ice crystal

unimpeded by AFP is equal in all directions, producing a smooth and circular disk of ice.

In contrast, ice growing in the presence of adhering AFP expresses flat crystal planes and

more irregular angular morphologies that are easily distinguishable from the smooth

disks observed in the absence of AFP. The expression of crystal planes and the direction

and patterns of crystal growth indicated the presence of AFP. Hexagons were taken as

evidence of detectable AFP activity. Irregular hexagons with pits or ridges were taken as evidence of more substantial AFP activity.

2.2.4 Blue Mussel AFP Activity: pH Variation Test

Concentrated acetone-precipitated proteins, from PE mussels, were prepared as 15 μL aliquots in microfuge tubes. To each aliquot either 1μL of 5 M sodium acetate, pH 4, 1 μL of 5 M CAPS, pH 10, or 1 μL of a 50:50 mixture of 5 M sodium acetate, pH 4, and 5 M CAPS, pH 10, was added. These solutions were allowed to incubate 1 hour at 4°C and analysed along with controls processed at the same time.

2.2.5 Blue Mussel AFP Activity: Heating Test

Concentrated acetone-precipitated proteins, from PE mussels, were prepared as 15 μL aliquots in a microfuge tube. The sample was brought to boiling in a water bath for 30 minutes, and then cooled to 4°C and analyzed along with controls processed at the same time.

2.2.6 Blue Mussel AFP Activity: Proteinase K Test

Concentrated acetone-precipitated proteins, from PE mussels, were prepared as 15 μL aliquots in microfuge tubes. To one sample proteinase K was added 1:10 by creating a stock solution of proteinase K (in 1 mM NH_4HCO_3 and 3 mM $CaCl_2$) and adding 1 μL to the 15 μL mussel sample. To a second 15 μL mussel sample 1 μL of 1 mM NH_4HCO_3 and 3 mM $CaCl_2$ was added as a control. A third microfuge tube contained 15 μL of 100

mM NH$_4$HCO$_3$ and 1 µL of the stock proteinase K solution was added. These samples were allowed to incubate overnight at 37°C and analyzed along with controls.

2.2.7 Gel Electrophoresis and Staining

SDS-PAGE gels were prepared either using EZ-Run^{TM} Protein Gel Solution (Fisher Bioreagents®) or using the standard method according to the procedure of Laemmli *et al.* (1970) with a Bio-Rad Gel Casting System (Bio-Rad Laboratories) as indicated by the manufacturers. All gels were stained using either silver staining (Swain and Ross, 1995) or GelCode Blue^{TM} Coomassie blue (Pierce). Imaging of gels was performed using a GS-800 gel scanning system.

Tricine gels were used following the method of Shagger and von Jagow (1987). Acrylamide for the tricine SDS-PAGE was prepared at 49.5% with a 32:1 ratio of acrylamide to bis (48g acrylamide, 1.5g bis-acrylamide per 100mL). The final gel concentration was 16.5%. The samples were resolved at 15 mA until they passed through the stacking gel and the current was increased to 30 mA and held until completion. Gels were stained using GelCode Blue^{TM} Coomassie blue (Pierce).

2.2.8 Con A Affinity Chromatography of Blue Mussel AFP

Con A affinity chromatography was performed based on that described in Guderley *et al.* (1985) with modifications. Guderley Con A procedure aliquots of the necessary buffers were both chilled, to approximately 4°C, and left at RT. To begin, 2-4 mussels (depending on size) were thawed on wet ice, then cut into small, 2.5 mm, pieces and

placed into a 50 mL falcon tube to which 5 mL of chilled Con A buffer (25 mM Tris-HCl, 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.2) was added and non-EDTA containing protease inhibitors. The Falcon tube was held in an ice water bath and the mussel homogenized using a Polytron homogenizer. The homogenate was then spun at 4000 x g for approximately 30 min in a chilled centrifuge. The supernatant was carefully transferred to a new 50 mL tube and the spin was repeated if necessary. The sample was then usable as the standard Con A mussel sample.

As an additional step the homogenates could be subjected to acetone precipitation to form a protein extract for the purpose of eliminating contaminants and concentrating. Approximately 0.1-0.15 g of acetone-precipitated proteins were then dissolved in 1.5 mL of Guderley Con A buffer and kept on ice until chromatography for same day use, or stored at -20°C for later. These were acetone-precipitated Con A samples.

Following sample preparation 2.5 mL of Con A-Sepharose® 4B beads (Sigma or GE Healthcare) were placed in a 15 mL screw cap tube and spun briefly at low speed. Any buffer above the beads was removed with a pipette and the beads then rinsed with 10mL of Con A buffer mixed by inversion. The beads were then spun again at 1000 x g and excess buffer removed. Cytochrome c, 1 mg prepared in 2.5mL of Con A buffer, was then added to the Con A beads and allowed to mix with the beads for 10 minutes, using a rocker table, as a blocking agent to prevent any non-specific binding of the sample protein. The Con A beads were then rinsed four times with 10 mL of Con A buffer, spun at 1000 x g, and the excess buffer removed. The mussel homogenate sample, either 1.5

mL of standard or acetone-precipitated Con A sample, was then allowed to mix with the
Con A beads for 30 minutes at 4°C on a rocker table.

During this period a disposable 10 mL chromatography column (Bio-Rad Laboratories)
was washed and equilibrated. The column was prepared with buffer level from the tube
outlet to just above the column end membrane. To the column, 2.5 mL of Con A buffer
containing 1mg of cytochrome c, was added. The buffer was then allowed to flow out
until the level was just above the end membrane. The column was then rinsed three times
by adding 5 mL of Con A buffer and allowing the buffer to flow out to just above the end
membrane as above.

The column was transferred to a cold box, at 8°C, and a volume of Con A buffer at the
same temperature was run through it to equilibrate the apparatus. The Con A beads and
mussel extract were added to the column and unbound material which passed though the
column collected until the buffer reached the column surface. The column was then
washed three times with 5 mL of the same Con A buffer, collecting the wash flow-thru in
different 15 mL screw cap tubes, allowing the buffer to flow down to the column surface
between additions of buffer. This was followed by the addition of 1.5 mL of elution
buffer (25 mM Tris-HCl, 0.5 M NaCl, 0.4 M mannose) to the column surface allowing
the buffer to flow out till the buffer level reached the column surface. The eluate was
collected in a 2 mL centrifuge tube. This elution step was repeated.

Following the elution, 1.5 mL of metal stripping buffer (25 mM Tris-HCl, 0.5 M NaCl, 50 mM EDTA) was then added to the column surface, following the same procedure as for the elution buffer. This step was also repeated. The four eluates produced by the addition of elution buffer and metal stripping buffer were then each concentrated by passing a 400 µL portion through a 3000 molecular mass cutoff spin filter (Amicon). Fractions were stored at -80°C.

2.2.9 Ice Affinity Purification

A cold finger apparatus was, as described by Kuiper *et al.,* 2003, employed in order to enrich the mussel AFP. The ice finger was largely composed of aluminum piping and was connected to a RTE-111 (Neslab) refrigerator unit filled with a 1:1 mixture of ethylene glycol and water. The cold finger was attached to the unit by plastic tubing to allow circulation of the coolant. Insulation for the sample containing beaker was made using Styrofoam.

The procedure was performed largely as described in Kuiper *et al.,* 2003, with volumes scaled up to account for the ready availability of mussel samples. Samples volumes were varied over the course of the procedure, ranging between 250 mL and 2 L. Samples were composed of standard homogenates and both heated and non heated acetone-precipitated proteins dissolved in the 100 mM NH_4HCO_3. Due to an inability to finely tune the temperature over a time course, freezing was performed at a single temperature sufficient to cause freezing, but requiring a long time period for a sufficient sample volume (approximately 50%) to freeze. The selected temperature was approximately -1°C, which

lays roughly central to previously used linear freezing gradients. Freezing required 6-12 hours.

Following freezing and removal of the frozen fraction from the liquid fraction, the ice was washed with 20-30 mL of chilled water, and then allowed to thaw into a clean beaker. This frozen fraction could be used in a second round of freezing, ideally further concentrating the AFP, immediately analyzed by various methods or concentrated by freeze drying.

2.2.10 Freeze Drying

Mussel protein samples produced by the ice affinity purification procedure were large volumes of dilute solution requiring a method such as freeze drying for effective concentration. Samples were freeze dried in 50 mL falcon tubes, containing approximately 25 mL of (mussel) sample from the ice affinity purification step. The top of the tubes were sealed with tin foil perforated with small holes, then placed in the freeze dryer. The freeze dryer was set to the program detailed in Table 1.

Following completion of the freeze drying process, the samples were stored at -20°C until required. Samples to be analysed were dissolved in approximately 25-100 µL of 100 mM NH_4HCO_3.

Table 1. Freeze dryer program utilized for concentration of ice affinity mussel samples.

Step	Temperature (°C)	Duration (hours)	Pressure (torr)
1	-40	0.3	500
2	-20	10	200
3	-5	10	100
4	5	10	70
5	20	10	50
6	25	10	50

2.3 RESULTS

2.3.1 Verification of AFP Activity in Blue Mussel Populations

The initial step in the plan to isolate the blue mussel AFP was to verify the presence of the AFPs within the mussels collected for this purpose. Mussels from three locations (NB, NS and PE) were homogenized and centrifuged to remove debris. The supernatant was then examined for AFP activity by viewing the morphology of growing ice crystals on a cooling stage with a microscope. A variety of methods, including filtration, centrifugation and acetone precipitation, were used to enhance both the clarity of the homogenates and the magnitude of AFP activity present within them.

The ice crystal morphologies in samples obtained by homogenization of 2-4 mussels from each population (Figure 6), as well as by analysis of individual mussels within a population (Figure 7) showed faceted morphologies consistent with the presence of antifreeze protein. The crystals generated in most of the homogenates could be easily distinguished from the control, which contained buffer alone and produced a smooth disk of ice with no faceted surfaces expressed. Based upon the qualitative assessment of faceting and bipyramidal morphology, the sample (or pool) from PE appeared to have the greatest antifreeze activity. Activities could not be quantified by thermal hysteresis due to instability in the osmometer and with unsuccessful total protein quantification, activity values could not have been compared directly, even if they were obtained.
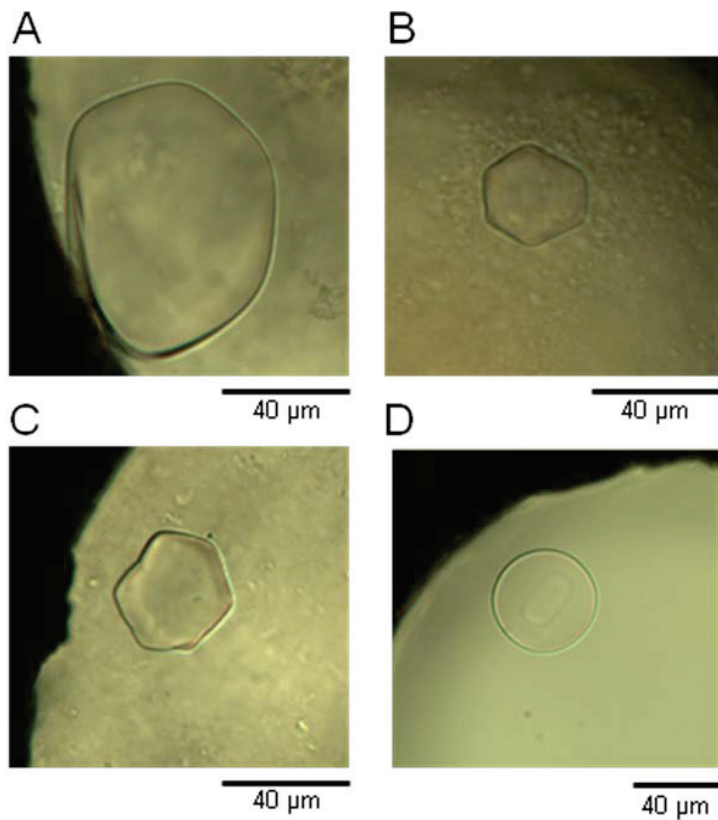
Figure 6. Ice crystal morphologies in homogenates of blue mussel (*M. edulis*) from three locations in eastern Canada. Standard homogenates from 2-4 mussels from NB (panel A), NS (panel B) and PE (panel C) are shown along with a buffer-only control (panel D). Homogenates were prepared by the standard protocol and analyzed using a Clifton nanolitre osmometer as described in the Materials and Methods.
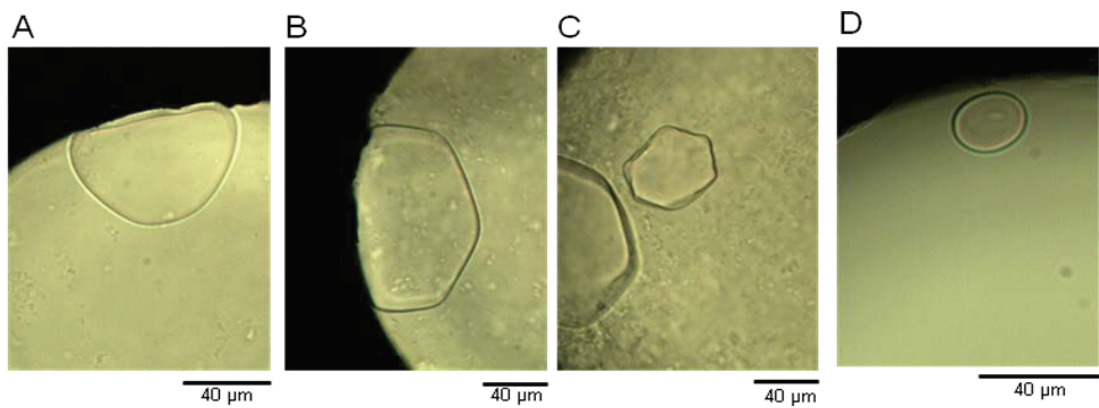
Figure 7. Morphologies of ice crystals grown in homogenates prepared from individual mussels from PE. Standard homogenates were prepared from individual mussels as indicated in the Materials and Methods, and growing ice crystal morphology observed in each homogenate (panels A, B and C) are shown along with a buffer-only control (panel D) using a Clifton nanolitre osmometer as described in the Materials and Methods.

2.3.2 Preliminary Characterization of the Blue Mussel AFP

Samples of the acetone-precipitated protein from PE mussels were adjusted to a range of pH values, and subjected to boiling and digestion with proteinase K in order to characterize the antifreeze agent and to begin devising a purification protocol.

Following the incubations, samples were qualitatively assessed for AFP activity by growing ice crystal morphology. Control samples of the PE acetone-precipitated protein, dissolved in 10 mM NH_4HCO_3, left untreated and 10 mM NH_4HCO_3 alone were used as positive and negative controls respectively.

The blue mussel acetone-precipitated protein showed clear activity at pH 4, 7 and 10 (Figure 8). The AFP appears to be at least partially boiling-stable, showing detectable but possibly less pronounced ice faceting following boiling (Figure 9). This would suggest that the protein is stable in comparison to many other proteins and this could be a useful characteristic. The proteinase K digestion did not result in appreciable change in activity (Figure 10), although SDS-PAGE analysis demonstrated extensive digestion by proteinase K (Figure 11).
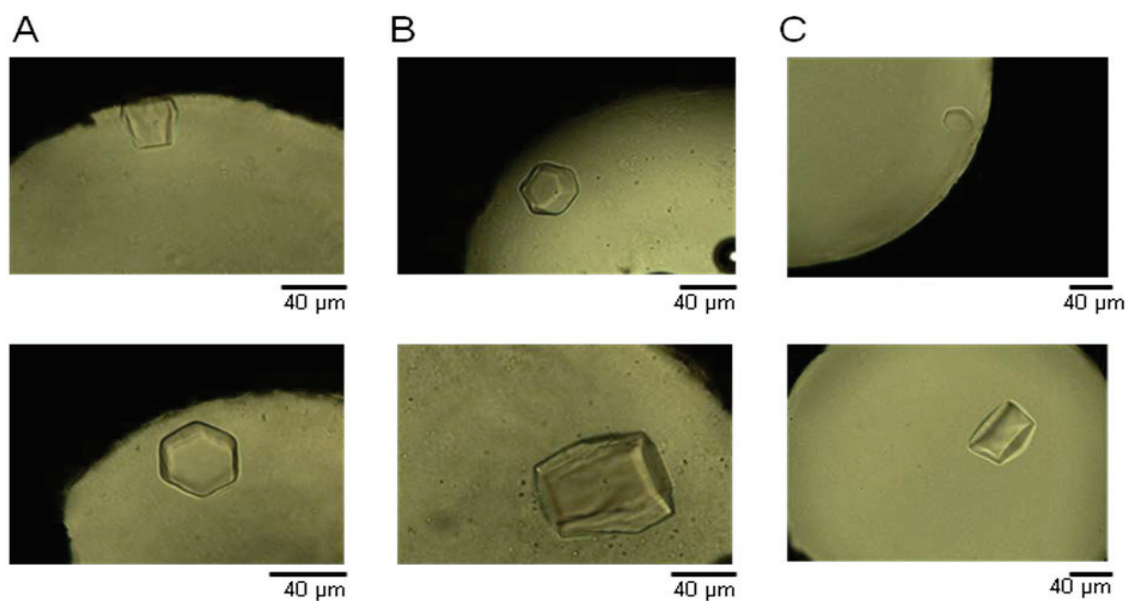
Figure 8. Morphologies of ice crystals in dissolved acetone-precipitated proteins of mussels adjusted to different pHs. An acetone-precipitated protein from a pool of 2-4 PE mussels was prepared as indicated in the Materials and Methods and growing ice crystal morphology observed in each pH adjusted sample using a Clifton nanolitre osmometer. Columns are as follows: (A) pH 4; (B) pH 7; (C) pH 10.

Figure 9. Morphologies of ice crystals in solutions of boiled and untreated protein from PE mussels. An acetone-precipitated protein of 2-4 PE mussels was prepared by as described in the Materials and Methods. A dissolved sample was then boiled for 5 minutes and allowed to cool to 4°C before observing growing ice crystal morphology with a Clifton nanolitre osmometer. Untreated mussel protein is shown in panel A, treated mussel protein in panel B and a buffer-only control in panel C.
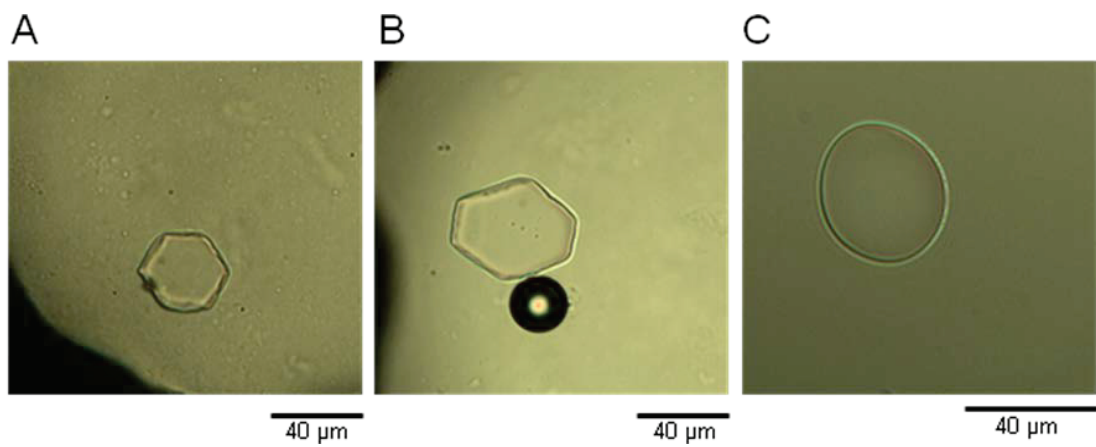
Figure 10. Morphologies of ice crystals in solutions of proteinase K-digested and untreated protein from PE mussels. An acetone-precipitated protein was prepared of 2-4 PE mussels as in the Materials and Methods. Untreated mussel protein is shown in panel A, treated mussel protein in panel B and a buffer only control in panel C.
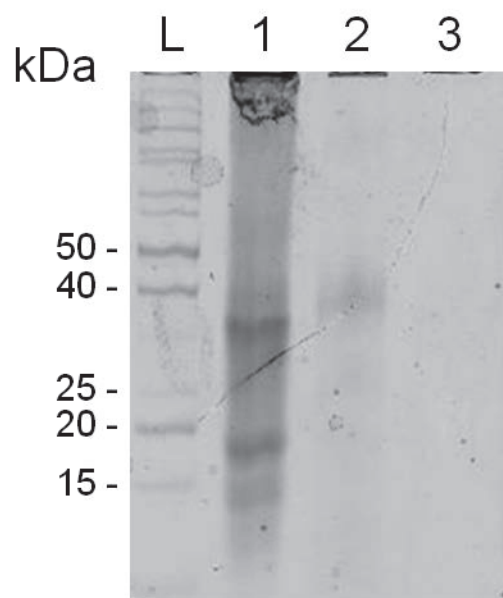
Figure 11. SDS-PAGE analysis of proteinase K-treated and untreated mussel protein. An acetone-precipitated protein preparation was obtained from 2-4 PE mussels as in the Materials and Methods. Samples were then resolved by SDS-PAGE (15%) and stained with coomassie blue. Lanes are molecular mass ladder (L), untreated protein (1), protein treated with proteinase K (2) and proteinase K incubated alone (3).

2.3.3 Chromatography and Affinity Enrichment of the Blue Mussel AFP

2.3.3.1 Affinity Chromatography using Immobilized Con A

Affinity chromatography using Con A-Sepharose® 4B was performed in order to enrich for the AFP because this process had been previously shown to do so (Guderley *et al.,* 1985). A sample homogenate pool isolated from 2-4 PE mussels was used in this instance; however, various preparations of a total mussel protein fraction, including homogenates from different mussel populations, were also tested. The fractions were prepared by a number of methods, standard homogenates and acetone-precipitated protein preparations both concentrated and not, and the Con A chromatography was repeated several times on fresh samples. Several parameters were adjusted, including volume of ConA beads used, volume of homogenate used (ranging from 5 mL to entire homogenates of approximately 10 mL) and wash volumes and numbers, which had no detectable effects.

Upon completion of the Con A chromatography, fractions were examined for enriched proteins and a reduction in candidate proteins using SDS-PAGE (Figure 12). The stained gel revealed enrichment of some bands with depletion of others, suggesting that the chromatography was at least partially successful in selecting for glycoproteins. Nonetheless, it appeared tentatively insufficient to provide promising enrichment. Without successful protein assays to quantify the yield of total protein obtained, direct comparison was difficult. Antifreeze activity, as evidenced using ice morphology, did on one occasion appear different between the starting material and eluted protein (Figure 13). Nonetheless, this positive result could not be repeated.
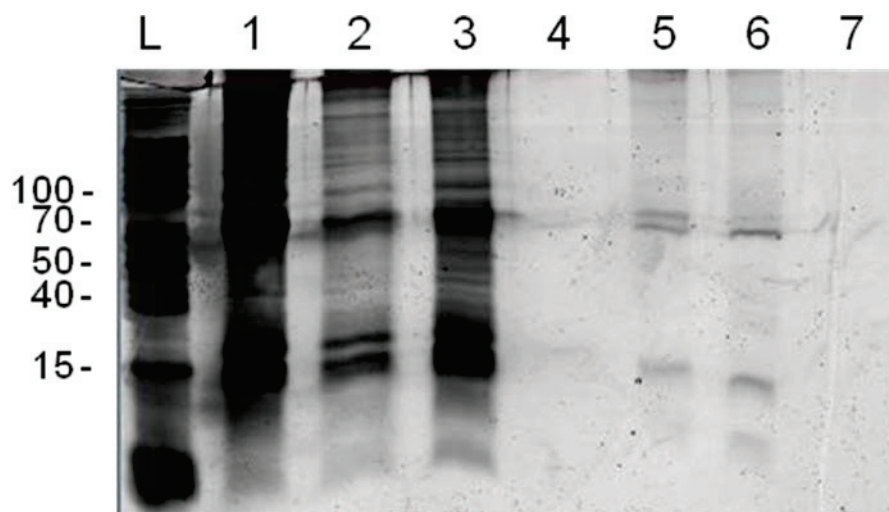
Figure 12. SDS-PAGE of fractions from standard mussel homogenate applied to Con A chromatography. The use of Con A affinity chromatography results in limited removal of candidate AFP proteins from blue mussel total protein extract. Lanes are the blue mussel homogenate applied to the column (lane 1), the unbound protein (lane 2), three wash steps (lanes 3, 4, 5), eluted protein (lane 6) and the metal stripping buffer used to clean the column (lane 7).
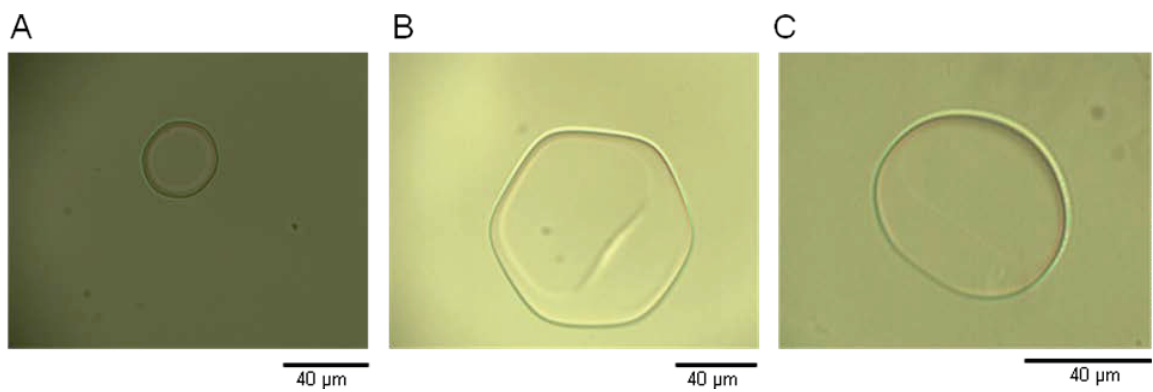
Figure 13.Morphologies of ice crystals from PE mussel standard homogenate before and after Con A chromatography. A standard homogenate for Con A was prepared from 2-4 PE mussels as indicated in the Materials and Methods. A control of 100 mM NH_4HCO_3 alone is shown in panel A. The mannose eluate from the Con A procedure is shown in panel B. The original standard homogenate is shown in panel C (a new preparation than found in Fig. 6).

2.3.3.2 Ice-Affinity Purification

Homogenates prepared from blue mussels obtained in PEI were diluted to volumes between 250 mL and 2 L (depending on the size of the original sample and the desired concentration). Samples were subject to slow, controlled freezing as described in the Materials and Methods in order to allow the AFP to bind the ice surface and become incorporated into the ice and then thawed to release the AFP. Some samples were subjected to a series (up to 4) of freeze and thaw events in an attempt to enhance sample purity and AFP enrichment. Samples were then freeze dried as described in the Materials and Methods. Analysis of the freeze-dried protein obtained from thawed ice and the starting material by tricine SDS-PAGE revealed protein to be present in the frozen fraction, but did not show detectable enrichment of any specific proteins relative to the liquid fraction (Figure 14).
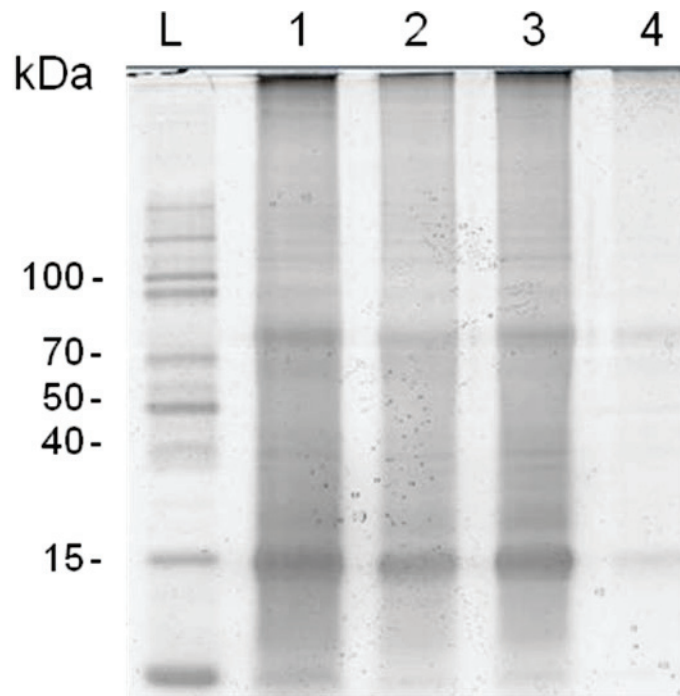
Figure 14. Tricine SDS-PAGE analysis of heated homogenate from PE mussels subject to ice-affinity chromatography. Blue mussel homogenates were diluted and subject to ice-affinity chromatography followed by freeze drying as described in the Materials and Methods. Tricine gels (16.5%) were stained with Coomassie Blue. Lanes are molecular mass ladder (L), starting sample (1), product of first freeze and thaw cycle (2), of the second cycle (3) and the final cycle (4).

2.4 DISCUSSION

2.4.1 Variability of AFP Activity amongst Blue Mussel Populations

AFP levels can differ substantially among populations of a species. For example, the winter flounder and wolffish (*Anarhichas lupus*) show stable population differences in maximal AFP levels that correspond to the severity of freezing risk in different locations and that appear to be the result of different gene copy numbers (Fletcher *et al*. 2001). Expression of AFP genes, and corresponding protein levels, can also vary in plants and animals according to environmental parameters such as temperature (Fletcher *et al*., 2001; Horwath & Duman, 1983; Griffith & Yaish, 2004). Previous detection of AFP in mussels employed samples from the Magdalen Islands (QC), (Guderley *et al*., 1985). With no access to mussels from that location, this project sought to sample mussels from 3 geographically distinct locations, all under the coldest winter conditions, in order to favour the identification of maximal AFP levels and thereby facilitate purification. Locations included the Bay of Fundy (NB), Sambro (NS) and the estuary of the Montague-Brudenell river watershed (PE). Qualitative screening of pooled mussel homogenates for antifreeze activity showed at least trace activity in the preparations from each location. The mussels from PE showed activity above trace levels, and therefore individual mussels from that location were examined to determine activity in individuals. In future, it would be interesting to determine in a larger study whether the PE mussels have quantifiably higher AFP activity and, if so, whether it is the result of a genetic difference, e.g. gene copy number or gene promoter strength difference, or a result of their growth environment on long lines under sea ice. If the blue mussel AFP is

successfully isolated and its cDNA cloned in the future, several experiments could be easily conducted to compare gene dosage, gene expression and protein levels among populations.

2.4.2 Characteristics of the Blue Mussel AFP

In order to determine which conditions could be used for isolation, experiments were conducted on the mussel preparations. The method used by Guderley (1985) to enrich for AFP included a heating step (65°C). Therefore, the boiling stability of the AFP activity was determined using a preparation from PE. The partial to full boiling stability of the mussel protein in this study suggested that heating without boiling might be valuable as a purification step, allowing many other proteins to aggregate and be removed from solution. Heating to 55°C allows the precipitation of many less stable proteins and it was therefore incorporated into our protein isolation protocol; however, boiling was avoided. The effect of pH on AFP activity was also assessed. The protein showed activity that was indistinguishable between pH 4 and 10, suggesting wide-ranging pH stability. In addition, since different buffers (sodium acetate, CAPS and Tris) were used at the distinct pH values, this suggests that the protein is not detectably inhibited by any of these buffers. Both heat and pH stability could be better assessed with a quantitative hysteresis assay.

The protease stability of the mussel AFP was examined for two reasons. First, the susceptibility of the AFP to protease degradation or resistance to it would be helpful in the design of an isolation protocol. Furthermore, a number of cold-adapted terrestrial

invertebrate taxa were shown to have a xylomannan glycolipid antifreeze (Walters *et al.,* 2011), raising the possibility that mussel may produce a similar non-protein antifreeze agent rather than the more familiar AFPs. Nonetheless, retention of the mussel AFP on a 3000 MW cutoff filter following Con A chromatography suggests that it may be larger than the xylomannan glycolipid, which has an upper mass limit of 2400 Da, based on the mass/charge ratio detection by matrix associated laser desorption mass spectrometry (Walters *et al.*, 2009). Of course, with the possibility that the mussel antifreeze is not a protein, it may not be logical to refer to it as AFP. Nonetheless, this acronym will continue to be used here for consistency. If the AFP is related to the glycolipid AFP, it may be feasible to purify it by organic extraction and this could be explored in future.

2.4.3 Methods of Isolation for the Blue Mussel AFP

2.4.3.1 Con A Affinity Chromatography

Con A affinity chromatography can be employed to isolate glycoproteins with terminal mannose or related sugars (Brewer & Bhattacharyya, 1985). The blue mussel AFP was previously shown to bind Con A (Guderley *et al*, 1985). Blue mussels contain a range of glycoproteins, some of which may be present at fairly high concentrations such as the histidine rich-glycoprotein which constitutes 60% of the total protein in blue mussel plasma (Devoid *et al.,* 2007) or mucous glycoproteins secreted from the gills of the mussel (Ahn *et al.,* 1988).While mussels would have a large number of different glycoproteins, the goal of Con A chromatography was to enrich for those with appropriate terminal sugars, including the AFP. While AFP was eluted from the Con A column in one instance, this could not be replicated. Many repetitions of the procedure

with the same or slightly modified conditions were unsuccessful.   It is possible that the

glycosylation of Magdalen Island mussels studied previously (Guderley *et al*., 1985) is

different from that of the mussels from PE. In the future, blue mussels from the northern

Quebec area could be used as the protein sample sources in order to more closely follow

the Guderley process and replicate these past findings as a positive control.

2.4.3.2 Ice Affinity Purification

Ice affinity purification is a remarkably simple concept for isolating AFPs based upon

their activity. The binding of AFP to ice can be exploited to isolate the protein. When ice

is grown slowly under equilibrium conditions, AFP can bind in a specific manner to the

ice surfaces while other proteins and small molecules are excluded by the advancing ice

front and remain in the liquid (Kuiper *et al.,* 2002). An AFP will bind directly to the ice

surface, which then will advance and grow over the bound AFP molecules, trapping them

within the ice. After a period of growth, the resulting ice can then be rinsed and allowed

to thaw, resulting in a solution that should largely be enriched with the AFP present

within the initial sample. In order to avoid accidental trapping of non-AFP molecules in

the ice, the concentrations of proteins and salts must be low and the freezing must be

slow, uniform and bubble-free. Clear, transparent ice indicates that the growth conditions

are appropriate, whereas opaque white ice indicates poor growth conditions. An

awkward aspect of this method is that volumes must be substantial in order to allow

sufficient dilution. Therefore, the resulting ice eluate must be concentrated. Our analyses

showed AFP to be present in the ice eluate, but its activity did not appear enriched

compared with the starting material and gel analysis showed that other proteins were

clearly abundant within the ice. Since total protein concentrations were not determined,

it is not possible to fully assess this method in this analysis. Nonetheless, means of improving the yield and enrichment in future are of interest. While time frames ranging from 4 to greater than 12 hrs were employed, freezing over 24-48 hrs may provide better results. The difficulty arises in monitoring the progress of freezing for this period and having a device capable being set to a very specific temperature and decreasing that temperature in a precisely timed manner over long periods because a progressively lower temperature is required for continued freezing as the ice grows outward from the cold finger. This is critical, as freezing too quickly or thawing – even briefly – is incompatible with this method.

2.4.4 Concluding Remarks

Blue mussel AFP was not isolated in this work. Judging by the ice morphology, if the protein has an activity on a concentration basis similar to most fish AFPs (Kao *et al*, 1986), then it is only present at trace levels in mussels. This is not surprising because AFP found in blue mussels does not act to prevent the organism from freezing, as the blue mussel survives by freezing gradually at high subzero temperatures. Interestingly, during the winter months when mussels encounter freezing conditions, they freeze at higher temperatures than during other periods of the year. During the summer, blue mussels may supercool (cool without freezing) to temperatures approaching -12.5ºC or lower, while only super cooling to approximately -5.5ºC or less during the colder months (Valliere *et al.,* 1990). Thus, at times where it would be expected that AFP levels would be at their highest, blue mussels also freeze more readily. This may reflect the overwintering strategy of the species, which seems to rely more on PINs to gently freeze

with many very small ice crystals while the AFPs act to prevent recrystallization of these small ice crystals into larger more damaging crystals. The concentration of AFP that would be required for this protection would be much lower than that required for effective freezing point depression in freeze-avoiding fish species. This would result in lower levels of AFP in mussels, which would make it more challenging to isolate than most other marine AFPs. Nonetheless, repetition of the ice-affinity chromatography on a more precisely regulated system should allow isolation of this AFP with minimal ensuing clean-up steps. Therefore, it is likely that other investigators would be able to quickly obtain this protein and continue this work.

In the process of working toward isolation, features of the AFP were determined. Perhaps the most intriguing is its resistance to protease. It is possible that this is a highly protease-resistant protein. The Atlantic herring AFP is remarkably protease resistant (Ewart *et al*., 1996), as are many other C-type lectin like domains, and this may be the case for the mussel AFP as well. Alternatively, the possibility that the mussel antifreeze is not a protein at all must be given due consideration now that it has been shown to be the case for other invertebrate antifreezes. Isolation of the active agent from mussels will settle these questions.

CHAPTER 3: CHARACTERIZATION OF THE WFLAFP6 AMYLOID-LIKE FIBRIL FORMATION

3.1 INTRODUCTION

The formation of amyloid-like fibrils by proteins is surprisingly common; however, rapid and inducible amyloid formation under mild and easily controlled conditions is unusual (Stefani & Dobson, 2003). The common AFP of winter flounder, wflAFP6, is interesting in that a freezing event, with sufficient levels of the protein present, appears to be sufficient for amyloid formation and the transformation occurs rapidly (Graether *et al*. 2003; Graether & Sykes, 2009). Characterisation of the amyloid-like fibril-forming activity of wflAFP6 may provide interesting and useful insight into amyloid formation processes, while providing a convenient and controllable assay for amyloid study. Therefore, it is important to gain a clear understanding of it.

The freezing-induced formation of amyloid-like conformation by an AFP was persuasively established by three methods. A 13C NMR analysis showed β-sheet structure in the amyloid, which was distinct from the α-helix normally adopted by the AFP in solution (Graether *et al*., 2003). The amyloid also bound ThT, and it showed typical amyloid-like fibrils when analyzed by TEM (Graether *et al.,* 2003). Nonetheless, intriguing questions emerge from this finding. The parameters required for amyloid formation, including protein concentration, solution properties and other features are unknown. The specificity of this process for this peptide is also undefined to date. Most importantly, it is unclear what aspect of the freezing process is triggering amyloid formation. Examination of the roles of specific aspects of freezing and of solution

properties on amyloid formation by wflAFP6 can be undertaken to better define the parameters that trigger or favour this process.

When a solution is subject to freezing, there are distinct events that might affect amyloid formation. One is a change in temperature, as the temperature is lowered several degrees. Another is a phase change, which is the transition from liquid water to ice. Finally, in the case of the AFP, there is the surface provided by ice during freezing upon which the protein can accumulate as it binds specifically to particular ice crystal faces. These three features of a freezing solution can be separated by targeted experiments because over a short time period in a small volume, solutions at mild subzero temperatures remain liquid unless there is template ice added because homogeneous ice nucleation, leading to solution freezing, does not occur readily under those conditions. Experiments can be designed that separate these aspects of the freezing process in order to determine which of them is necessary for amyloid formation by the AFP. Solution characteristics are also important in the behaviour of other amyloid-forming peptides and proteins such as Aβ and many others where specific pHs may be required to induce amyloid formation or maintain fibril stability (Barrow & Zagorski, 1991; SchmittSchmitt & Scholtz, 2003).

The goal of this project was to determine what is inducing amyloid formation by the antifreeze protein and the conditions required for this to occur.

3.2 Materials and Methods

3.2.1 WflAFP6 Sources

Preliminary experiments were conducted using readily available wflAFP isolated from winter flounder serum. This product was a mixture of wflAFP isoforms 6 and 8, which differ at positions 18, 22 and 26 (Figure 2). The protein was previously isolated by gel filtration and isocratic DEAE ion exchange chromatography of winter flounder (*P. americanus*) blood serum obtained in Newfoundland (Figure 15). The presence of both AFP isoforms was confirmed by mass spectrometry. Gel filtration was performed by A/F Protein Canada Inc. This was followed by acetone treatment and ion exchange chromatography of acetone-soluble peptides (K.V. Ewart, unpublished) to obtain pure AFP. Samples were subjected to electrospray mass spectrometry at the Proteomics Core Facility (Dalhousie University), by Elden Rowland, to confirm the identity and determine the isoforms present in the preparation.

In order to further confirm the identity of the wflAFP present in the winter flounder serum isolated AFP, amino acid analysis was employed to determine the amino acid composition of the AFP and to determine the proportion of the two major isoforms (wflAFP6 and wflAFP8) in the preparation. Approximately 1 mg of the serum-isolated wflAFP was transferred to a centrifuge tube and sealed for transport with parafilm. The sample was analysed for amino acid content using the Waters Pico-Tag system by the Advanced Protein Technology Centre at the Hospital for Sick Children in Toronto.
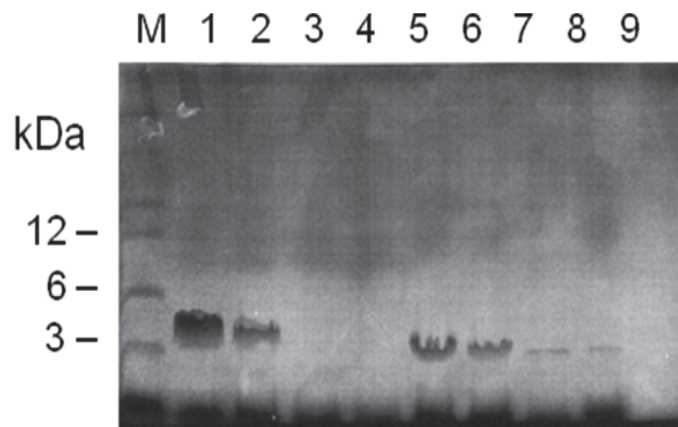
Figure 15. Resolution of wflAFPs from winter flounder serum using tricine SDS-PAGE. Gel filtration followed by DEAE chromatography under isocratic conditions was the procedure initially used to isolate the proteins from winter flounder serum. The gel was negatively stained with $CuCl_2$. (K.V. Ewart, unpublished)

This procedure involves the hydrolysis and pre-column derivatization of the hydrolyzates using phenyisothiocyanate (PITC) followed by reverse phase HPLC using a Pico-Tag column.

Lyophilized synthetic wflAFP6 was purchased from Sheldon Biotechnology Centre and Biomatik. Both mass spectrometry and HPLC analysis were provided by the suppliers and the data confirmed the identity and purity of the peptides. Samples were stored at -20°C until required for use. Antifreeze activity of synthetic wflAFP6 samples, 0.1 mg/mL in 10 mM NH_4CH_3COO pH 6, was examined by growing ice morphology as previously found in the Materials and Methods.

3.2.2 Standard Method of WflAFP6 Sample Preparation

Tubes containing wflAFP6 were removed from the -20°C freezer and allowed to equilibrate to room temperature before opening. The desired mass of protein was weighed on an analytical balance and transferred to a 500 µL centrifuge tube. The wflAFP6 was then diluted in distilled water to 1 mL and filtered through a prepared Sep-Pak® C18 cartridge (Waters) to remove trifluoroacetic acid and other possible contaminants. The Sep-Pak was preconditioned with 2 mL of methanol followed by a 5 mL distilled water rinse, using a 5 mL syringe.

The protein sample was injected into the Sep-Pak using a 5 mL syringe, followed by a 2 mL rinse with distilled water, while taking care to maintain a flow rate below 3 mL/min. The wflAFP6 sample was eluted in a 1.5 mL microcentrifuge tube in 1 mL of methanol.

The methanol was then evaporated from the sample using a speed-vac (Vacufuge®, Eppendorf). Once the sample had reached a suitable volume (approximately 3-400 µL), the sample containing methanol was transferred to a pre-weighed 500 µL centrifuge tube. Following the evaporation of all methanol the final mass of wflAFP6 protein was then determined.

The wflAFP6 sample was then either stored at 4°C for later use or diluted to 15 mM (1 mg/ 20 µL), in 10 mM NH_4CH_3COO pH 6, for immediate use. If other concentrations were desired for a specific experiment, that was accomplished at this step. If different buffers were desired, that would also be accomplished at this step. The amount of time samples were maintained at high concentrations in solution was minimized, as the AFP can form an amorphous non-amyloid gel upon extended storage (Graether *et al.,* 2003). A 15 mM concentration appeared to limit the occurrence while providing a suitable concentration for later experimentation.

3.2.3 Simple Cooling, Ice Interaction, and Phase Change of WflAFP6

WflAFP6 samples were prepared using the standard sample preparation method. For wflAFP6 samples undergoing ice interaction, 5 µL of distilled water was first frozen in 500 µL centrifuge tubes on dry ice. For samples undergoing phase change 5 µL of 15 mM wflAFP6 sample was frozen on dry ice in 500 µL centrifuge tubes. For simple cooling, 5 µL of 15 mM wflAFP6 sample was placed in 500 µL centrifuge tubes. The tubes were then placed in a refrigeration unit at -2°C. The simple cooling sample tubes were monitored visually for changes in opacity or growing ice, in an effort to ensure that

no control samples froze. All sample tubes were then placed in a refrigeration unit (RTE-III, NesLab), at the same time, at approximately -2°C. For the samples undergoing ice interaction freezing 5 µL of wflAFP6 sample was added to the tubes containing frozen water (Figure 16). For the purposes of statistical comparisons all experiments were performed in triplicate at minimum.

The samples were then allowed to incubate 1.5 hours at -2°C. Sample tubes were removed from the refrigeration unit and placed at 4°C to thaw, for approximately 30 minutes. A portion of each sample was then diluted to 0.1 µg/µL with 10 mM NH_4CH_3COO, pH 6, for use in ThT assays. A sample of the 15 mM wflAFP6, kept at 4°C, was also diluted to 0.1 µg/µL, using 10 mM NH_4CH_3COO pH 6 as buffer, to act as a negative control.

ThT assays were carried out largely as described by Levine (1999), Graether *et al.* (2003) and Reinke *et al.* (2010). Work using ThT was performed in low light conditions, with just enough light to see clearly, to preserve the fluorescence of ThT. A 50 mM stock solution of ThT dye was prepared in distilled water and filtered through a 0.45 µm syringe filter into a 15 mL screw cap tube. Stock solutions were maintained in the dark covered in foil for up to one month at 4°C.

For the working ThT assay dye solution (50 µM ThT, 50 mM glycine-NaOH at pH 8.5), the stock ThT solution was mixed at 1 µL per 1 mL of 50 mM glycine-NaOH, pH 8.5. In a black 96 well-plate, 1 µg of sample protein, or 10 µL of the 0.1 µg/µL wflAFP6

samples prepared during the various experiments, was added to the desired wells,

ensuring to mix the samples using the pipette prior to dispensing. ThT assay dye, 250 μL,

was then added to all sample containing wells using the pipette to mix the sample. Wells

containing only the ThT sample dye were also prepared as negative controls. Samples

were read on a SPECTRAmax® GEMINI XS dual-scanning microplate

spectrofluorometer (Molecular Devices) with excitation set to 450 nm and emission set to

482 nm.
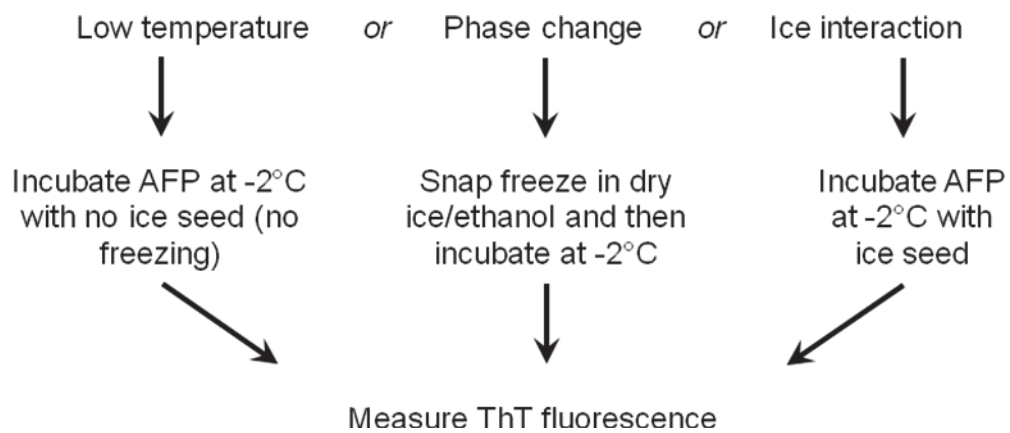
Figure 16. Flow scheme for experiment to compare wflAFP6 subjected to various freezing/cooling conditions. Incubation steps were accomplished using a refrigeration unit.

3.2.4 The Role of Denaturation in Amyloid-Like Fibril Formation by WflAFP6

For testing the effect of denaturation, 5 µL of 15 mM wflAFP6 sample were placed in a 500 µL centrifuge tube. The tube was then placed in a boiling water bath for approximately 5 minutes and then chilled to 4°C for approximately 30 minutes and a portion diluted to 0.1 µg/µL with 10 mM NH_4CH_3COO pH 6, if possible, for use in ThT assays. The heat treated samples formed an amorphous gel, making exact volume measurements difficult. Identical samples of the 15 mM wflAFP6, kept at 4°C, were also diluted to 0.1 µg/µL with 10 mM NH_4CH_3COO, pH 6, to act as negative controls.

3.2.5 Effect of pH on Amyloid-Like Fibril Formation by WflAFP6

WflAFP6 samples were prepared using the standard sample preparation protocol until the preparation of the final sample solution following precipitation by Speedvac. Samples were instead dissolved to 15 mM in either 10 mM NH_4CH_3COO (pH 6), 10 mM $NaCH_3COO$ (pH 4), 10 mM CAPs (pH 10), or 10 mM Tris-HCl (pH 8). Prepared samples were then frozen using the procedure indicated for ice interaction and prepared for ThT assay, ensuring triplicates. A sample of 15mM wflAFP6 was also diluted to 0.1µg/µL using 10mM NH_4CH_3COO, pH 6, to act as a negative control.

3.2.6 Effect of WflAFP6 Concentration on Amyloid-Like Fibril Formation

WflAFP6 samples were prepared using the standard sample preparation protocol until the preparation of the final sample solutions following precipitation by Speedvac. Samples

were instead prepared as 15 mM, 10 mM, 5 mM, and 1 mM wflAFP6 concentrations with 10 mM NH_4CH_3COO, pH 6.

 The various wflAFP6 sample concentrations were then frozen using the procedure indicated for ice interaction and prepared for ThT assay. A portion of each original wflAFP6 sample concentration, held at 4°C, was also diluted to 0.1 µg/µL wflAFP-6 using 10 mM NH_4CH_3COO, pH 6, to act as negative controls.

3.2.7 Specificity of Freeze-Induced Amyloid to WflAFP6

Two proteins, soy trypsin inhibitor and the AFGP from Atlantic cod, were used in order to test the specificity of freeze-induced amyloid formation. Solutions of the protein, in 10 mM NH_4CH_3COO pH 6, were prepared having an equi-molar concentration of amino acid residues in comparison to 15 mM wflAFP6. Approximately 1.2 mg of protein, producing a 1.9 mM solution of soy trypsin inhibitor and 2.2 mM for the AFGP, was transferred to a 500 µL centrifuge tube, and then dissolved in 30 µL of 10 mM NH_4CH_3COO pH 6 to act as a stock solution. WflAFP6 samples were prepared using the standard sample preparation protocol for use in freezing experiments, then frozen using the procedure indicated for ice interaction and prepared for ThT assay, ensuring triplicates. Portions of the original unfrozen stock solutions were also diluted to 0.1 µg/µL, to act as negative controls. Samples of 15 mM wflAFP6, subjected to ice interaction, freezing were used as a positive control.

3.2.8 Tricine SDS-PAGE

Tricine gels were used following the method of Shagger and von Jagow (1987), to resolve the wflAFPs. Acrylamide for the tricine SDS-PAGE was prepared at 49.5% with a 32:1 ratio of acrylamide to bis (48g acrylamide, 1.5g bis-acrylamide per 100mL). The final gel concentration was 16.5%. The samples were resolved at 15 mA until they passed through the stacking gel and the current was increased to 30 mA and held until completion. The gels were stained using negative copper staining (Lee *et al.,* 1987), which employs 0.3 M CuCl$_2$.

3.2.9 Protein Analysis by TEM

WflAFP6 samples at 15 mM were subjected to the ice interaction and unordered freezing protocols and a volume of 15 mM wflAFP6 was also left untreated. The frozen samples were then allowed to thaw at 4°C for 30 minutes. Following thawing, samples were diluted to produce a volume of approximately 30 µL or 2.5 mM. Working under a fume hood, the samples (ice interaction freezing, rapid phase-change freezing and untreated) were dispensed as drops into different wells of 16 well plates lined with parafilm.
A carbon coated copper TEM grid was then made to float on a sample drop for 5 minutes, removed and allowed to dry for approximately 10 minutes. The sample grids were then made to float on drops of 45 µm syringe-filtered 2% uranyl acetate dye pH 4.0 for approximately 5 minutes then washed by floating on a series of 4 drops of 45 µm syringe filtered distilled water for approximately 1 minute each. The samples were then gently dried by touching Whatman filter paper to the side of the grids and allowing the grids to

sit for approximately 10 minutes. Sample grids were viewed under the TEM following drying.

3.2.10 Size Exclusion to Determine Amyloid Molecular Weight

A 16/60 Hiprep Sephacryl S-200 HR column (GE Healthcare) was purchased for use with the Acta-FPLC system. The column was washed with multiple volumes of filtered water, and then equilibrated with 10 mM NH_4CH_3COO, pH 6, filtered and degassed by vacuum. Molecular mass standards used to calibrate the column included γ-globulin, BSA, ovalbumin, trysinogen and cyctochrome-C, providing a wide range of masses. Each sample was run twice for comparison. Standards contained a protein mass of approximately 2-10 mg, and were dissolved in 2 mL of 10 mM NH_4CH_3COO, pH 6. Flow rate was maintained at the recommended column maximum of 0.5 mL/min and 2 column volumes were used to ensure complete removal of one standard before addition of the next. Each standard was run twice.

Several 15 mM wflAFP6 samples, subjected to the standard ice interaction freezing protocol, were combined in order to obtain over 1 mg of protein. The sample volume was then brought to 2 mL with 10 mM NH_4CH_3COO, pH 6 for use with the column. Proteins were detected exiting the column using a UV based detector set to 214 nm, to detect the peptide bond, as wflAFP6 does not contain any aromatic amino acids that would absorb light at 280 nm. Comparison between protein samples were made based on peak retention times on the column, using the known mass of the employed standards to from curve. This was performed twice.

Several samples of 15 mM wflAFP6, which had never undergone ice interaction, were combined in order to obtain over 1 mg of protein. These samples were fairly old at approximately 6 months. The sample volume was then brought to 2 mL with 10 mM NH_4CH_3COO, pH 6 for use with the column. Protein samples were detected at 214 nm as described above. This was performed only once.

3.3 RESULTS

3.3.1 Identification of Known AFPs from Winter Flounder Plasma and their Amyloid-Like Conformational Change

To begin studying amyloid-like structural conversion of AFP, a supply of the protein was required. To begin with, serum-derived protein was used. Freeze-dried samples of protein previously isolated from winter flounder, and identified as a possible mixture of wflAFP6 and 8 isoforms by SDS-PAGE analysis and antifreeze activity as AFP, were confirmed here to be a mixture of isoforms wflAFP6 and wflAFP8 by mass spectrometry (Figure 17). Therefore, amino acid analysis was performed. The wflAFP8 sequence is identical to that of wflAFP6, except at 3 positions out of the total 37 residues (Figure 2). The two peptides contain the same amino acid composition, with the exception of a Glu residue that is only found in wflAFP6. WflAFP8 has an additional Ala, giving the same number of residues overall. This fact allowed the use of amino acid analysis to determine an approximate ratio of wflAFP6 to wflAFP8 in the preparation, based upon the mol% of Glu in the total composition. Serum derived wflAFP was compared to the known sequences of both wflAFP6 and wflAFP8. The amino acid analysis showed that the sample was a mixture of wflAFP6 andwflAFP8 (Table 2). Analysis was performed as described in the Materials and Methods.

Using this mixed isoform preparation, a proof-of-concept study was performed to determine if ThT binding, diagnostic of an amyloid-like transition, could be generated. It showed clear evidence of increased fluorescence in the frozen samples, with particularly elevated fluorescence in the sample that was allowed to interact with ice (Figure 18).

Synthetically derived wflAFP6 was examined for AFP activity by growing ice crystal

morphology and high AFP activity was found (Figure 19).
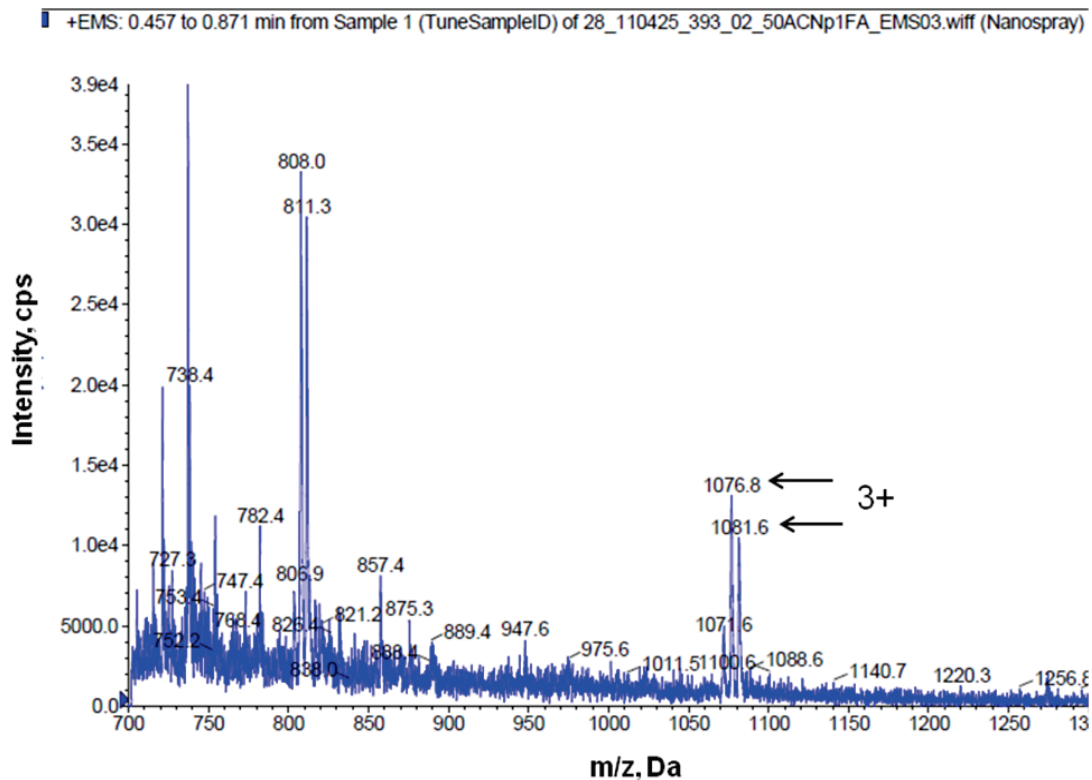
Figure 17. Mass spectrum of serum-isolated winter flounder AFP. WflAFP samples isolated from the serum of winter flounder contain both the wflAFP6 and -8 proteins (as indicated by the pair of peaks). WflAFP samples were dissolved to a concentration of 15 mM in 10 mM ammonium acetate pH 6.5 and sent to the Proteomics Core Facility (Dalhousie University) for analysis by mass spectrometry using the nanospray method.

Table 2. Amino acid composition of winter flounder serum-derived AFP

	wflAFP6	wflAFP8	wflAFP Sample
Amino Acids		% mols	
D	5.7	8.6	12
E	**2.9**	**0.0**	**2**
S	2.9	2.9	3
G	0.0	0.0	1
H	0.0	0.0	0
R	2.9	2.9	3
T	11.4	11.4	10
A	65.7	65.7	59
P	0.0	0.0	0
Y	0.0	0.0	0
V	0.0	0.0	1
M	0.0	0.0	0
C	0.0	0.0	0
I	0.0	0.0	0
L	5.7	5.7	5
F	0.0	0.0	0
K	2.9	2.9	2
Total:	**100**	**100**	**100**
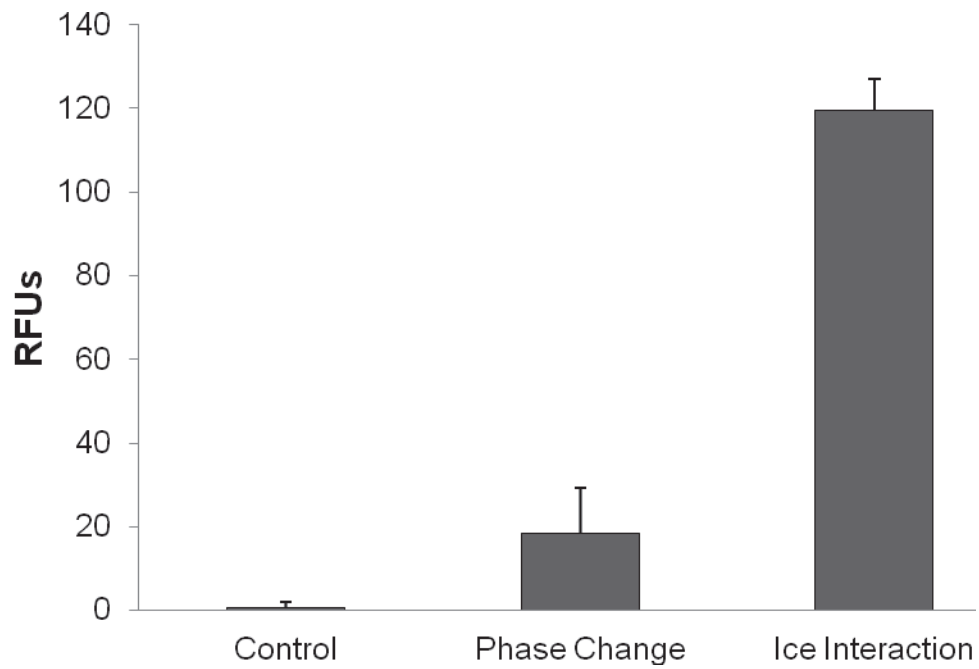
Figure 18. Fluorescence of ThT dye in the presence of serum-isolated wflAFP incubated under different freezing conditions. Samples of wflAFP were prepared to and fluorescence was measured as described in the Materials and Methods. Fluorescence levels in wflAFP samples that were snap frozen and then maintained at – 2°C (phase change), frozen at -2°C with seed ice (ice interaction) or untreated (control) prior to dye addition are shown. (Values shown are means +/- SEM of 3 samples.)
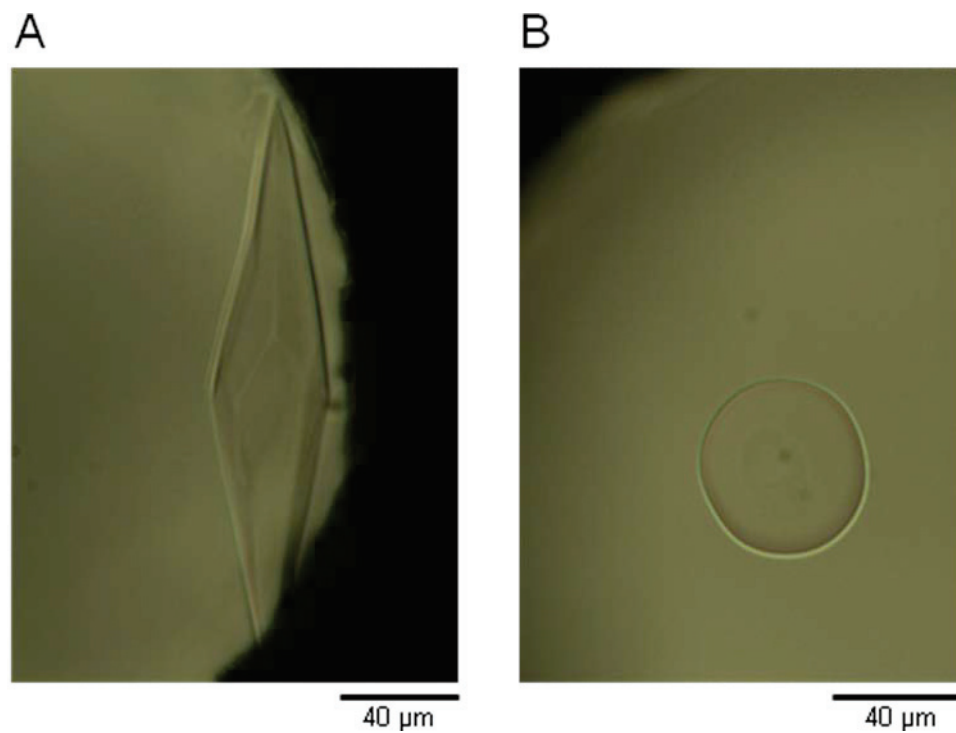
Figure 19. Ice crystal morphology of dilute wflAFP6 sample. A dilute, 1 mg/mL, sample of wflAFP6 (panel A) and buffer-only (panel B) are shown. WflAFP6 sample was prepared by the standard protocol and analyzed using a Clifton nanolitre osmometer as described in the Materials and Methods.

3.3.2 WflAFP6 Amyloid-Like Fibril Formation

3.3.2.1 Effect of Ordered versus Disordered Freezing and Cooling Without Freezing

Any solute, and particularly an antifreeze protein that adheres to ice, will undergo several changes during freeze/thaw. Therefore, the freezing process was dissected out into its component aspects in order to more precisely determine the trigger for amyloid formation during freeze/thaw. Three events occur during the cooling of an antifreeze protein under specific conditions: (1) the temperature declines, (2) the solute phase changes (freezing and thawing) and (3) ordered interaction of AFP with the ice surface may take place. Experiments were designed to examine these events separately.

Samples of synthetic wflAFP6 were frozen under controlled conditions that either produced a slow freezing process with an orderly advancing ice front to which AFP could bind by pre-seeding with ice, or a snap-freezing process that would allow phase change without any appreciable AFP-ice interaction. Samples were also prepared that would undergo the same incubation temperature, but without ice. Binding of the samples to ThT was then measured using a fluorescence assay to determine if amyloid-like fibrils were present following these events (Figure 20). Ordered freezing of the wflAFP6 protein at a concentration of 15 mM followed by ThT binding gave a strong fluorescence result, while disordered freezing and simple cooling produced a much weaker signal. This indicates that it is the ordered binding of AFP to ice that favours amyloid-like structure formation by wflAFP6, rather than temperature or phase changes that occur during the freezing process. The low temperature and phase change controls showed fluorescence levels indistinguishable from control samples that had not undergone freezing or cooling

events. With the suggestion of amyloid-like structure formation by the strong fluorescence in the ThT assay, the samples were subsequently examined by TEM to determine whether fibrils or related aggregates were present. Samples that underwent freezing with ice interaction (ice-seeded freezing) revealed amyloid-like fibrils and oligomers as well as large aggregates (Figure 21). These were not present in identical control samples that were held unfrozen at 4°C. This suggests that the ThT fluorescence emerging after AFP-ice interaction is a result of amyloid fibrils.

Two control proteins were then examined in ice interaction (ice-seeded freezing) experiments in order to determine whether this effect is specific to the wflAFP. The controls were a non-homologous antifreeze protein (the antifreeze glycoprotein of Atlantic cod) and a non-antifreeze protein with a pI and molecular mass within the range of the wflAFP. ThT fluorescence was indistinguishable from controls for these proteins subject to ice interaction (Figure 22), suggesting specificity to freeze-induced amyloid formation to the wflAFP.
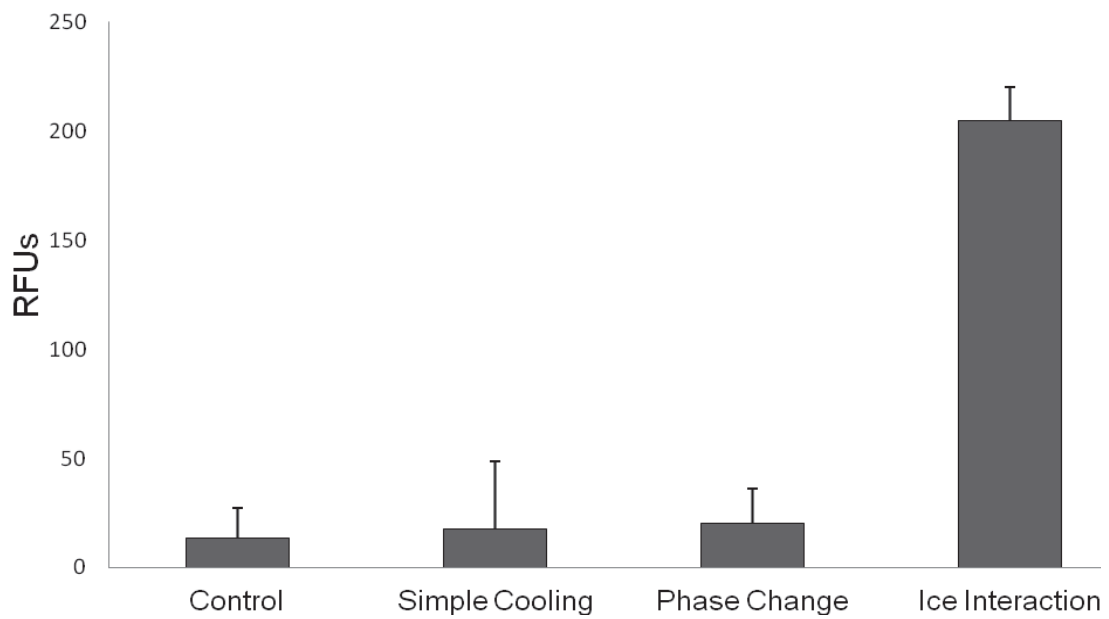
Figure 20. Fluorescence of ThT dye in the presence of wflAFP6 incubated under different freezing and non-freezing conditions. Samples of wflAFP6 were prepared and fluorescence was measured as described in the Materials and Methods. Fluorescence levels in wflAFP samples that were maintained at 4°C (controls), cooled to -2°C (simple cooling), snap frozen and then maintained at – 2°C (phase change) or frozen at -2°C with seed ice (ice interaction) prior to dye addition are shown. (Values shown are means +/- SEM of 3 samples.)
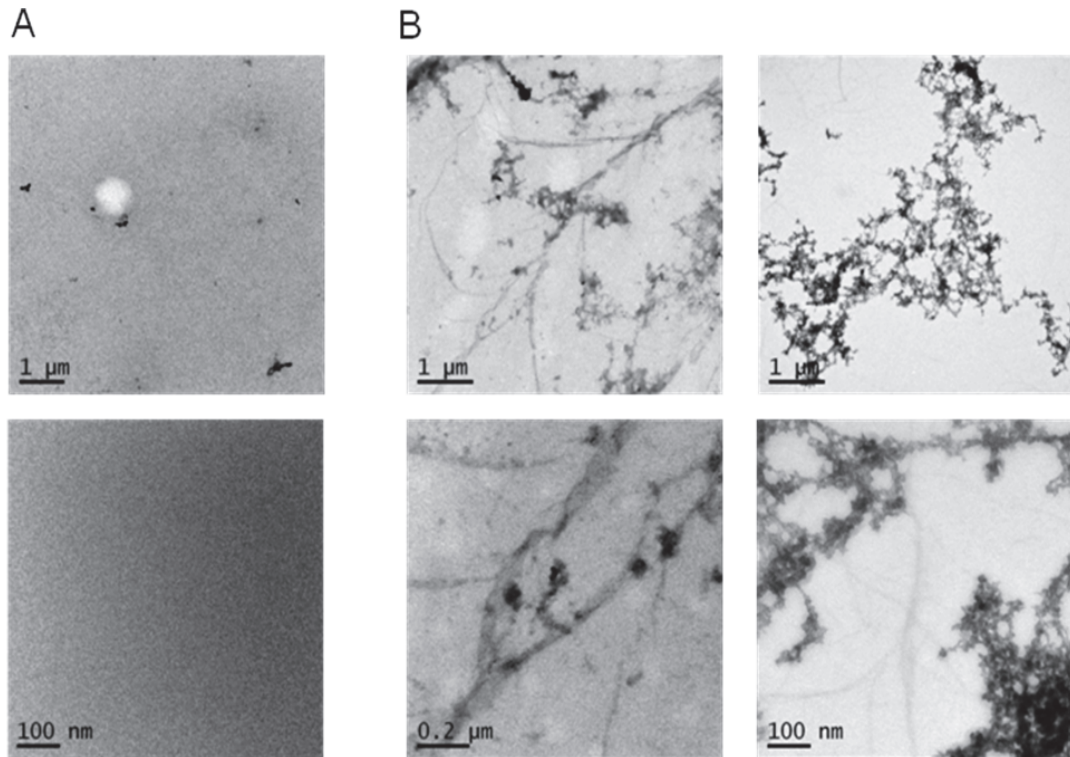
Figure 21. TEM analysis of wflAFP6 subject to ice interaction. WflAFP6 samples that have previously undergone freezing with ice interaction and controls maintained at 4°C were diluted to approximately 2.5 mM and analyzed by TEM with uranyl acetate staining, as described in the Materials and Methods. Panels are a control sample of wflAFP6 (A) and two samples of wflAFP6 having undergone freezing with ice interaction (B). In B, upper and lower panels in each column are from single samples viewed at different magnifications.
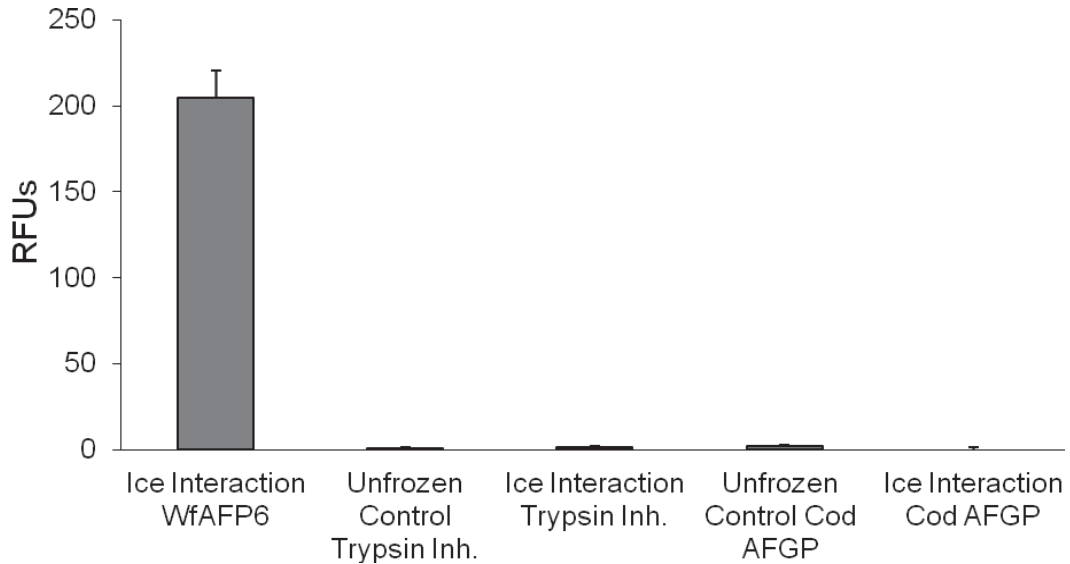
Figure 22. Fluorescence of ThT dye in the presence of wflAFP6 and other proteins. Two proteins, soybean trypsin inhibitor and the Atlantic cod AFGP, do not show signs of amyloid formation, by ThT fluorescence, following ordered freezing. Samples of the proteins were prepared to contain equi-molar concentrations of amino acids to 15 mM wflAFP6. The trypsin inhibitor and AFGP were then subjected to ice interaction and analyzed for the presence of amyloid by ThT assay. (Values shown are means +/- SEM of 3 samples.)

3.3.2.2 Effect of Denaturation

A second consideration of the wflAFP6 amyloid-like fibril forming activity is whether denaturation of the protein fosters amyloid formation. Temperature can play an important role in protein stability and activity. The samples were heated and then prepared for ThT assay to determine if amyloid-like fibrils were present (Figure 23). The boiled sample showed no increase in ThT fluorescence, suggesting that denaturation of the AFP during ice interaction is unlikely to be the trigger for amyloid formation.

3.3.2.3 Effects of pH

The pH of a solution can affect charge interactions within and among peptides. Therefore, the effect of pH on amyloid-like conformation of the wflAFP6 was investigated. Identical wflAFP6 solutions with different solution pH values were subjected to ice interaction and analysed. Flourescence was increased at pH 4, 6 and 8 with the greatest observed level at pH 6, suggesting it to be in the optimal range (Figure 24). In contrast, there was no fluorescence at pH 10. The pH of the wflAFP6 samples should have no direct effect on the efficacy of the ThT assay, as the wflAFP6 samples constitute 10 µL in the 260 µL total volume of a sample well, with 250 µL being 50 mM glycine-NaOH pH 8.5.These results suggest that wflAFP6 forms amyloid-like fibrils at acidic and neutral pHs, while high pHs seem disruptive to the process.
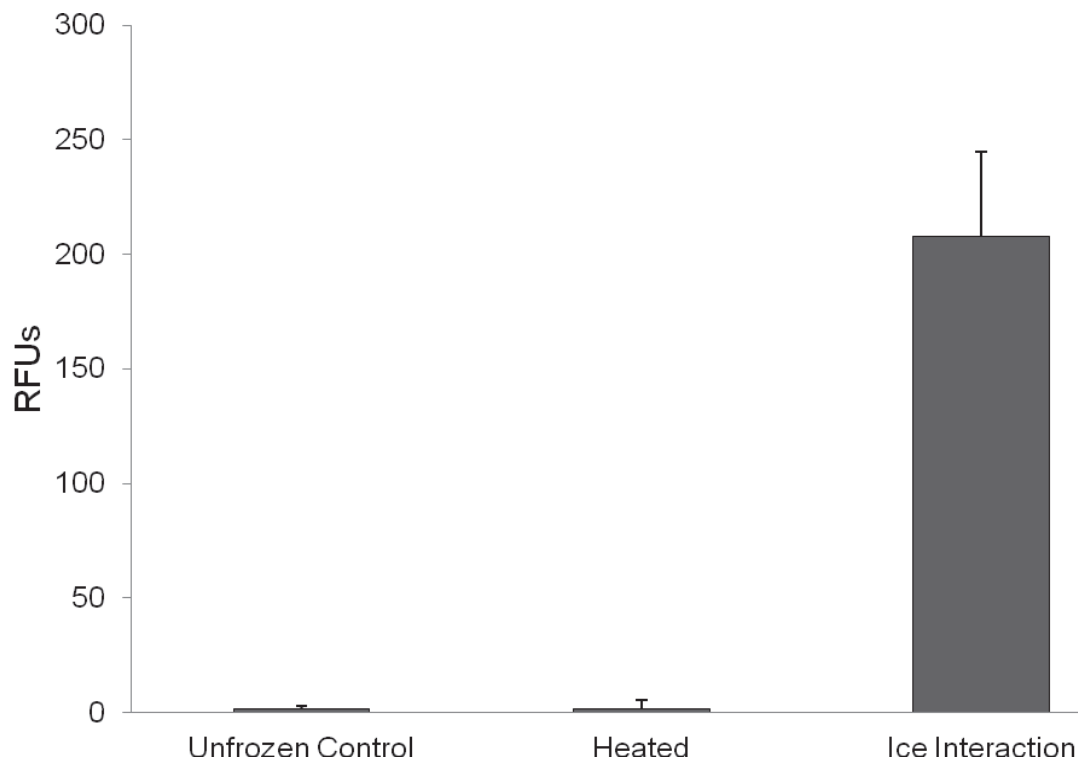
Figure 23. Fluorescence of ThT dye in the presence of wflAFP6 subject to denaturation by heating. Samples of wflAFP6 were prepared at 15 mM in 10mM ammonium acetate pH 6.5, then subjected to either ordered freezing or heating and then assayed using ThT as indicated in the Materials and Methods. The heated sample was measured along with positive (ice interaction) and negative (unfrozen/ untreated) controls. (Values shown are means +/- SEM of 3 samples.)
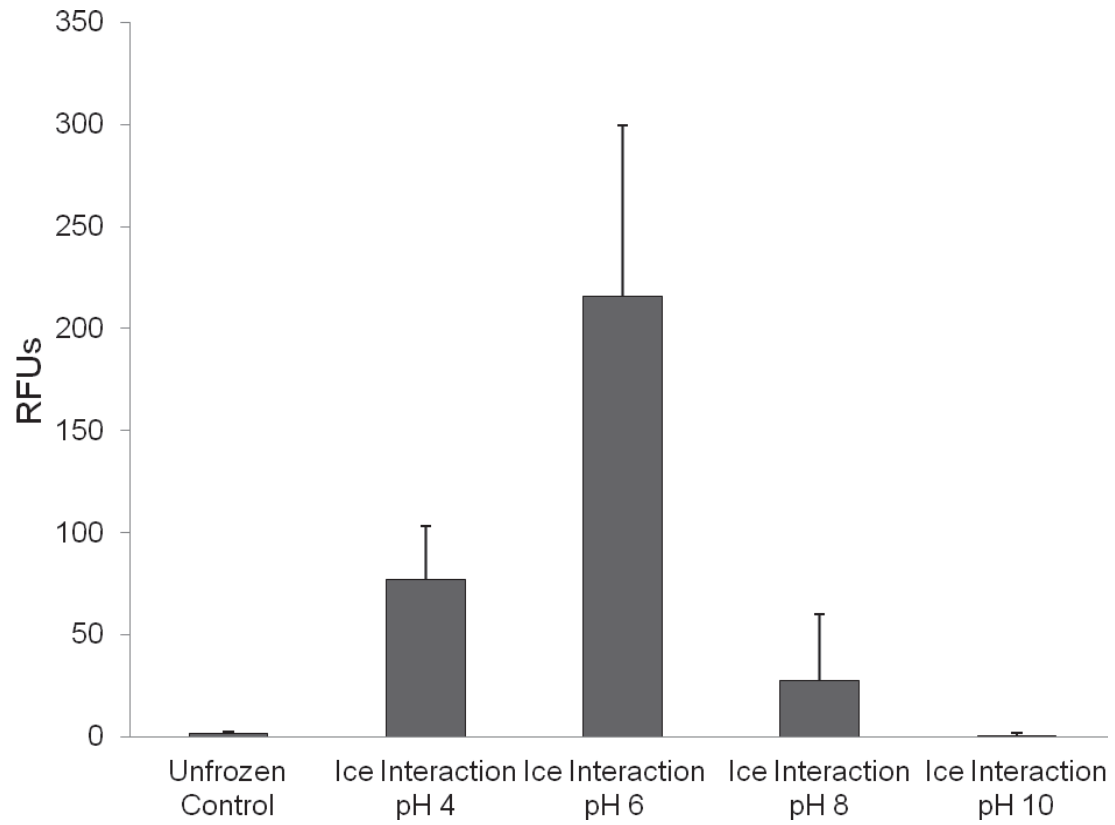
Figure 24. Fluorescence of ThT dye in the presence of wflAFP6 incubated at different pH values. Samples of wflAFP6 were prepared at 15 mM in either 10 mM sodium acetate pH 4, 10 mM ammonium acetate pH 6, 10 mM Tris-HCl pH 8 or 10 mM CAPS pH 10, subjected to ordered freezing and then assayed using ThT as indicated in the Materials and Methods. (Values shown are means +/- SEM of 3 samples.)

3.3.2.4 Effects of Concentration

In order to determine the effect of wflAFP6 concentration on amyloid-like fibril formation, samples at a range of concentrations were subject to ice interaction and then assayed using ThT. Results suggested a changing efficiency of amyloid-like fibril formation, with increased concentration leading to increased efficiency (Figure 25). ThT fluorescence in solutions of 10 and 15 mM AFP appeared equivalent, suggesting a protein concentration beyond which the efficiency of amyloid formation no longer increases, perhaps due to the AFP concentration reaching a critical level. There were other concentration effects in the protein that occured over time. At high concentrations (15 mM and greater), wflAFP6 gradually formed an amorphous gel during storage at 4°C without any ice interaction. This gel was distinct from the amyloid-like conformation, as it did not increase ThT fluorescence (Figure 26) and has been shown by NMR to have no β formation (Graether *et al.,* 2003). A number of wflAFP6 sample solutions (ranging 25 mM to 1 mM) were examined for this gel forming activity and no gel formation was seen in samples below 10 mM and infrequently at 15 mM with frequency and rapidity increasing sharply at 20 mM.
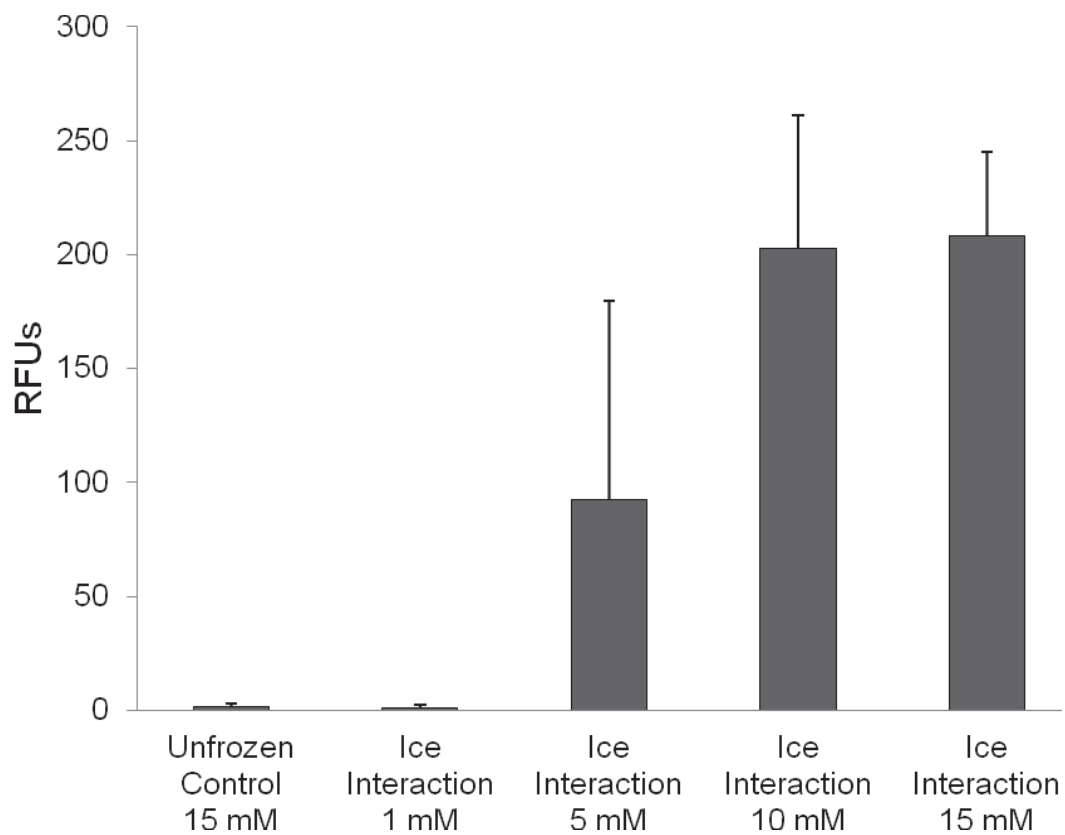
Figure 25. Fluorescence of ThT dye in the presence of wflAFP6 subject to ice interaction at different concentrations. Samples of wflAFP6 were prepared at 15 mM, 10 mM, 5 mM, and 1 mM concentrations, subjected to ice interaction and then assayed using ThT as indicated in the Materials and Methods. A control sample of stock 15 mM wflAFP6, held at 4°C, was also measured. (Values shown are means +/- SEM of 3 samples.)
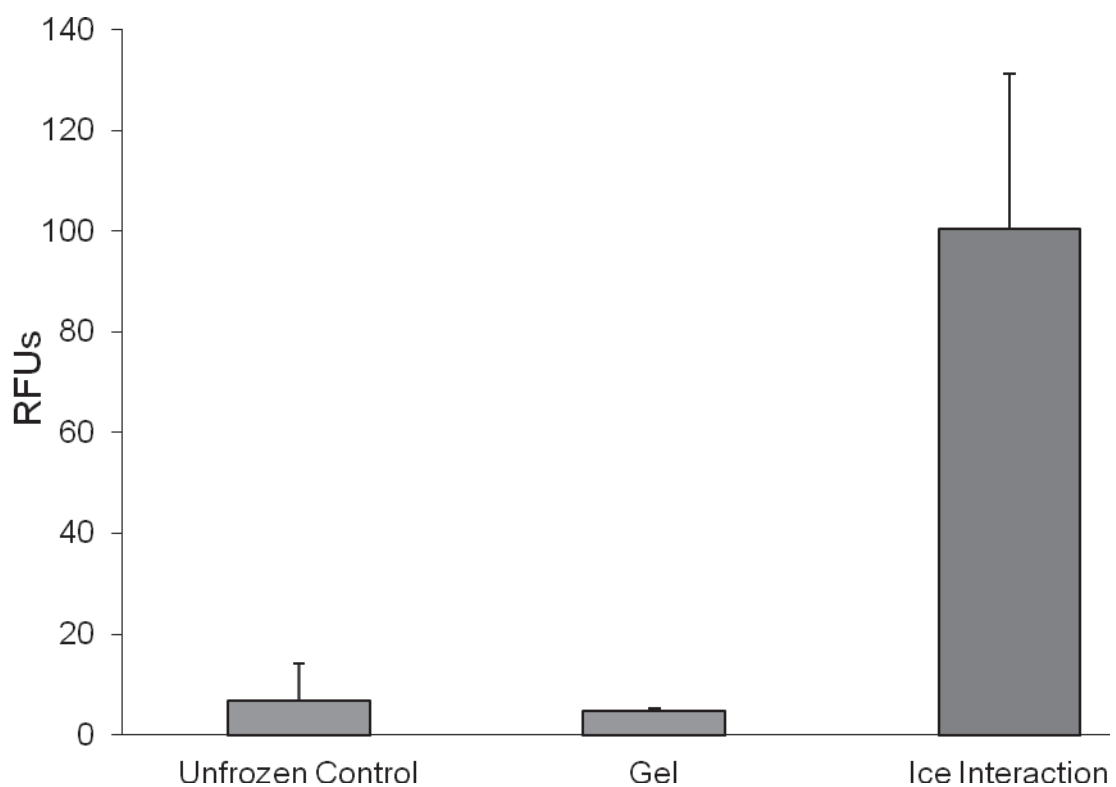
Figure 26. Fluorescence of ThT dye in the presence of wflAFP6 allowed to form a gel. WflAFP6 forms an amorphous clear gel following storage at 4°C at concentrations ≥ 15 mM. A ThT assay was conducted on this material in order to determine if the gel shows amyloid-like characteristics. (Values shown are means +/- SEM of 3 samples.)

3.3.3 Determining the Size of Oligomers

Size exclusion chromatography of the wflAFP6 revealed three major peak sets. Based on the calibration curve and elution volumes, peaks 1 and 2 correspond to the void space of the column. This would indicate a size greater than 66 kDa, although an exact value is impossible to determine. These peaks may represent larger oligomers, which appear to come in several lengths. A second peak appears to be approximately 32 kDa (Peak 3) and it could represent a number of arrangements of the wflAFP6 protein. It is possible that these peaks are representing smaller pre-fibril oligomers. Lastly there is peak 4, corresponding to the size of 10 kDA, which is likely the monomeric form of wflAFP6. There also appears to be several peaks between the approximately 10 kDA and 32 kDA peaks, which may represent various small oligomers (Figure 27). While peaks consistently appear within this range, the individual peaks, as seen in this representative chromatogram, are not constant between sample runs. Sizes were estimated using a standard curve derived from the control proteins utilised as indicated in the Materials and Methods (Figure 28).
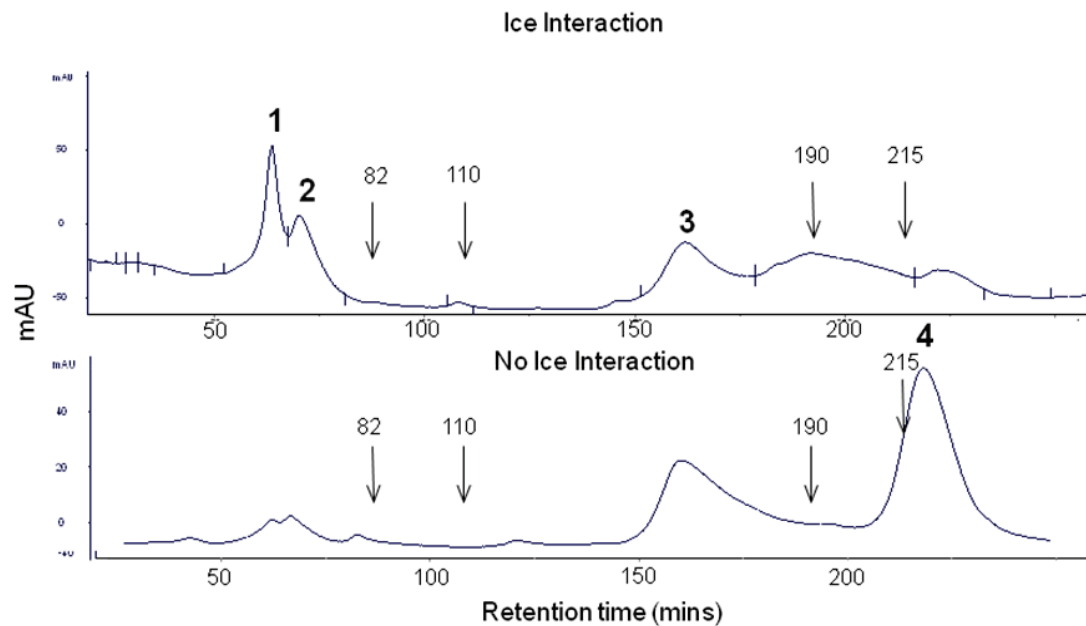
Figure 27. Size exclusion chromatograms of wflAFP6 samples with and without ice-interaction. Size exclusion chromatography was performed on wflAFP6 samples treated as indicated in the Materials and Methods. The elution times of the standard proteins are indicated by arrows and peaks of interest are numbered (1-4).
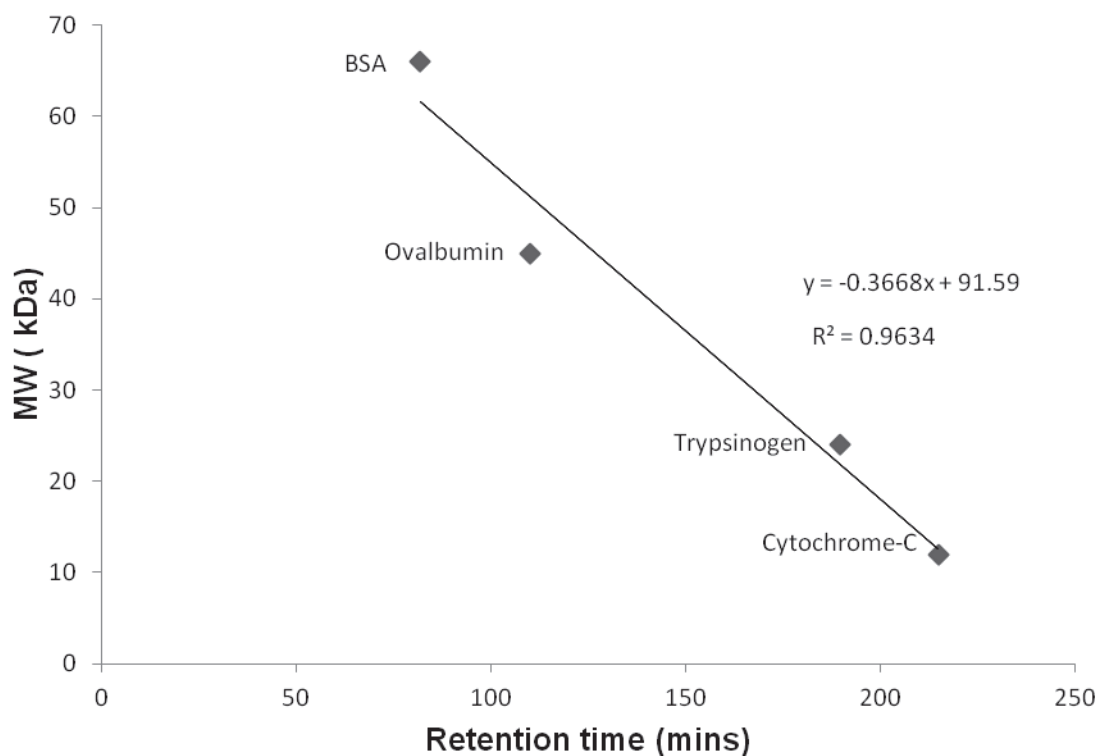
Figure 28. Standard curve of size exclusion standard proteins. Curve represents a comparison of molecular weight versus the standard proteins retention time during size exclusion chromatography. Both the standard curve formula and a standard confidence test are shown, along with standard protein names. Size exclusion was performed as indicated in the Materials and Methods. (Values shown are means of 2 samples)

3.4 DISCUSSION

3.4.1 Serum-Isolated and synthetic WflAFP6

The short (< 40 residue) winter flounder AFPs are encoded by a multigene family (reviewed in Fletcher *et al.*, 2001). As a result of minor variation among gene copies, there are several isoforms of these proteins, with wflAFP6 and 8 predominant. The discovery of amyloid-like structure formation by the AFP was made using synthetic wflAFP6. Nonetheless, as a first step toward studying amyloid transition, it was important to ensure that this could be appropriately replicated in this laboratory before procurement of synthetic AFP for detailed studies. Therefore, initial evaluation of freeze-induced amyloid-like conformation was performed using AFP isolated from flounder plasma. These experiments showed clear indication of amyloid-like structure. Therefore, it was of interest to determine whether this material might be suitable for the entire study. Nonetheless, MS and amino acid analyses revealed the presence of both wflAFP6 and wflAFP8 in the preparation. Therefore, further work was performed on synthetic AFP to avoid the potentially confounding effects of an isoform mixture and of trace fish serum components.

3.4.2 Characteristics of WflAFP6 Amyloid-Like Fibril Formation

3.4.2.1 Ice-Templated versus Non-Templated Freezing and Simple Cooling

The discovery of AFP conversion to an amyloid-like conformation was initially made in a repeatedly frozen and thawed preparation (Graether *et al.,* 2003) and follow-up studies suggested that a single freezing event would be sufficient to trigger this conversion

(Graether & Sykes, 2009). Nonetheless, the freezing process was not controlled or tested

to determine if equilibrium ice-templated freezing or non-equilibrium rapid freezing

would both promote this conversion. Along the same thought process, it was not

determined whether the phase change (i.e. ice formation) or temperature change (i.e.

cooling) was playing the critical role in wflAFP6 forming amyloid-like fibrils.

Controlling for the freezing process and sample phase changes would be helpful in

revealing which component event triggers the rapid conversion to an amyloid-like

conformation in this AFP.

Equilibrium freezing essentially refers to a process in which ice advances gradually from

a preexisting template at mild subzero temperatures, which depend upon the solute

concentration of the solution. The term equilibrium is used because the system is not

displaced far from its equilibrium freezing point (Olien, 1967), where ice and water are in

equilibrium with the rate of ice formation equal to the rate of its melting. In this

situation, the ice template also provides a surface where wflAFP6 may bind, resulting in

an increased local concentration of the protein. In non-equilibrium freezing, very low

temperatures are used to promote spontaneous ice nucleation without any need for an ice

template to induce very quick freezing of the entire sample. This leaves little time for the

migration of molecules in solution, and no initial ice surface for wflAFP6 to bind. It

would be expected that the distribution of salts and proteins within this samples would be

nearly homogeneous, whereas the same may not be the case in equilibrium freezing, as

salts and molecules are partially excluded from slowly growing ice (Kuiper *et al.,* 2002).

Cooling wflAFP6 samples to the temperatures at which equilibrium freezing samples are

held without an ice template, results in supercooling of the sample solution, but no ice formation. It was found that amyloid-like structure only occurred in the samples subjected to equilibrium ice-templated freezing. This implies that ice binding may play a role in rapid transformation of wflAFP6 to an amyloid-like structure. It is also notable that wflAFP6 subjected to either non-equilibrium (rapid non-ice templated) freezing or to cooling without ice showed no amyloid-like structure (Figure 20).

ThT fluorescence appeared slightly elevated in some of the rapidly frozen non-ice templated samples. It is possible that low levels of amyloid fibrils could occur due to transient alignment of wflAFP6 on the ice surface during thawing.   It is not clear if the freezing-induced amyloid-like formation of wflAFP6 bears any mechanistic similarity to the Aβ seed formation by freezing (Levine, 1999). Furthermore, given a suitable length of time at suitable concentrations, wflAFP6 may be capable of assembly into amyloid fibrils without the freezing process. However, the time scale for this process could easily be on the order of weeks to months and that was not explored.

The ability of wflAFP6 to form amyloid upon ice-templated freezing most likely indicates that this process requires a suitable concentration of wflAFP6 and proximity between the individual protein molecules. In this instance the binding of wflAFP6 to an ice surface may act as a method to achieve this concentration and proximity. The binding of wflAFP6 to ice may also orient the protein molecules favourably for amyloid formation.

3.4.2.2 Effects of Protein Denaturation

High temperatures often denature proteins and, in some cases, cause their precipitation from solution. If unfolding of wflAFP6 is a requirement for amyloid-like conversion as it appears to be for known amyloid-forming proteins (Uversky *et al.*, 2008), boiling may have a similar effect to freezing of the sample. Results suggested that this is not the case for wflAFP6. Following boiling, wflAFP6 did form an amorphous gel in solution, but this material produced no signal in ThT assays. Thus, the gel formed by boiling appears to correspond to the amorphous gel distinct from amyloid that was reported previously (Graether et al., 2003; Graether & Sykes, 2009). As such, denaturation did not appear to be the process promoting amyloid-like conversion during ice interaction.

3.4.2.3 Effect of pH Change

The propensity of proteins to form amyloid, and the stability of the resulting fibrils, is often greatly affected by the sample solution pH (SchmittSchmitt & Scholtz, 2003). This change in stability is likely due to changes in charge of various amino acids, which may result in the loss of various salt bridges and changes in protein solubility. By examining the effects of pH on the formation of amyloid-like fibrils by wflAFP6, comparing these to the potential changes in point charges in wflAFP6, a preliminary prediction of the groups important to amyloid formation may be possible.

It was found that amyloid-like conformation was detectable at pH 4, 6 and 8 while not at pH 10. This would suggest that charged amino acid groups, namely lysine 18 having the only pKa value that might indicate a relative change in charge strength and potential loss of charge at ~pH 10, and solubility could play a role in the ability of wflAFP6 to convert

to an amyloid-like structure. In this investigation, it also appears that amyloid formation is highest at pH 6, which is above the pI of wflAFP6 of 4.6. It has been noted previously that for Aβ amyloid, formation is greatest at pHs close to the isoelectric point (Hortschansky *et al.*, 2005). WflAFP6 is also slightly less helical at pH 10, but maintains full activity, while helical structure is steady between pH 4 and 8 (Chakarabartty & Hew, 1985).

It has previously been suggested by modeling that wflAFP6 may be capable of forming dimers (Nguyen *et al.,* 2004). Lys 18 and Glu 22, as well as the C- and N-terminal groups, are capable of forming stabilizing interactions when protein molecules are in an anti-parallel configuration. While this energy of association is theoretically counterbalanced by the actions of solvation and entropy, an environment of high enough wflAFP6 concentration could thermodynamically favour an interaction of this sort. The optimal pH of 6 for amyloid-like fibril formation is also ideal for this interaction. At high pH, this interaction would be lessened with the eventual loss of charge on Lys 18 and the loss of helical structure. Thus, it is interesting to consider the possibility that such an association might favour wflAFP6 conversion to an amyloid-like structure.

3.4.2.4 Effect of Protein Concentration

The discovery of wflAFP6 conversion to an amyloid-like conformation was initially made in a solution of 23 mM peptide (Graether *et al.,* 2003) and follow-up studies were made in a solution of 15 mM peptide (Graether & Sykes*,* 2009). Local concentration of protein in solution can have a large impact on the stability of proteins. The effect of concentration on stability is readily observable within the environment of a cell during

protein folding, where local protein, and various other molecule concentrations, can be very high (Ellis & Minton, 2003).Thus circumstances that result in high local protein concentration could induce flexible/ amyloidogenic proteins to change conformation. Misfolded protein may then act as template, to induce this same conformational change to natively folded protein (Eisenberg & Jucker, 2012, Nelson *et al.,* 2005).

In the case of wflAFP6 it was found that elevated concentrations of protein are required for efficient amyloid-like fibril formation. At concentrations of 10 mM or greater, substantial amyloid-like conformation is apparent; however, this is much lower at lower protein concentrations. A caveat here is that the efficiency of amyloid formation may not be as different in the low concentration samples as it appears based upon this experiment. Obviously, with less wflAFP6 in solution, a similar efficiency (on a molar basis) of conversion to a ThT-binding form will result in lower ThT fluorescence levels. The concentration dependence appears to level off, with no appreciable difference in amyloid-like structure formation between 10 mM and 15 mM solutions, indicating little increase in the efficiency of converting native protein to amyloid-like fibrils upon reaching a critical concentration. This leveling off is unlikely to be a result of limited ice binding surfaces, as the ice seed used was prepared to have a maximal surface area proportional to the solution and this should not have been limiting. Nonetheless, the concentration dependence of this process may be an informative area of future study.

3.4.3 WflAFP6 Oligomer Size

The size of the oligomers and fibrils involved in wflAFP6 amyloid-like fibril formation was difficult to discern. However, size exclusion chromatography has provided indications as to size distributions in samples of wflAFP6 that have undergone freezing with ice interaction and samples that are several months old. There is some difficulty in linking these peaks to specific oligomeric arrangements due to the native structure of wflAFP6, a rod-like α-helical protein. While ~3 kDa in size, its extended helical structure makes its elution volume correspond to that of a 10 kDa protein, as observed in this study and previously documented (Davies *et al.,* 1982). This relationship would not necessarily continue past the monomer of the protein. In other words, a dimer would likely not appear as a 20 kDa protein and the same difference in size relationship might apply when the protein is in an amyloid-like form.

While these findings may prove very interesting with further work, there is also the possibility that this technique may not prove a suitable diagnostic for amyloid oligomer/fibril size.

3.4.4 WflAFP6 Amyloid-Like Fibril Formation

3.4.4.1 WflAFP6 Structure

WflAFP6 shares several characteristics with some of the better known amyloid proteins (Tzotzos & Doig, 2009). The amino acid composition of wflAFP6 is however heavily biased towards Ala, and does not contain any of the bulky aromatic amino acids often associated with structures such as a discordant helix (Kallberg *et al*., 2000), which is an

α-helix with a sequence that would favour a β-sheet in isolation. There exist other predictors of amyloid forming proteins such as chameleon sequences, short sequences of amino acids found in multiple types of secondary structure, namely α helices and β sheets (Gendoo & Harrison, 2011). These sequences provide potential flexibility in a protein to change secondary structure.

 Interestingly, the high alanine content of wflAFP6, and more specifically two stretches of the proteins sequence which are AAAA or AAAAA (Figure 2), have been determined as potential chameleon sequences (Gendoo & Harrison, 2011). However, chameleon sequences alone may not strongly predict a protein that would readily form amyloid (Gendoo & Harrison, 2011). Several amyloid-forming proteins have been shown to contain both discordant helices and chameleon sequences, and the presence of both these sequence types seem to be a stronger indication of the amyloid-forming potential of a protein (Gendoo & Harrison, 2011). It was also determined that the C-terminus of wflAFP6 appears to retain localized α-helical character though decreased, when in amyloid-like fibrils, indicating the protein may not be involved with the amyloid structure in its entirety (Graether & Sykes, 2009). The extent of this α-helical section is unknown, and would be an interesting area to study, as one of the potential chameleon sequences is found at residues 28-34.

The presence of known chameleon sequences within wflAFP6 coupled with the observations made during this study provides great insight into the ability of wflAFP6 formation of amyloid-like fibrils. Amyloid-forming proteins are a large and diverse

group, with many differences in structure. The examination of any protein that forms amyloid may also open opportunities in discovering molecular mechanisms and potentially open many doors in research (Eichner & Radford, 2011).

3.4.4.2 WflAFP6 Activity

A notable feature of wflAFP6's ability to form amyloid-like fibrils is the length of time required for this conversion. While this study made use of incubation times between 1-1.5 hrs, it has been determined that as little as 15 minutes may be sufficient to seed wflAFP6 for amyloid-like fibril formation and detect early fibril formation by NMR (Graether *et al.,* 2009). The extended 1-1.5 hrs, with ice interaction, provides enough time for amyloid-like fibrils easily detectable by ThT. Without resorting to the use of harsher treatments, or seeding with protein already in amyloid conformation, the time frame for fibril formation with most amyloid proteins is often much longer, days or weeks.

One obvious difference between most amyloid-forming proteins in disease and wflAFP6 is that, being an AFP, the protein interacts with and binds to ice. This could play several important roles in acting as a catalyst for amyloid formation. The ice surface may act to bring many wflAFP6 molecules into close proximity, increasing the probability of interaction between molecules. The ice surface may also act to direct the wflAFP6 molecules into a favorable orientation for interaction. Thirdly, the close proximity of many wflAFP6 molecules in the ice-water interface layer may result in an environment where the protein is transiently more flexible. A combination of these effects is likely at play during rapid wflAFP6 amyloid-like fibril formation. However, it is possible that a

suitably high concentration of wflAFP6 incubated for sufficient time in the absence of freezing may also meet the requirements of this protein for amyloid-like conversion over a sufficient length of time. It cannot be distinctly stated at this time that ice interaction is an absolute requirement for amyloid-like fibril formation with wflAFP6; however, for rapid amyloid-like structure formation over very short time periods, this appears to be the case. A time course providing extended examination of solutions of wflAFP6 at various concentrations could help answer this question.

3.4.5 WflAFP6 for use in Amyloid Assays

WflAFP6 could be of use in studying several facets of amyloid-like fibril formation due to the rapidity and mild conditions under which it undergoes this conversion. WflAFP6 is also a fairly small protein that can be synthesized, increasing ease of use. Alternatively, protein preparations from appropriate flatfish sera may allow very low cost assay material for certain applications. Primarily, wflAFP6 could be useful in testing the effects of protein folding modulating agents on amyloid fibril formation and stability. Large numbers of potential candidates can be screened in well plates by ThT assay. The speed at which assays can be performed also allows for greater sample sizes, which may prove important when considering the inherent variability seen in amyloid formation by most other proteins.

WflAFP6 also presents several difficulties however, as the protein is not readily detectable by means conventionally used for other proteins. The protein does not interact with standard protein staining dyes such as Coomassie blue and BCA and also contains

no aromatic amino acids, a situation which prevents fluorescent detection by UV at 280nm. Alternative techniques such as negative $CuCl_2$ staining for electrophoretic gels and UV absorbance measurement at a wavelength such as 214 nm (to detect the peptide bond) can overcome these obstacles if necessary.

3.4.6 Concluding Remarks

This study determined the effects of many variables in the formation of amyloid-like fibrils by wflAFP6 and revealed that freezing of the protein with ice interaction allowed rapid fibril formation, where a phase change and simple cooling did not. Knowledge of variables that promote the formation of amyloid-like conformational change for wflAFP6 could prove of great use in the development of novel assays and as a model for amyloid studies.

This study is a first step in answering the questions that surround the ability of wflAFP6 to form amyloid-like fibrils. It would be of great interest to determine whether the AFP activity of wflAFP6 is a required feature or catalyst of amyloid-like fibril formation, further explore how the various sample conditions (freezing conditions, pH, etc.) mechanistically affect wflAFP6 amyloid-like structure formation, determine what the structure of wflAFP6 is when present in amyloid fibrils and the overall structure of wflAFP6 fibrils. A number of possibilities exist to explore these features, such as mutational studies, and NMR analysis.

There is also a question of whether wflAFP6, once induced to form amyloid by ice-templated freezing, can then act as a template to induce the conformation change of non-amyloid wflAFP6 to amyloid. This behavior is seen quite commonly in other amyloid proteins, such as the prion protein associated with multiple prion disordered and Aβ (Toyama & Weissman, 2011; Bhak *et al.*, 2009). WflAFP6 induced into fibrils, by freezing with ice interaction, could be added to non-amyloid samples of wflAFP6. If positive this may simplify the use of wflAFP6 as a candidate for screens, testing chemical chaperones for example. It would also indicate that wflAFP6 amyloid behaves similarly to many amyloids.

A great number of questions remain to be answered concerning the nature of wflAFP6 to form amyloid-like structure. Further experiments and future work will determine the intricacies of this protein interaction, and may also open important doors in life sciences research involving amyloid.

CHAPTER 4: DISCUSSION

The aim of this study was to generate new understanding of marine AFPs, specifically the characteristics of the blue mussel antifreeze molecule and several interesting facets of the formation of amyloid-like fibrils by wflAFP6. The path has also been laid for future experimentation concerning these topics.

Previously, little was known concerning the blue mussel antifreeze molecule, apart from its existence and its apparent binding to Con A lectin. This study has determined several features which were not previously known. The antifreeze molecule is capable of activity at a wide range of pH values, pH 4 to pH 10, and appears to be protease resistant. It also appears to be at least partially heat stable. These findings allow future research aimed at isolating the AFP from mussels to proceed more easily; for example, use of a pH gradient for ion exchange chromatography would not cause any problems for this AFP. Further attention could be brought to the ice affinity chromatography. If successful, that could yield a nearly pure AFP, which could then be efficiently cleaned up and characterized fully. Isolation of the blue mussel antifreeze molecule could provide an entirely new type of AFP or an informative homolog of existing AFPs, which would be informative in terms of mussel biology, AFP evolution and AFP structure/function.

The formation of amyloid-like fibrils by wflAFP specifically following a freezing event has been analyzed by this study to build upon the informative work that was previously done. In past studies, only two concentrations and a single form of freezing event have been employed (Graether *et al*., 2003; Graether & Sykes, 2009). In this study the freezing

event has been dissected into its component parts, temperature change, phase change and ice interaction, to determine the effect these elements have on fibril formation. This work showed that ice interaction is critical to the rapid formation of amyloid-like fibrils by wflAFP6; phase change and temperature change alone are insufficient. There are several avenues that could be pursued next. For example, rapid assay analysis could be developed based upon this protein's rapid amyloid-like transition. First, though, it will be important to determine how representative this wflAFP6 amyloid-like structure is of others that are of interest in neurodegenerative diseases or other disorders.

AFPs continue to be intriguing field of study, both for their potential commercial uses and interesting activities. The pursuit of novel antifreeze molecules, in the case of blue mussels, and interesting activities of a protein, in the case of wflAFP6, may offer greater understanding of these proteins along with novel practical applications. This study had endeavoured to make progress toward both.

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