CHARACTERIZATION OF SKIN AND PLASMA TYPE I

ANTIFREEZE PROTEINS FROM ATLANTIC

(Liparis atlanticus) AND DUSKY (Liparis gibbus) SNAILFISH

ROBERT PHILIP EVANS

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CHARACTERIZATION OF SKIN AND PLASMA TYPE I ANTIFREEZE

PROTEINS FROM ATLANTIC (*Liparis atlanticus*) AND

DUSKY (*Liparis gibbus*) SNAILFISH

By

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ABSTRACT

Atlantic snailfish (*Liparis atlanticus*) and dusky snailfish (*Liparis gibbus*) belong to a large family of benthic and pelagic marine fishes that inhabit northern regions of the Atlantic Ocean. Both species spawn during the winter months in ice-laden inshore coastal regions around Newfoundland. Due to their harsh winter environment, snailfish are prime candidates for production of antifreeze proteins (AFPs).

Initial results confirmed that plasma from both species contain alanine rich, α-helical, type I AFPs that are significantly larger (>9.3 kDa) than all previously described type I AFPs. Surprisingly, their skin tissues produce AFPs that are identical to those which circulate in blood. While all snailfish consistently express antifreeze mRNA in skin tissue, there is extreme individual variation in liver expression – an unusual phenomenon that has never been reported previously. Molecular analyses revealed that snailfish AFPs are products of multigene families that consist of at least ten gene copies per genome. It is unclear if liver and skin antifreeze mRNAs are expressed by the same gene or a separate subset of genes which is typical of other fish that produce skin-type AFPs.

Although the 113 residue snailfish AFPs are unusually long, their amino acid composition, highly α-helical secondary structure and the bipyramidal ice-crystals they create are characteristic of all type I AFPs. However, unlike other type I AFPs, snailfish proteins do not contain any obvious amino acid repeats or a continuous hydrophobic face that typify the structure of most other type I AFPs. These structural differences might have implications for their ice crystal binding properties. Biochemical experiments

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demonstrated that physiological concentrations of normal salts are responsible for a significant increase in thermal hysteresis activity in antifreeze proteins and glycoproteins. The colligative effects of these salts can account for the supplementary freezing point depression of blood required to ensure survival of marine fish in ice-laden seawater during winter.

Two cDNA clones were identified from a snailfish liver cDNA library that code for fish eggshell proteins while a third one codes for a type II keratin. However, all three of these clones contain sections with substantial amino acid and nucleotide sequence similarity to snailfish type I AFPs. It is plausible that one or more of these proteins represent the ancestral proteins of snailfish type I AFPs.

Novel type I AFPs were isolated and partially characterized from skin tissues of cunner (*Tautogolabrus adspersus*). Type II AFPs that are identical to those expressed in liver for export into blood were isolated from sea raven (*Hemitripterus americanus*) skin tissue extracts. Taken together the data generated in this thesis has strengthened and widened the scope of the hypothesis that skin represents the primordial source of AFP expression. It is clear that epithelial tissues are a primarily important source for antifreeze expression to enhance the complement of AFPs that protect fish from freezing in extreme cold environments.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AFGP	antifreeze glycoprotein
AFP	antifreeze protein
bp	base pair
CD	circular dichroism
cDNA	complementary DNA
Da	Dalton
DIG	digoxigenin
DNA	deoxyribonucleic acid
DTT	1,4-dithiothreitol
ESI-MS	electrospray ionization mass spectrometry
HPLC	high performance liquid chromatography
IBM	ice binding motifs
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS-222	3-aminobenzoic acid ethyl ester
MW	molecular weight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
RACE	rapid amplification of cDNA ends
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
TH	thermal hysteresis
UTR	untranslated region
UV	ultraviolet

CHAPTER 1:

General Introduction

1.1 Background – Adaptations to Cold and Freezing

The ability of animals to survive extreme cold or freezing conditions has intrigued the scientific community for centuries. Early investigations sought to develop a rational explanation for the seemingly extraordinary observations of cold or frozen animals in nature. Since the first scientific endeavors more that three centuries ago, much has been learned regarding how organisms cope with cold and freezing (Smith 1961). A number of these adaptations probably evolved from responses to other stressors such as dehydration and osmotic stress. Now, researchers are beginning to unravel how unicellular and multicellular organisms coordinate their numerous and varied responses to extreme cold. While clearly some of the responses are at the whole animal level (e.g. behavioral, physiological etc.), adaptation at the cellular level in response to temperature fluctuation occurs by alterations in metabolism, membrane composition, and gene expression.

Water is a key component in biochemical reactions and is integral for the hydration of most structurally important biological macromolecules (Franks et al 1990, Mazur 1984, Somero et al 1992, Zachariassen 1991). For a cell, extracellular freezing is essentially a form of dehydration stress. Furthermore, the physical damage (i.e. to cell membranes) that could result from uncontrolled ice formation is lethal. With the inherent risks, it is surprising that organisms thrive in cold oceans and in polar and north temperate regions where temperature ranges around the freezing point of water. Whereas endothermic animals that populate cold habitats produce heat to elevate their temperature well above freezing, ectotherms remain at the temperature of their immediate environment and must utilize methods to survive freezing stress. Studies of ectothermic

species have revealed an array of adaptations that reduce the risk of intra- and extracellular freezing.

To cope with exposure to temperatures below the freezing point of their body fluids, ectothermic animals use one of two general strategies: freeze-avoidance or freeze-tolerance. While both strategies include adaptations at behavioral, physiological, and biochemical levels there is one fundamental difference. Freeze-avoiding animals maintain body fluids in liquid form even at very low temperatures by substantially increasing their low molecular weight solute concentration (e.g. glycerol) but freeze-tolerant animals allow ice to form in extracellular space and only require cellular cytoplasm to remain unfrozen (Storey and Storey 1988, Storey 1989, Storey and Storey 1996, Storey and Storey 1999). The type of adaptation utilized varies among species in accordance with their specific environment.

Three different mechanisms are utilized in freeze avoidance strategies. The first involves accumulation of ions and low molecular weight solutes to colligatively lower the freezing point of extracellular fluids (Storey and Storey 1988, Storey and Storey 1996). In the second method, the organism lives in a supercooled state without freezing by eliminating contact with ice nucleators (Storey and Storey 1999, Sidell 2000). Finally, the freezing point of extracellular fluids can be lowered non-colligatively by synthesis of antifreeze proteins, which inhibit ice crystal growth (Davies et al 1988, Fletcher et al 1998, Fletcher et al 2001).

1.2 The Discovery of Antifreeze in Marine Fish

While adaptations for either freeze strategy are available to terrestrial ectotherms, the biology of teleost fish necessitates a freeze avoidance strategy. Teleost fish from the Northern hemisphere that inhabit extremely cold seawater in winter, (as low as -1.8°C) but do not come in contact with ice can live in a supercooled state. Indeed, fish colligatively lower the freezing point of their blood plasma to help deal with low seawater temperatures. For example, winter flounder seasonally elevate their extracellular sodium and chloride ions and rainbow smelt synthesize large quantities of glycerol during winter months to help raise blood osmolality to nearer that of seawater (Fletcher 1977, Fletcher 1981, Raymond 1992). However, the combination of supercooling and ice contact is lethal. Therefore, fish which live in shallow areas that are ice laden can come into contact with ice nucleating crystals must avoid ice crystal formation at all costs.

In the 1950s, Scholander and his colleagues traveled to Labrador to determine how Arctic fish avoid freezing in apparently deadly conditions. They observed that while some fish appeared to exist in a supercooled state and quickly died when contacted by ice, others survived in icy seawater with blood plasma freezing points the same as the seawater (Smith 1961, Gordon et al 1962, Scholander and Maggert 1971). Further experiments established that a plasma solute(s) was responsible for the protective effects. They named the solute "antifreeze" but it took another 10 years before the nature of these freeze protecting solutes was determined to be proteins/polypeptides and glycoproteins (Scholander and Maggert 1971, Fletcher et al 2001).

Fish antifreeze proteins/polypeptides and glycoproteins [AF(G)Ps] lower the freezing point of plasma non-colligatively by irreversibly binding to certain surfaces of ice crystals, modifying their structure and inhibiting further growth – a mechanism known as adsorption inhibition (Raymond and DeVries 1977, Hew and Yang 1992, Davies and Hew 1990). Essentially, the freezing point of a solution is lowered by AF(G)Ps while the melting point (equilibrium freezing point) is unaffected. The interval between the freezing and melting points is termed thermal hysteresis and can be measured *in vitro* using a Clifton nanoliter osmometer. AF(G)Ps are 200 to 300 times more effective in lowering the freezing point than would be expected on the basis of colligative properties alone and do not alter osmotic properties of the plasma. Therefore, marine fish that produce AF(G)Ps can maintain extracellular freezing points equal to seawater but with much lower solute concentrations.

Interestingly, the strategy of synthesizing AFPs to help avoid freezing is not the sole purview of marine fishes. In fact, they have been discovered in organisms from most phyla, in species ranging from fish to insects to plants and fungi. The structures of these AFPs are quite diverse but all seem to have evolved to serve the same function by a common mechanism. However, the effectiveness of the different forms of AFPs does relate somewhat to the environment in which the organism inhabits. For example, the insect AFPs are very highly active providing up to 8 degrees of freeze protection (Duman 1982, Graham et al 1997, Liou et al 1999, Duman 2001). This level of activity would be necessary considering the extreme cold that they may face during the winter months.

1.3 Structural Classification of Fish AF(G)Ps

Species from diverse taxonomic groups produce AFGPs and AFPs that are

classified as four distinct types based on their physical characteristics - termed type I, II,

III and IV (Davies and Sykes 1997, Cheng 1998, Ewart et al 1999, Fletcher et al 2001).

Type I AFPs are usually polypeptides that have high alanine content (>60 mol %) and an

entirely amphipathic α-helical secondary structure. These proteins were originally

identified in winter flounder and were later observed in other right-eye flounders and in

certain unrelated sculpin species (Harding et al 1999, Fletcher et al 2001) (see table 2-2).

Typically, type I AFPs are small polypeptides (3.3 – 4.5 kDa) but can be much larger as

in the case of shorthorn sculpin (Low et al 1998). The prototypical type I AFP contains a

specific 11 amino acid repeat motif (Thr-X_2-Asn/Asp-X_7), where X is usually alanine or

may be another amino acid that favours helix formation but variations on this theme have

been observed recently.

Type II AFPs are cysteine-rich globular proteins that contain disulfide bonds and

a mixed secondary structure. These proteins range in size from 14 to 24 kDa and share an

evolutionary relationship with C-type lectins (Fletcher et al 2001). Type II AFPs have

been isolated and characterized from sea raven, smelt and herring. Type III AFPs are

smaller proteins (6 – 7 kDa) that contain short β-strands and one helix turn that gives it a

unique flat-faced globular fold. The proteins are found in several families of Zoarcids

including many eelpout species (i.e. ocean pout) and wolffish. The recently discovered

Type IV AFP from the longhorn sculpin (*Myoxocephalus octodecimspinosis*) consists of

four amphipathic alpha-helices of similar length which are folded into a four-helix

bundle. The 12 kDa protein has sequence similarity to certain plasma apolipoproteins (Deng et al 1997, Deng and Laursen 1998, Zhao et al 1998).

The AFGPs, which are produced by Antarctic notothenioids and Northern cods, are made up of many tandem repeats of Ala-Ala-Thr. These proteins are folded into an amphipathic polyproline type II helix with a disaccharide attached to each threonine. Regardless of protein or polypeptide structure, all classes of fish AFPs lower the freezing point of plasma by the same adsorption inhibition mechanism (Fletcher et al 1998, Fletcher et al 2001).

1.4 Physiology and Function of Antifreeze Proteins

1.4.1 Liver type AFPs

The winter flounder, *Pseudopleuronectes americanus* (formerly *Pleuronectes americanus)*, has provided an excellent model for developing an understanding of AFP function and regulation. Winter flounder type I AFP is synthesized in the liver as an 82 amino acid preproprotein which contains a secretion signal sequence. After the pre-section is removed post-translationally, protein is secreted into the blood where the proprotein is finally cleaved to produce the mature 37 amino acid AFP (Fletcher et al 1989, Chan et al 1993, Gong et al 1995, Fletcher et al 1998). The circulating extracellular AFP protects the entire fish from freezing since the integrity and spatial structure of cell membranes reduce the risk of intracellular ice formation. Furthermore, skin and scales are effective barriers to the propagation of ice into the fish from the external environment. In fact, experimental evidence has demonstrated that the freezing point of blood plasma closely predicts the freezing point of the whole fish (Fletcher et al 1988, Goddard and

Fletcher 1994, Fletcher et al 2001). In extremely cold seawater, ice crystals enter the fish but without causing lethal consequences because extracellular plasma AFPs bind to and prevent them from uncontrolled growth.

Winter flounder liver AFP (wflAFP) gene expression and protein translation follows a seasonal cycle that is primarily controlled by photoperiod (see reviews by (Chan et al 1993, Fletcher et al 1989, Fletcher et al 2001). In fish from waters around Newfoundland, AFP appears in the plasma during November as the water temperature declines below 8°C and reaches peak levels of 10–15 mg/ml during winter. Regulation of AFP production is mediated through the pituitary gland in response to day-length and is repressed by growth hormone production. Water temperature is not a major factor in initiating AFP mRNA or protein synthesis in the fall, nor does it appear to be involved in terminating AFP production in the spring. However, water temperature must be sufficiently low for AFP mRNA to accumulate to high levels in winter to ensure elevated protein synthesis.

Some of the molecular mechanisms and important transcription factors regulating winter flounder liver AFP gene expression are beginning to be better understood. Studies have even identified the DNA elements controlling winter flounder liver-type AFP gene transcription. Current knowledge is comprehensive enough that a model has been proposed involving a repression mechanism to explain the complex seasonal, hormonal and tissue-specific regulation of the wflAFP genes (Miao et al 1998, Miao et al 2000, Miao et al 2002). While the regulation of AFP production in other fish species is not as well understood as in winter flounder, it is known that the controlling factors are not

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necessarily similar. In northern cod for example, water temperature plays a much more significant role in determining the onset and level of AFGP synthesis (Goddard et al 1992, Fletcher et al 1987, Goddard et al 1994).

1.4.2 Skin type AFPs

Until the past decade, it was generally accepted that the synthesis of AFPs was confined solely to liver tissue for secretion into blood for extracellular freeze protection. A paper published in 1982 that reported the isolation of AFP from the skin of European shorthorn sculpin was largely disregarded (Schneppenheim and Theede 1982). Almost 10 years later, Valerio et al (1990) found evidence of antifreeze activity in the epithelial tissue of cunner, but its significance was also unrecognized. However, interest was piqued with a report of AFP mRNA transcripts in winter flounder and ocean pout epithelial tissues (Gong et al 1992). Follow-up reports detailed the expression of winter flounder AFPs in numerous epithelial tissues which were related to, but distinct from, protein synthesized in liver (Gong et al 1995, Gong et al 1996). Recent publications of skin-type AFP isolation from shorthorn and longhorn sculpins indicate that the up-regulated production of AFP in peripheral epithelial tissues may be a common trait in many fish species (Low et al 1998, Low et al 2001).

Sequence analysis of clones isolated from a winter flounder skin cDNA library revealed that the epithelial protein lacks a peptide signal sequence which suggested that the protein remains intracellular (Gong et al 1996). In fact, subsequent immunohistochemical experiments have hinted that winter flounder skin-type AFP is restricted to the cytoplasm of the gill epithelial cells (Murray et al 2002). However, the

same authors found that in skin tissue, AFPs were localized outside of the cells despite the lack of secretion signal sequence. The skin-type AFPs presumably were exported out of cells via alternative pathways for protein secretion that circumvent the usual endoplasmic reticulum-Golgi pathway (Mignatti et al 1992, Menon and Hughes 1999).

The somewhat controversial hypothesis that skin-type AFPs remain intracellular, perhaps to protect cell membranes, is supported by some experimental evidence that AF(G)Ps might have functions other than freeze resistance (Wang 2000). It has been reported that AF(G)Ps could protect cold sensitive mammalian cells membranes from cold damage perhaps by blocking ion channels responsible for passive leakage across cell membranes (Negulescu et al 1992, Rubinsky et al 1992). Furthermore, new evidence suggests that fish AF(G)Ps stabilize cells during hypothermic storage and can afford thermal protection to model membranes by binding to and preserving membrane lipid order (Wu et al 2001, Wu and Fletcher 2001, Tomczak et al 2002a, Tomczak et al 2002b).

It is apparent that a different gene family codes for skin-type AFPs and these genes are expressed constitutively with much less seasonal variation than liver-type (Gong et al 1995, Gong et al 1996). In addition, the skin-type AFP mRNA levels do not appear to be influenced by removal of the pituitary gland (hypophysectomy). Overall, results indicate that the two sets of genes are not controlled by the same regulatory mechanisms. However, in contrast to the liver-type AFP genes, much less of the physiological or molecular regulatory mechanisms that control the expression of skin-

type AFPs have been fully deciphered. More work will need to be completed before the complex nature of antifreeze protein gene expression can be decoded.

1.5 Genetics and Evolution of AF(G)Ps

A puzzling aspect of the distribution of different classes of fish AF(G)Ps is that it does not correspond to teleost taxonomic groups very well. As illustrated in Fig 1-1, closely related species can produce widely variant proteins while at the same time single AFP types can be found in different Orders or even Super-Orders. The significant diversity in AFP structure in closely related species has been rationalized by the idea that the requirement for antifreeze arose relatively recently after current speciation had been established (Scott et al 1986). This theory suggests that recent sea-level glaciation selected for proteins which had ice binding potential and could potentially protect fish from freezing. The lateness of AFP evolution from pre-existing proteins was responsible for species developing radically different AFPs to control ice growth. Furthermore, the multifaceted structure of ice crystals could present a variety of surfaces for different proteins to bind (Davies et al 2002).

Figure 1-1. Phylogenetic tree illustrating the evolution and distribution of plasma AFPs and AFGPs in marine fishes. Adapted from a figure in Fletcher et al (2001). Tree distances are not drawn to scale.

[a]Type I AFPs have also been isolated from skin tissues of winter flounder, shorthorn and longhorn sculpins.

[b]Based on results from this study (Evans and Fletcher 2001).

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Subdivision				**TELEOSTEI**						
Infradivision	Clupeomorpha				Euteleostei					
Super-Order		Protocanthopterygii	Paracanthopterygii				Acanthopterygii			
Order				Pleuronectiformes	Perciformes			Scorpaeniformes		
Sub-Order					Labridae	Zoarcids	Notothenids			
Family	Clupeids	Osmerids	Gadids	Pleuronectidae				Cottidae		Cyclopteridae
Species	Herring	Smelt	Northern cods	Right-eyed[a] flounders	Cunner	Eelpouts	Antarctic Notothenids	Sculpin[a]	Sea Raven	Snailfish[b]
AF(G)P Type	**II**	**II**	**AFGP**	**I**	**I**	**III**	**AFGP**	**I, IV**	**II**	**I**

The original hypothesis however, does not explain the occurrence of similar

AF(G)Ps in unrelated fishes that could inhabit different hemispheres. In the case of

AFGPs for example, proteins from Antarctic Notothenioids are so similar to those from

Northern cods probably due to recent convergent evolution (Chen et al 1997a, Chen et al

1997b, Cheng and Chen 1999). While the Notothenioid gene appears to have evolved

from elements of a trypsinogen-like protease gene, the northern cod gene seems to be

evolutionarily unrelated. However, even convergent evolution cannot entirely explain the

descent of type II AFPs from the C-type lectin superfamily of proteins. Three species of

fish from diverse orders seem to have evolved their AFPs separately from C-type lectins

at different times in their history – a type of convergent evolution in parallel (Ewart et al

1992, Ewart and Fletcher 1993, Fletcher et al 1998, Ewart et al 1998, Ewart et al 1999).

The same convergence in parallel hypothesis is a reasonable explanation of the presence

of dissimilar type I AFPs in diverse species. Overall, it seems that the selective pressure

derived from recent geological cooling events has resulted in many different protein

adaptations to help fish species cope in new, cold water environments.

It is clear from genetic analysis of marine fish that rapid environmental cooling

has led to the amplification of liver specific AFP genes. In fish species with significant

levels of antifreeze activity, AF(G)Ps are encoded by large gene families that reflect

differences in environmental selective pressure (Scott et al 1987, Scott et al 1988).

Winter flounder contain a set of at least 30-50 gene copies arranged in tandem repeats

while another Pleuronectid, the yellowtail flounder, possess only 10 copies without the

same repeat structure. Estimates in other species range from 80 copies in wolffish, to 150

copies in one population of ocean pout (Fletcher et al 2001). It appears that skin specific

AFP genes are also encoded in multigene families that are just as extensive and complex

as the liver specific types (Gong et al 1996).

1.6 Description of Experimental Fish Species

The snailfishes belong to the family Cyclopteridae, which includes a large number

of benthic and pelagic marine fishes inhabiting northern regions of the Atlantic, Pacific

and Arctic oceans. This family is closely related to sculpins, which belong to a different

family of the same order Scorpaeniformes. The Atlantic snailfish (*Liparis atlanticus*) is a

very small species, with mature adults ranging in length from 9.5 to 14 cm. The body is

elongate with a small head, 31-35 dorsal fin rays and 25-29 anal fin rays (Scott and Scott

1988). Their colour varies from olive to reddish brown with some occasional lighter

coloured bars. They inhabit inshore waters of the northwest Atlantic Ocean from Ungava

Bay in northern Quebec and south to New York (Scott and Scott 1988). During the winter

months, January to March, fish move further inshore where females spawn small egg

masses, in depths less than 2 metres.

Dusky snailfish (*Liparis gibbus*) is a larger species, averaging 11cm in length but

adult females can reach up to 50 cm. The elongate body is compressed posteriorly with a

large rounded head and snout. These fish contain 40-45 dorsal fin rays and 34-37 anal fin

rays. Body colour is variable from dark to light brownish with many dark bands. Their

habitat extends from the Canadian Arctic Ocean, across to Greenland and down to the

coast of Newfoundland where they live mainly on muddy bottoms at depths up to 200 m

(Scott and Scott 1988).

1.7 Proposed Research and Thesis Objectives

It is known that closely related species from the order Scorpaeniformes produce type I, II and IV AFPs in liver for export into blood plasma. Because of the phylogenetic relationship of snailfish to other fish in this order, species from this genus were chosen to identify their constituent AFPs. Initially, the unknown snailfish AFPs would be isolated and characterized which could be useful to understanding protein structure/function relationships. The second stage would involve sequence and expression analysis of corresponding snailfish AFP genes. This information would help to clarify the evolutionary relationship between AFPs in the order Scorpaeniformes.

The primary objectives of the study are as follows:

(1)   Purification and characterization AFPs from both species of snailfish using a variety of standard biochemical and analytical techniques.

(2)   Construction and screening of cDNA libraries for both snailfish species in order to obtain the nucleotide sequence corresponding to isolated AFPs.

(3)   Generate a reasonable hypothesis to describe the evolutionary origins and to clarify some of the relationships between the antifreezes from fish in the order Scorpaeniformes.

CHAPTER 2:

Isolation and Characterization of Antifreeze Proteins from Blood Plasma of Atlantic and Dusky Snailfish

Preface

This chapter consists of a manuscript previously published in the journal *Biochimica Biophysica Acta* [Evans, R.P. and Fletcher, G.L. (2001). Isolation and characterization of type I antifreeze proteins from Atlantic snailfish (*Liparis atlanticus*) and dusky snailfish (*Liparis gibbus*). *Biochim Biophys Acta*. 1547:235-244]. The experimental research and data analysis were performed by RPE. The manuscript was written by RPE with editorial input from Dr. Fletcher. Some sections of the original manuscript have been altered to provide clarity for thesis presentation.

2.1 Introduction

Atlantic snailfish (*Liparis atlanticus*) and dusky snailfish (*Liparis gibbus*) inhabit

northern regions of the Atlantic ocean (see chapter 1). Because their harsh natural

environments would frequently expose both of these snailfish species to ice laden

seawater, they were considered excellent candidates for possession of AFPs. It was

originally hypothesized that the genus *Liparis* might produce either type I or type II

AFPs, since closely related species from the order Scorpaeniformes produce both classes

of AFPs. Shorthorn sculpin, *Myoxocephalus scorpius* and grubby sculpin, *M. aenaeus*

have type I AFP while sea raven, *Hemitripterus americanus* has type II AFP in their

blood plasma. Furthermore, it has recently been shown that another Scorpaeniforme, the

longhorn sculpin *Myoxocephalus octodecimspinosis*, produces an entirely unrelated

antifreeze protein in its blood plasma - type IV (Deng et al 1997, Deng and Laursen 1998,

Zhao et al 1998). For these reasons, we have purified and characterized AFPs from

snailfish to help elucidate their evolutionary origins and to clarify some of the

relationships between the antifreezes from fish in the order Scorpaeniformes.

2.2 Materials and Methods

2.2.1 Sample collection

Sixty-five Atlantic snailfish, *L. atlanticus*, were collected by divers near Logy

Bay, Newfoundland between December 1995 and August 1996. Three specimens of

dusky snailfish, *L. gibbus*, were collected from Placentia Bay, Newfoundland during the

winter of 1995. The species were distinguished by size, colour and their unique number

of dorsal and anal fin rays. The Atlantic snailfish specimens ranged from 7.5-14 cm while

the dusky snailfish specimens were up to 29 cm long. The live fish were brought into the laboratory and placed into holding tanks supplied with ambient temperature seawater prior to blood collection. Fish were anaesthetized using MS-222 and blood was collected from the caudal vein using a heparin containing syringe and 23-gauge needle. Blood samples were centrifuged at 2000 rpm for 10 minutes and the plasma was removed with a pipette.

2.2.2 Isolation and purification of plasma AFPs

Pooled plasma samples from Atlantic snailfish or from individual dusky snailfish (1 ml each) were applied to a Sephadex G-150 gel filtration column (0.9 x 60 cm) and eluted with 0.1M NH_4HCO_3. Protein fractions that exhibited antifreeze activity were collected and lyophilized. These were redissolved in 0.1M NH_4HCO_3 and applied a second time to the G-150 column. Following dialysis in 0.1M NH_4HCO_3 and lyophilization, samples were further purified by reverse phase HPLC. The partially purified AFPs were separated on a Nucleosil C8 column (0.46 x 25 cm). A gradient of 40-62% acetonitrile (solvent A) and 0.1% trifluoroacetic acid (solvent B) was used with a flow rate of 1 ml/min. Individual peaks were collected, lyophilized and redissolved in 0.01M NH_4HCO_3 for activity measurements. The purified proteins were separated on 15% polyacrylamide gels in a Tris-Tricine buffer system (SDS-PAGE) and stained with 0.1% Coomassie® Brilliant Blue R-250. Amino acid analysis and amino terminal sequencing were performed by the Biotechnology Service Centre; Hospital for Sick Children, Toronto, and mass spectrometry (MS) was performed by the Carbohydrate Centre, University of Toronto.

2.2.3 Measurement of antifreeze activity

Antifreeze activity was measured as thermal hysteresis using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY), following the procedure of Kao et al (1986). Thermal hysteresis is defined as the difference between the melting and freezing temperatures (in °C) of a test solution. *L. atlanticus* AFP (designated La-AFP) and *L. gibbus* AFP (designated Lg-AFP) were dissolved in 0.1M NH₄HCO₃ and centrifuged before use. For each sample, measurements were made in triplicate, and the average value taken. Video recordings were made of ice crystals for analysis of crystal morphologies. In some cases, individual frames were captured and the digital images were used to make photographs.

2.2.4 Circular dichroism spectroscopy

Circular Dichroism (CD) spectra were recorded on an Aviv Circular Dichroism Spectrometer model 62DS (Lakewood, NJ) in the Department of Medical Biophysics, University of Toronto. Spectra were obtained from 200 to 260 nm, with a 0.5 nm step, 1-nm bandwidth, and 20 seconds collection time per step. For thermal denaturation profiles, ellipticity was measured at 222 nm and data was collected at a rate of 1 sec per point for 300 data points. Samples were cooled at a rate of 1°C every 16 seconds. Lyophilized protein was dissolved in 0.01 M NH₄HCO₃ (pH 8.5). The protein concentration was approximately 0.35 mg/ml (La-AFP) and 0.15 mg/ml (Lg-AFP1). CD measurement was carried out using a cuvette of 0.1-cm path length. The following equation was used to calculate the predicted mean residue ellipticity for 100% helix:

$$[\theta]^n = [\theta]^\infty \left(1 - \frac{k}{n}\right)$$ Equation 2-1   (Low et al 1998)

Where n is the chain length and k is a wavelength dependent factor (2.57 at 222 nm) and

$[\theta]^\infty$ = 39,500 degree•cm^2•dmol^{-1}. The percentage of helix for AFPs was determined from

the average mean residue ellipticity at 222 nm.

2.3 Results

2.3.1 Purification and analysis of snailfish AFPs

Analysis of blood plasma from both snailfish species indicated that fish contained

antifreeze activity during the winter months from January to March. Atlantic snailfish

had an average thermal hysteresis measurement of 0.73 ± 0.15 °C (n = 65) while dusky

snailfish plasma was measured as 0.92 ± 0.20 °C (n = 3). Additionally, a seasonal cycle

was observed for Atlantic snailfish antifreeze activity since it peaked during February and

subsequently decreased during the spring to where it was nonexistent in August (0.05 °C).

The antifreeze activity found in blood plasma was first isolated and then purified

using a variety of standard techniques. Partially purified protein isolated from plasma by

Sephadex G-150 gel filtration chromatography, could be further resolved by HPLC (Fig

2-1, 2-2). A single peak from *L. atlanticus* (designated as La-AFP) was isolated and 2

separate peaks were resolved for *L. gibbus* (designated as Lg-AFP1 and Lg-AFP2). These

peaks were collected and analyzed by SDS-PAGE. Based on these results, it appeared

that the collected fractions were purified to homogeneity since there was a single band on

the gel for each of these three protein peaks (Fig 2-1, 2-2). The minor peaks observed on

some HPLC profiles were also collected and analyzed for the presence of antifreeze

activity. Initially, these small peaks appeared to contain some possible antifreeze activity but this activity was subsequently lost when the fractions were lyophilized and re-dissolved in 0.1M NH4HCO3. Additionally, these fractions did not alter ice crystal structure and were not consistently observed between HPLC runs whereas the major AFP peaks were always seen. For these reasons it appears that all AFPs are contained within the major peaks collected and that the small ones were probably due to the presence of salts and other small blood solutes.

On SDS-PAGE, the relative molecular weights of La-AFP, Lg-AFP1 and Lg-AFP2 were 6.2 kDa (Fig 2-1, 2-2). Also based on SDS-PAGE, it appeared that the HPLC peaks collected during protein purification contained single proteins. However, upon further analysis by mass spectrometry this was concluded not to be accurate. It was determined that the single band from La-AFP on the gel was actually 2 different proteins having molecular weights of 9344 Da (major) and 9415 Da (minor). Amino terminal sequencing of the first 17 amino acid residues, by Edman degradation (see Table 2-2), indicated that the 71 Da difference was due to a single alanine residue that was missing from the major protein form at the amino terminus. The sequence suggests that the two different forms of La-AFP are probably due to differences in post-translational processing of the individual proteins, possibly during signal peptide cleavage. Analysis of Lg-AFP1 and Lg-AFP2 by SDS-PAGE also indicated that the single bands were individual proteins.

Figure 2-1. Outline of the purification of *Liparis atlanticus* AFPs. Panel (A) shows a typical Sephadex G-150 profile of crude blood plasma with fractions that contained antifreeze activity indicated. (B) Sephadex G-150 purified antifreeze from plasma were separated on a Nucleosil C8 column (25 x 0.46 cm); flow rate was set at 1 ml/min with a 40-62% acetonitrile, 0.1% trifluoroacetic acid gradient. The sole AFP peak found is labelled as *L. atlanticus* AFP (La-AFP). Panel (C) is the SDS-PAGE separation of *L. atlanticus* AFP. A 15% polyacrylamide gel was used with the Tris-Tricine buffer system. Lane 1, pooled G-150 column fractions of *L. atlanticus* AFPs (approx. 150 μg of total protein); Lane 2, HPLC purified protein (approx. 75 μg of protein).

A
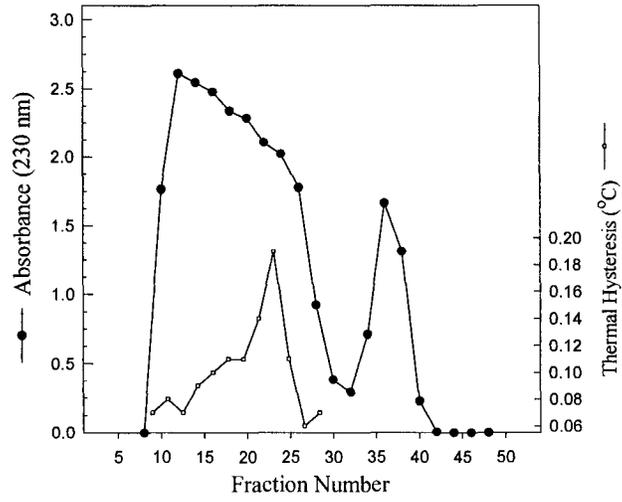
B
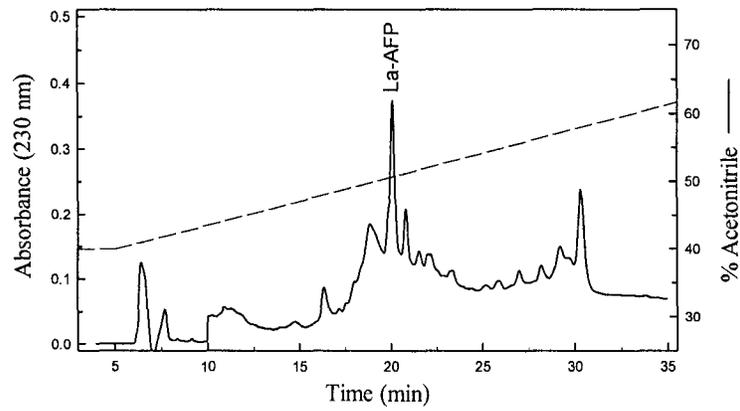
C
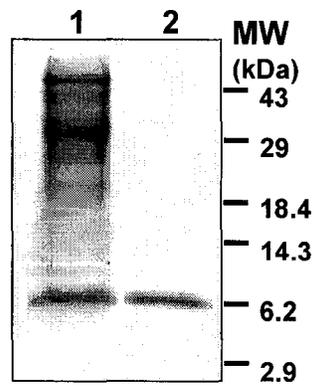
Figure 2-2. Outline of the purification of *Liparis gibbus* AFPs. Panel (A) shows a typical Sephadex G-150 profile of crude blood plasma with fractions that contained antifreeze activity indicated. (B) Sephadex G-150 purified antifreeze from plasma were separated on a Nucleosil C8 column (25 x 0.46 cm); flow rate was set at 1 ml/min with a 40-62% acetonitrile, 0.1% trifluoroacetic acid gradient. The AFP peaks found are labelled as *L. gibbus* peak 1 (Lg-AFP1) and *L. gibbus* peak 2 (Lg-AFP2). Panel (C) is the SDS-PAGE separation of *L. gibbus* AFPs. A 15% polyacrylamide gel was used with the Tris-Tricine buffer system. Lane 1, pooled G-150 column fractions of *L. gibbus* AFPs (approx. 200µg of total protein); Lane 2, HPLC purified Lg-AFP1 (approx. 100 µg of protein); Lane 3, HPLC purified Lg-AFP2 (approx. 80 µg of protein).

A

B

C
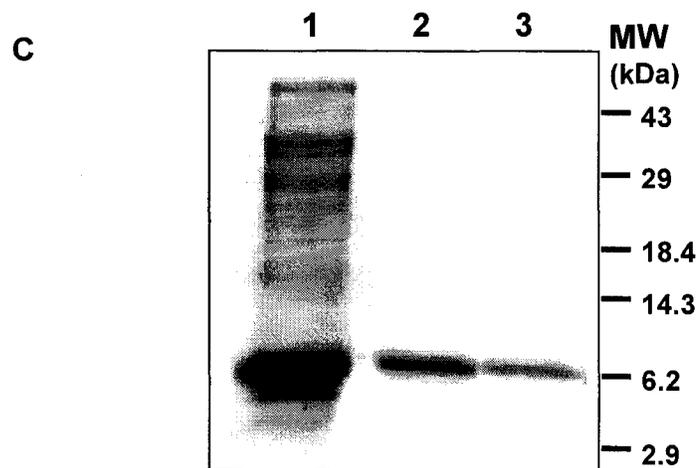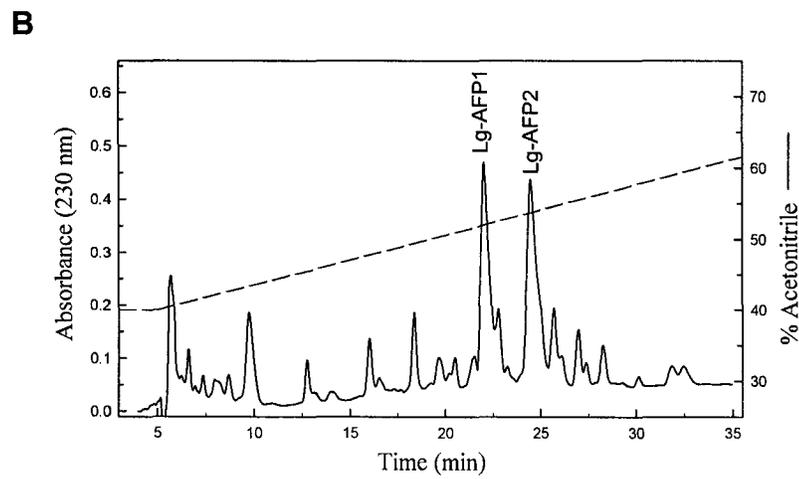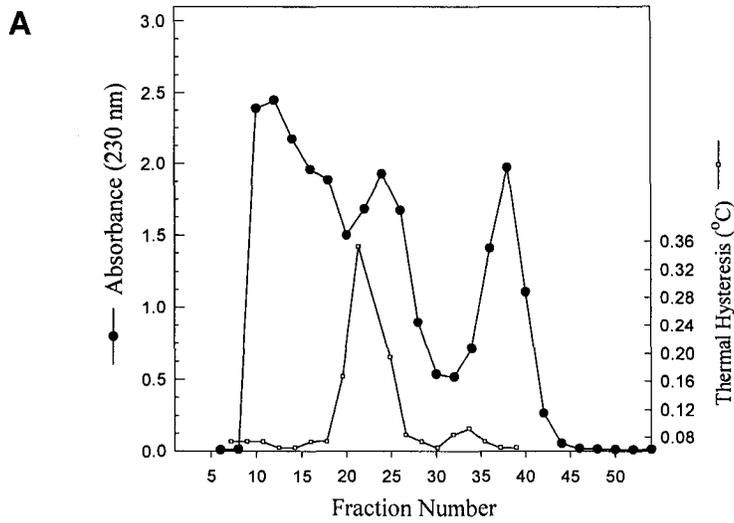
However, analysis by mass spectrometry revealed that it was more complicated than initially surmised. Lg-AFP1 contained three isoforms; 9646 Da (major) and 2 minor isoforms of 9514 Da and 9814 Da. Additionally, the Lg-AFP2 band contained a major isoform with a mass of 9573 Da and a minor one of 9742 Da. The observed discrepancy in molecular mass between electrophoretic and mass spectrometry data may be a direct consequence of the amino acid content of these proteins. The large number of alanine residues would give rise to a larger than average number of SDS molecules associated with the proteins which would affect gel mobility (Hew et al 1980). Repeated attempts to separate the protein mixtures into their individual protein isoforms by HPLC or SDS-PAGE proved to be unsuccessful.

2.3.2 Amino acid composition

The amino acid compositions of snailfish AFPs (La-AFP, Lg-AFP1 and Lg-AFP2) are given in Table 2-1. These proteins are typical of all type I AFPs in that they have a high alanine content, which accounts for between 51 to 59 mol%. The abundance of alanine residues is similar to winter flounder liver/skin AFPs which are between 60-62% alanine (Gong et al 1996) but is somewhat lower than shorthorn sculpin skin AFP which is ~70% alanine (Low et al 1998). An interesting aspect of these antifreeze proteins is their proline content which is 2.5 mol% for La-AFP and ~ 4.2 mol% in Lg-AFP1, 2 (Table 2-1). This would correspond to between 3 and 5 proline residues for each of these AFPs, which may disrupt the helix content of the protein backbone since proline residues are known to disrupt α-helices (Chakrabartty and Baldwin 1995).

Table 2-1. Amino acid composition (Mol %) and molecular mass of *Liparis* AFPs

Amino Acids	*Liparis atlanticus* La-AFP	*Liparis gibbus* Lg-AFP1	*Liparis gibbus* Lg-AFP2
ASP	3.63	5.41	5.46
GLU	2.95	2.61	2.63
SER	2.80	2.03	2.05
GLY	4.56	3.85	3.88
ARG	1.57	1.75	0.87
THR	10.26	8.93	9.01
ALA	58.79	51.22	51.68
PRO	2.50	4.18	4.21
VAL	5.57	8.41	8.48
ILE	1.28	1.74	1.75
LEU	2.55	2.31	2.33
PHE	0.12	1.00	1.01
LYS	3.43	6.57	6.63
Mol Mass* Da	9344 (major) 9415**	9646 (major) 9514; 9814	9573 (major) 9742

*Based on Mass Spectroscopic analysis
** 15% of protein molecules contain 1 fewer Ala residue at amino terminus

2.3.3 Antifreeze activity and ice crystal morphology of snailfish AFPs

All of the snailfish AFPs tested here, (La-AFP, Lg-AFP1 and Lg-AFP2) display concentration-dependent thermal hysteresis activities that are not significantly different from each other. As shown in Figure 2-3, the thermal hysteretic ability of snailfish AFPs is intermediate on a molar basis, with activity lower than that of winter flounder liver HPLC-6 (wflAFP-6) but higher than winter flounder skin (wfsAFP-2). Owing to the fact that snailfish AFPs are nearly three times larger than wflAFP-6, their activity on a weight basis is substantially lower than this winter flounder AFP, as would be expected. However, the activity of snailfish AFPs is also lower on a weight basis when compared to recombinant shorthorn sculpin skin AFP (sssAFP-2) that has a similar molecular mass (9700 Da) (Low et al 1998). At low protein concentrations, snailfish AFPs impart the typical hexagonal bipyramidal shape to ice crystals that other type I AFPs give (Fig 2-4A, B) and these are subsequently elongated/sharpened into spicule-like forms at high protein concentrations (Fig 2-4C), a characteristic typical of active AFPs.

2.3.4 Secondary structure of snailfish AFPs

CD spectral analyses of La-AFP and Lg-AFP2 (Fig 2-5A, C) show strong minima at 208 and 222 nm, which are typical of an α-helical secondary structure. Calculation of helix content indicates that La-AFP is 79% α-helical and Lg-AFP2 is 83% α-helical when measured at 0 °C.

Figure 2-3. Comparison of thermal hysteresis activity curves on (A) molar basis and (B) weight basis. HPLC purified AFPs from *Liparis atlanticus* (La-AFP) and *Liparis gibbus* peak 1 (Lg-AFP1) and 2 (Lg-AFP2). Data for winter flounder plasma (HPLC-6) AFP were taken from (Kao et al 1986) while winter flounder skin AFP (wfsAFP-2) data was taken from (Gong et al 1996). Shorthorn sculpin skin (sssAFP-2) data are from (Low et al 1998, Chadwick et al 1990).
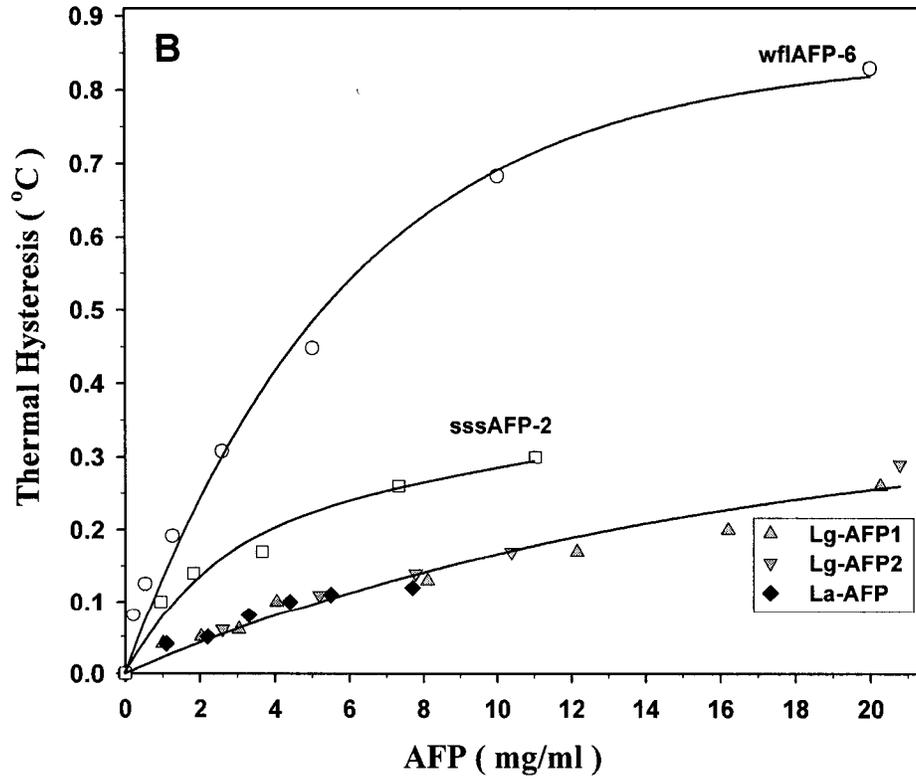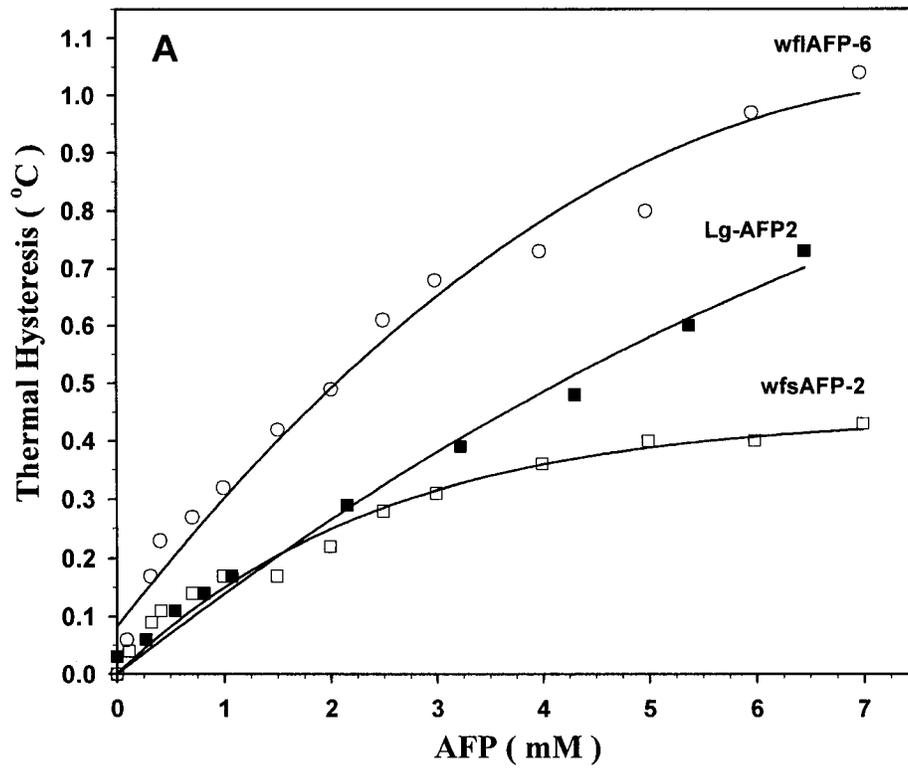
Figure 2-4. Ice crystal morphology in the presence of AFPs. Crystals were videotaped at temperatures below their melting point after they had remained a constant size for at least ten seconds. Individual frames were captured from tape and converted to image files. (A) La-AFP used at a concentration of approximately 1.25 mM (thermal hysteresis 0.16 °C). (B) Lg-AFP2 used at a concentration of approximately 1.5 mM (thermal hysteresis 0.2 °C) or (C) at 5 mM concentration (thermal hysteresis 0.6 °C).
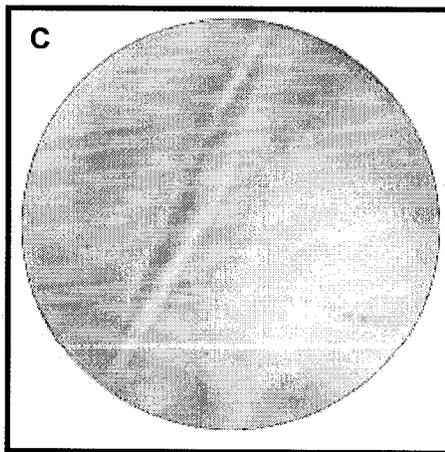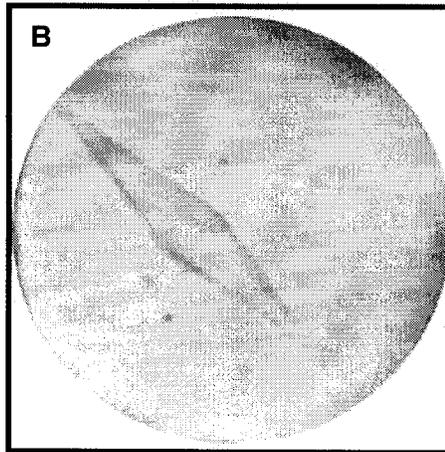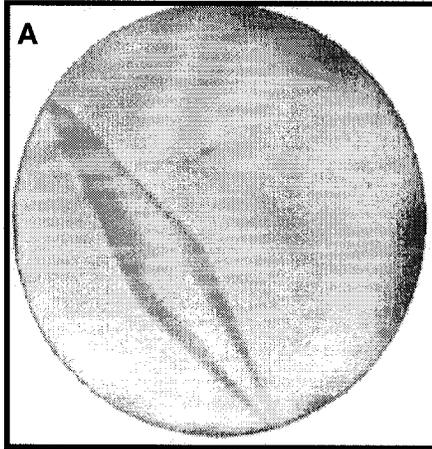
Figure 2-5. CD spectra and thermal denaturation profiles of *L. atlanticus* and *L. gibbus* AFPs. (A) CD spectrum and (B) thermal denaturation curve for *L. atlanticus* AFP (La-AFP). The protein concentration was approximately 0.35 mg/ml in 0.1 M NH_4HCO_3 (pH 8.5) and cell length was 0.1 cm. (C) CD spectrum and (D) thermal denaturation curve for *L. gibbus* peak 1 (Lg-AFP1). The protein concentration was approximately 0.15 mg/ml in 0.1 M NH_4HCO_3 (pH 8.5) and cell length was 0.1 cm.

A

[θ] x 10⁻³ (deg·cm²·dmol⁻¹)

0 °C

40 °C

Wavelength (nm)

B

[θ]₂₂₂ x 10⁻³ (deg·cm²·dmol⁻¹)

Temp	% Helix
0 °C	79.2
5 °C	78.6
20 °C	54.7
40 °C	9.9

Temperature (°C)

C

D

Temp	% Helix
0 °C	83.7
5 °C	85.5
20 °C	82.9
40 °C	29.4
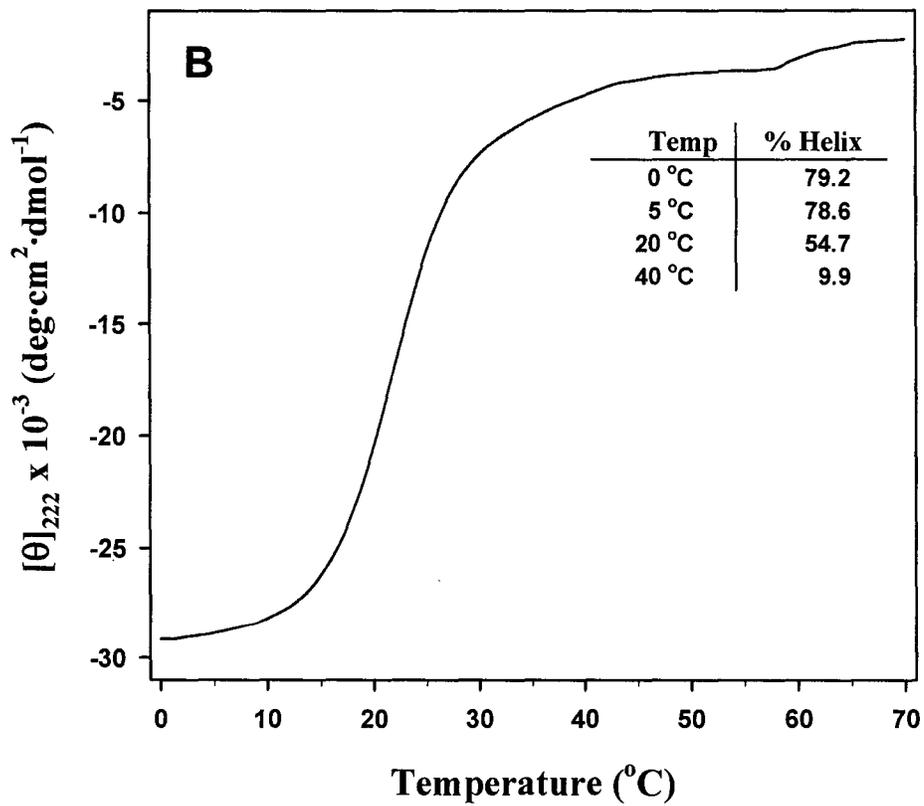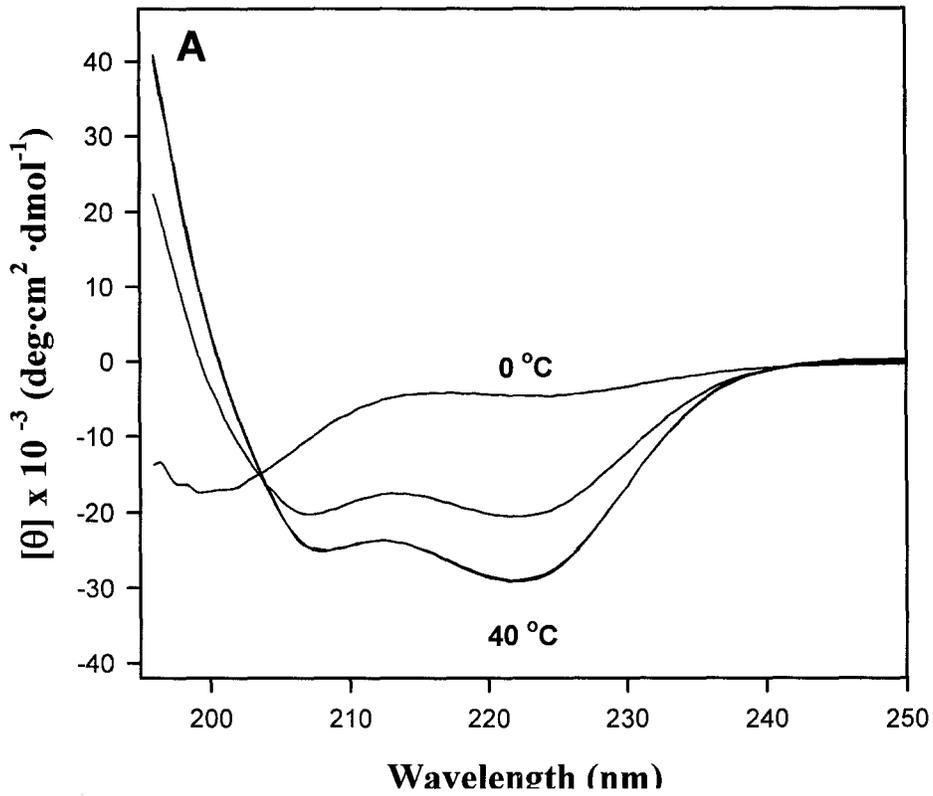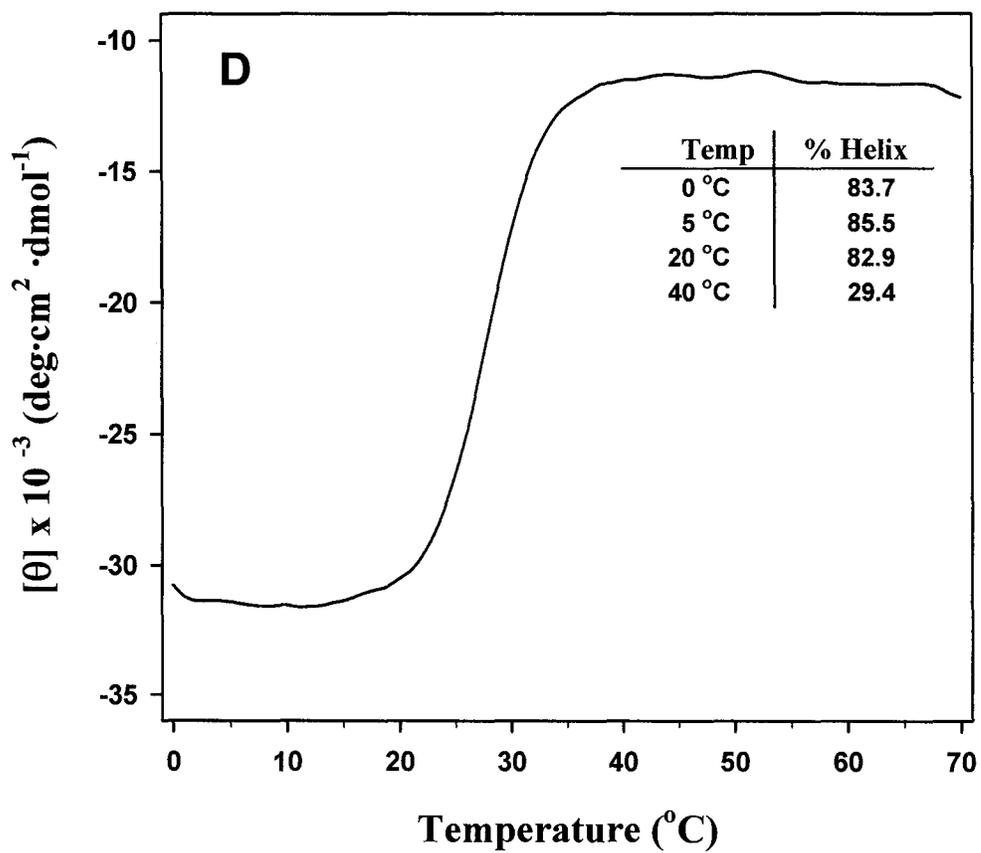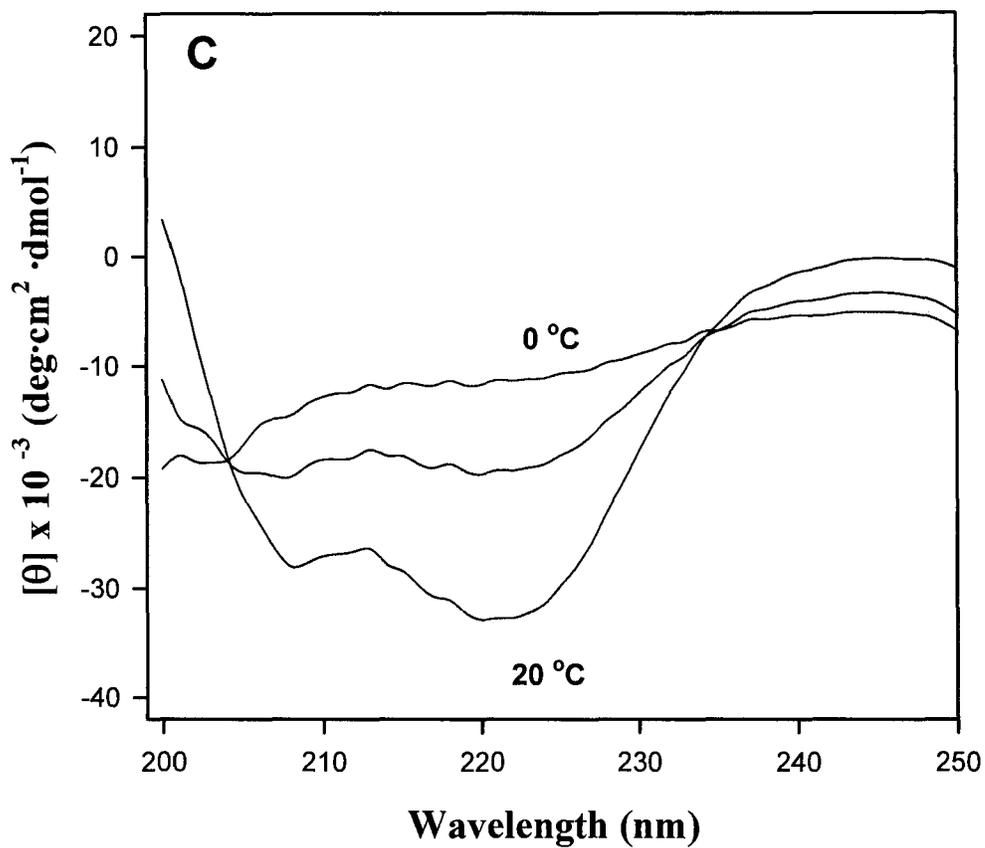
The helix content begins to disappear as the temperature increases and the proteins are fully denatured after 40 °C but will regain their full helix content when cooled back down to 0 °C, indicating reversible thermal denaturation is occurring. These AFPs become irreversibly unfolded after 70 °C. The midpoint of thermal denaturation (Tm) was calculated as 22 °C for La-AFP and 28 °C Lg-AFP2 which is similar to that of winter flounder liver AFP (Wen and Laursen 1992). The thermal denaturation profile (Fig 2-5B, D) for *Liparis* AFPs appears to be cooperative in nature as indicated by the CD spectral data. This property of these AFPs may be associated with the proline residues in the proteins that are known to disrupt helical proteins (Chakrabartty and Baldwin 1995) and seems to indicate that these antifreeze proteins are unfolding as separately distinct helices.

2.4 Discussion

The results of the present study clearly demonstrate that snailfish produce AFPs during winter. These proteins are similar to type I AFPs in their high alanine content and α-helical secondary structure, but are significantly larger than all type I AFPs isolated from any other source. Table 2-2 gives an overall comparison of the major type I AFPs investigated to date indicating the species from which they originate. Fish from the Teleostei Superorder Acanthopterygii contain all known types of fish AFPs / AFGPs and diverse species from two of its orders (Scorpaeniformes and Pleuronectiformes) produce all type I AFPs discovered thus far (Cheng 1998, Harding et al 1999). Here

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Table 2-2. Species origin and physical properties of Type I AFPs.

Species	Protein / Peptide Name	M_r (kDa)	Helix Content (%)	Activity (°C)	Amino Acid Sequence	Refs
Winter flounder	HPLC6	3.2	85	0.68	DTASDAAAAAALTAANAKAAAELTAANAAAAAAATAR	1
	HPLC8	3.3	85	0.60	DTASDAAAAAALTAANAKAAAKLTADNAAAAAAATAR	1
	AFP9	4.3	-	-	DTASDAAAAAAATAATAAAAAAATAVTAAKAAALTAANAAAAAAATAAAARG	2
Yellowtail flounder	YTAFP	4.0	-	-	DTASDAAAAAAATAAAAAKAAADTAAAAAKAAADTAAAAAEAAAATAR	3
Alaskan plaice	AP	3.5	-	-	DTASDAAAAAAATAAAAKAAAEKTARDAAAAAAATAAAAR	4
Shorthorn sculpin	SS3	2.9	45	0.39	MNAPARAAAKTAADALAAAKKTAADAAAAAA	5, 6
	SS8	4.0	73	0.67	MNGETPAQKAARLAAAAALAAKTAADAAAKAAAKAAAIAAAAASA	
Grubby sculpin	GS5	3.0	-	-	MDAPAIAAAKTAADALAAAKKTAADAAAAAAKP	7
	GS8	3.4	-	0.68	MDGETPAQKAARLAAAAALAAKTAADAAAKAAAIAAAAA	
Arctic sculpin	AS1	3.4	-	-	MDGETPAGKAARLAAAAALAAKTAADAAAKAAAIAAAAA	8
	AS3	2.9	-	-	MDAPARAAAKTAADALAAANKTAADAAAAAAA	
Atlantic snailfish	La-AFP	9.3	79	0.15	[a](A)ATPAQRAAATATAAAA...	
Dusky snailfish	Lg-AFP2	9.6	83	0.15		
Winter flounder	wfsAFP2	3.4	81	0.45[c]	MDAPAKAAAATAAAAKAAAEATAAAAAKAAAATKAGAAR	9
Shorthorn sculpin	sssAFP-2	7.7[b]	74	0.30	MAAAAKAAEAAAMAAANAAEAAATKAADAAASAAAAAIAAIAEAAEAAEA AATKSANVAAAAAATSAAAAAKATANAAAAASAAAAAAAVA	10

[a]15% of protein molecules contain 1 fewer Ala residue at amino terminus
[b]Mass of native protein. Reported mass of 9.7 kDa for recombinant protein
[c]Activity at 7 mg/ml protein

[1](Duman and DeVries 1974); [2](Chao et al 1996); [3](Scott et al 1987); [4](Knight et al 1991); [5](Hew et al 1980); [6](Hew et al 1985); [7](Reisman et al 1987); [8](Fletcher et al 1982); [9](Gong et al 1996); [10](Low et al 1998)

we have demonstrated that another family from the order Scorpaeniformes; namely

Cyclopteridae, produces type I AFPs that circulate in snailfish blood plasma.

The type I AFPs found in snailfish plasma are interesting since although there is

high similarity amongst snailfish species they are unlike closely related sculpin plasma

AFPs in their amino terminal sequence and their large size. This indicates that the

snailfish AFPs derived from the same progenitor protein prior to the differentiation of

these species but that the sculpin AFPs derived from another one. However, we also have

evidence that snailfish skin AFP sequences are very similar to those from shorthorn

sculpin skin AFPs both at the DNA and amino acid levels (Evans, R.P. and Fletcher, G.L.

unpublished data). Taken together, this information suggests that type I AFPs have

emerged on more than one occasion from a common progenitor. Only after an ancestral

protein (or proteins) that is common to all type I AFPs is discovered will we know for

certain. Furthermore, it would also be helpful to determine the primary nucleotide

sequence of snailfish plasma AFPs in order to confirm the presence of any repeat regions

and to identify possible ice binding sites or surfaces to see how they compare to others.

The activity of snailfish plasma AFPs is quite low when measured on a weight

basis (mg/ml) compared to wfl-AFP6 and even sssAFP-2 (Fig 2-3). However, when their

molecular mass is taken into account when measurements are made (mM), the ability of

these AFPs to lower solution freezing points is intermediate compared to other type I

AFPs (Kao et al 1986, Ewart and Fletcher 1990) but still lower than sssAFP-2 (Fig 2-3).

These observations suggest that although the snailfish AFPs are very large they may

possess fewer ice-binding surfaces or motifs than do the sssAFP-2 and wfl-AFP6. This

relationship appears to be consistent for the large type I AFPs from snailfish and shorthorn sculpin since these proteins have lower thermal hysteresis activities than their smaller type I counterparts (Low et al 1998). One puzzling fact that arises from these results is the apparent inability of plasma AFPs to protect snailfish from freezing in ice-cold seawater that they would be exposed to in winter. Even at very high concentrations of 20 mg/ml, purified proteins would provide approximately 0.25 °C of freeze protection, which does not meet required levels (up to 0.7 °C). This suggests that some other factor is augmenting the plasma AFPs that protect these fish from freezing in extreme environmental conditions. We have evidence that physiological concentrations of typical plasma salts can significantly enhance the thermal hysteresis activity of purified type I AFPs from winter flounder (see chapter 3). These data have clear implications for studies that attempt to quantify the 'true' antifreeze capability of a particular type I AFP and when comparisons are made between AFPs isolated by different groups. Other studies have demonstrated that some common blood solutes (i.e. sorbitol, glycerol, alanine etc.) can significantly enhance the activity of beetle, *Dendroides canadensis*, AFP (Li et al 1998) while rainbow smelt *Osmerus mordax* synthesize glycerol as an additional, colligative antifreeze in blood plasma (Raymond and Driedzic 1997, Driedzic et al 1998). More work needs to be done to determine which mechanism(s) is (are) involved in snailfish.

The results of this research, and that of others, suggest that it may be necessary to simplify the definition of type I antifreeze proteins. Since their discovery, this class of AFPs has been defined as small helical proteins/polypeptides (less than 4.5 kDa) that

have a specific 11 amino acid repeat motif (Thr-X2-Asn/Asp-X7), where X is usually alanine or may be another amino acid that favours helix formation (Fletcher et al 1998, Ewart et al 1999, Harding et al 1999). It was also proposed that the repeating nature of type I AFPs matched the ice-crystal lattice structure in such a way that polar residues interacted through hydrogen bonding while the hydrophobic side was exposed to the water molecules which prevented further crystal growth (Fletcher et al 1998, Ewart et al 1999, Harding et al 1999). However, recent studies have suggested that non-polar interactions may also play important roles in ice binding (Chao et al 1997, Cheng and Merz, 1997) while other groups have examined the roles of putative ice binding motifs (IBM) and the properties are necessary to allow an AFP to function correctly (Lin et al 1999a, Lin et al 1999b). It has also been speculated that motifs that were originally thought to be important for ice binding are actually functioning in protein solubility, and a new site for ice binding has been proposed that involves hydrophobic interactions between the ice surface and the protein hydrophobic surface (i.e. alanine residues) (Harding et al 1999, Baardsnes et al 1999, Loewen et al 1999). The research presented here and other work on shorthorn sculpin skin AFP (Low et al 1998) has shown that both plasma and skin type I AFP can be significantly larger than previously thought and not necessarily contain the 11 amino acid repeat sequence. Essentially, this class of antifreeze proteins is defined by its high alanine content and α-helical secondary structure.

CHAPTER 3:

The Importance of Dissolved Salts to the *In Vivo* Efficacy of

Antifreeze Proteins

3.1 Introduction

The blood plasma of most marine teleost species freezes at around -0.7°C

(Holmes and Donaldson 1969). However, some teleost fish inhabiting icy seawater are

able to synthesize AF(G)Ps for protection against extracellular freezing. Since seawater

temperatures can fall to -1.8°C in winter, a level of thermal protection near this

temperature would be necessary to survive in such extreme conditions. In addition to the

non-colligative (thermal hysteretic) action of AF(G)Ps, the colligative effects of

dissolved plasma solutes contribute to overall freeze protection.

In chapter 2 (Evans and Fletcher 2001) it was noted that the standard curves of

thermal hysteresis (TH) activity for purified snailfish AFPs suggested that the proteins

had inherently low activity that did not relate well to plasma freezing points. Therefore, a

physiological concentration (~10 mg/ml) of the AFPs could not protect the fish from

extracellular freezing at temperatures below -0.9°C. Over the years, many researchers

have been puzzled by the discrepancy between plasma freezing points and TH activity

measured using solutions of purified AFPs. Moreover, numerous reports have indicated

that TH is diminished during AFP purification as solutes are removed from the protein

solution. Such observations suggest that AFP activity is improved by compounds in the

plasma that are removed during the purification process.

DeVries et al (1970) attributed the difference between plasma and pure AFP

solution freezing points strictly to the colligative effects of plasma solutes (particularly

Na^+ and Cl^- ions). This has been the paradigm that researchers in the field have worked

under since then. However, the additional colligative freezing point depression cannot

explain the phenomenon of increased AFP thermal hysteresis. Experiments have demonstrated that high molecular weight solutes could have "superadditive" effects on the measured TH activity of fish AF(G)Ps in solution (Caple et al 1986, Kerr et al 1985). A more recent study has shown that low molecular weight solutes can enhance the antifreeze activity of *Dendroides canadensis* larvae AFP (Li et al 1998). However, in all cases the effects were observed using highly concentrated solutions of the test solutes which make it unclear if there is any physiological significance of the enhanced activity.

To address the problem more fully, experiments were designed here to measure thermal hysteresis of AFPs dissolved in salt solutions at concentrations similar to fish plasma. The purpose is to formulate a general mechanism describing the effects of salts on TH activity that is applicable to all antifreeze protein classes.

3.2 Materials and Methods

3.2.1 Plasma sample collection

Winter flounder (*Pseudopleuronectes americanus*) and ocean pout (*Macrozoarces americanus*) were collected by gillnet and brought to the fish hatchery in Wesleyville, Trinity Bay, Newfoundland. These two teleost species produce type I and type III AFP, respectively. For AFGP isolation, rock cod (*Gadus ogac*) were collected as by-catch from lobster traps. Live fish were brought into the laboratory and placed into holding tanks supplied with ambient temperature seawater prior to blood collection. Fish were anaesthetized using MS-222 and blood was collected from the caudal vein using a heparin containing syringe and 23-gauge needle. Blood samples were centrifuged at 2000 rpm for 10 minutes and the plasma was removed with a pipette.

3.2.2 Isolation and purification of plasma AFPs

Winter flounder or ocean pout plasma was applied to a Sephadex G-75 gel filtration column, eluted with 0.1M NH_4HCO_3, and collected in a fraction collector. Fractions that exhibited antifreeze activity were pooled, lyophilized and redissolved in 0.1M NH_4HCO_3 Proteins were further purified on a Sephadex G-75 column, re-lyophilized and stored at -20°C over desiccant in a sealed container until required.

Antifreeze glycoproteins (AFGP) were purified from the blood plasma of rock cod using the method of Wu et al (2001). Plasma was treated with 50% ethanol at 4°C and the precipitated proteins were removed by centrifugation. The resulting supernatant containing AFGP was dialyzed against 2.5mM Tris-HCl (pH 9.4) using Spectropore 3 dialysis tubing with a molecular weight cut off of 3500. The dialyzed proteins were applied to a DEAE-Bio-gel ion exchange column and eluted with a stepwise gradient of 2.5-250 mM Tris-HCl (pH 9.4). Fractions that exhibited antifreeze activity as assessed with the Clifton Nanolitre Osmometer (see below) were pooled, lyophilized, and stored at -20°C over desiccant in a sealed container until required.

3.2.3 Preparation of salt solutions

Molecular biology grade NaCl, KCl and LiCl were purchased from Fisher Scientific. The Stock solutions of each salt were prepared in distilled water at the beginning of each experiment and diluted to twice the final desired concentration. Stock solutions of each AFP in 10 mM NH_4HCO_3 (or pure water) were made such that the final desired concentration was achieved when mixed with an equal volume of test salt solution.

3.2.4 Measurement of antifreeze activity

Antifreeze activity was measured as thermal hysteresis using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY), following the procedure of (Kao et al 1986). Thermal hysteresis is defined as the difference between the melting and freezing temperatures (in °C) of a test solution. For each sample, measurements were made in triplicate, and the average value taken.

3.3 Results

Figure 3-1 shows the freezing and melting curves of three types of AF(G)Ps measured in increasing concentration of NaCl, KCl and LiCl solutions. Since there was no significant difference between the data points for the individual salt solutions, they were combined for the regression analysis and displayed as a single line for clarity. Clearly the addition of salts to the AF(G)Ps lowers the freezing points of the solutions in a linear fashion that almost parallels the melting curves. The difference between the freezing and melting curves is attributable to the thermal hysteresis (TH) or antifreeze activity of the particular AFP or AFGP in the solution. However, as the salt concentration increases, the lines deviate increasingly from parallel, illustrating that the effect of salt on the freezing temperature of AF(G)P solutions is not strictly colligative (additive). There is an effect which enhances the activity of the antifreeze proteins. The results were similar when a divalent salt ($MgCl_2$) was used for comparison purposes (data not shown).

As is evident in figure 3-2, the measured thermal hysteresis activity of each AF(G)P increased concomitantly with salt concentration. Overall, thermal hysteresis of

Figure 3-1. The effects of increasing salt concentration on melting and freezing curves of AF(G)Ps. Linear regression lines for melting and freezing curves are drawn through grouped values of three individual salt solutions. Regression analysis was performed using SigmaPlot™ software. Thermal Hysteresis (TH) is calculated as the difference between freezing and melting curves. The dashed line represents the calculated freezing point from added salt solution based on colligative effects alone. Type I and III AFP (5 mg/ml); AFGP (10 mg/ml).

Type I AFP

r² = 0.9767

(AFP + salt)

Freezing

ΔT = TH

r² =0.9705

Melting

Type III AFP

r² = 0.9704

(AFP + salt)

Freezing

ΔT = TH

r² =0.9790

Melting

AFGP

r² = 0.8812

(AFP + salt)

Freezing

ΔT = TH

r² =0.8694

Melting

Salt Concentration (mM)
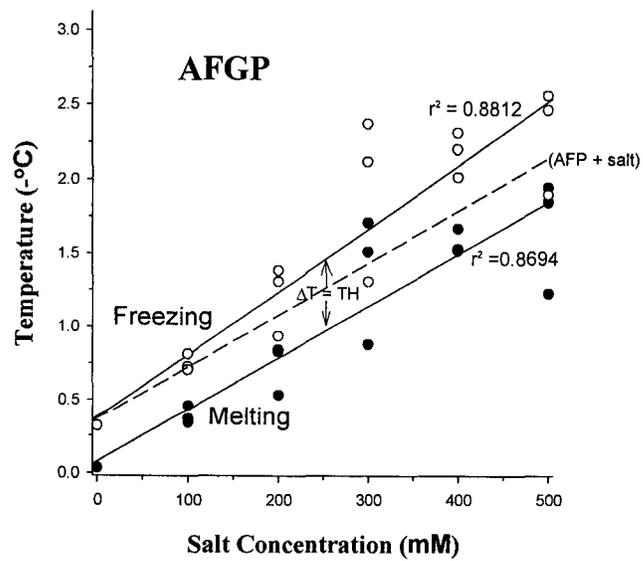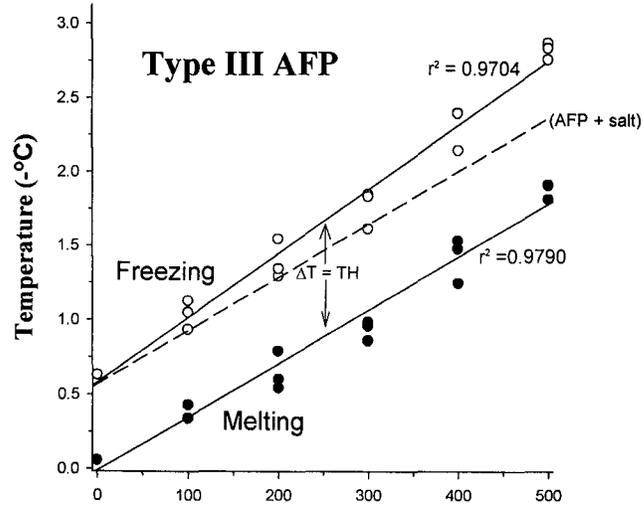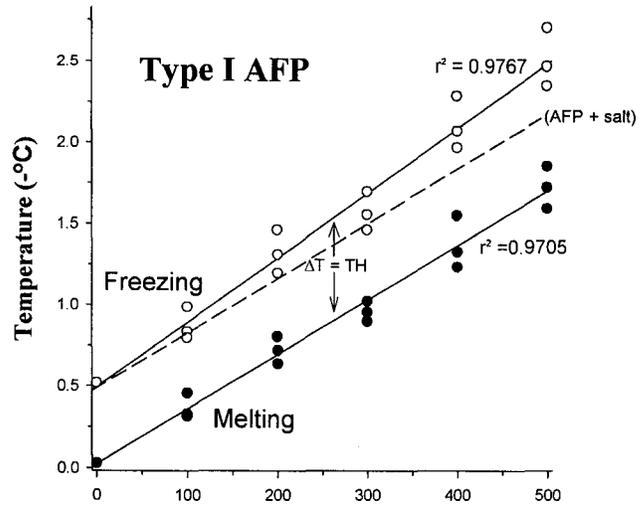
Figure 3-2. The effects of increasing salt concentration on thermal hysteresis activity of AF(G)P solutions. Individual linear regression lines are drawn for each protein in the individual salt solutions using SigmaPlot™ software. Type I and III AFP (5 mg/ml); AFGP (10 mg/ml).
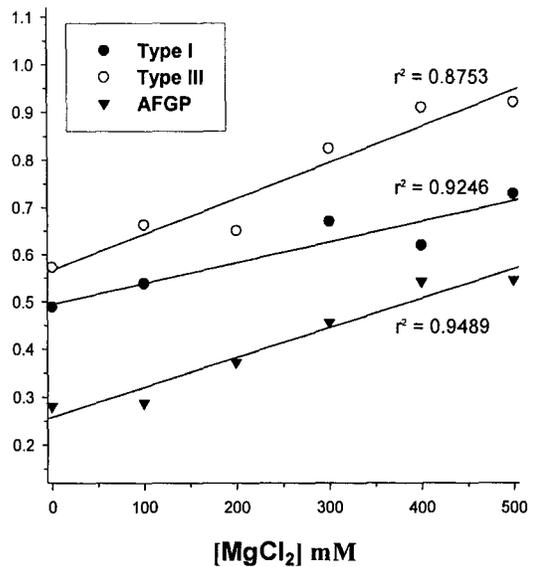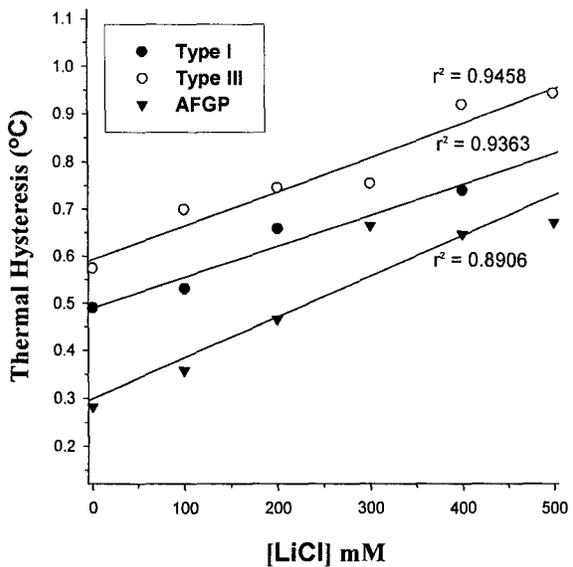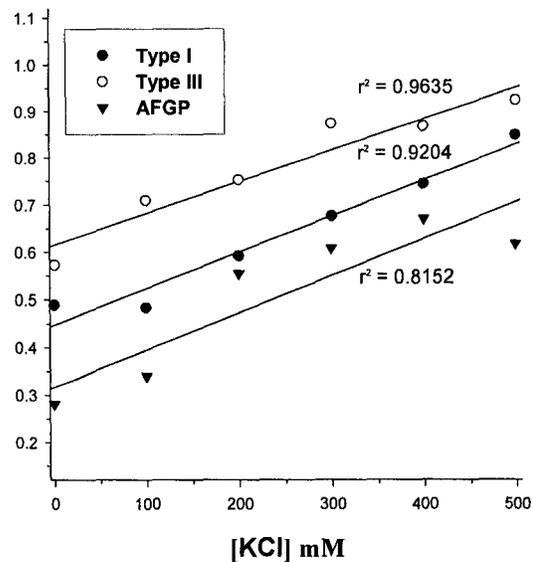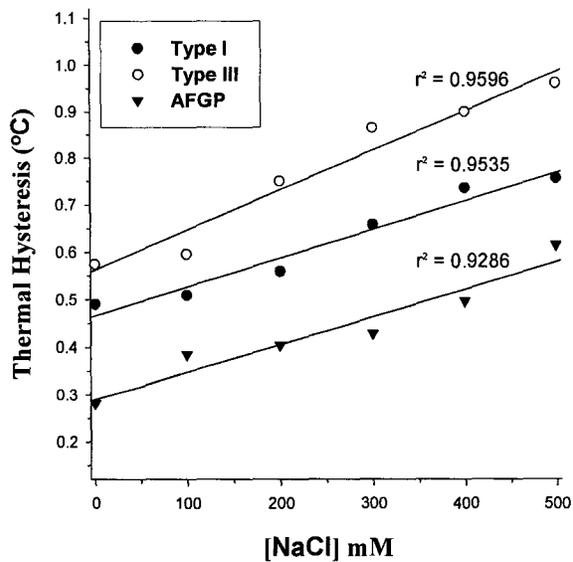
type I AFP rose by approximately 57%, type III AFP by 64% and AFGP by 118% between zero salt and the maximum concentration tested. Comparing high and low salt conditions, the increase in measured thermal hysteresis levels of type III AFP and AFGP is consistent regardless of protein concentration (Fig 3-3).

3.4 Discussion

It is evident from these data that the observed freezing point depression produced by AF(G)Ps is linearly related to the concentration of salts present in the solution. Figure 3-4 illustrates that at higher salt concentrations, colligative effects are responsible for an increasing proportion of the total freezing point depression (FDP). At 300mM, approximately 1°C (57-67%) of FPD is provided by dissolved salts while the remaining 33-43% is derived from thermal hysteresis of the AF(G)Ps. In these nonideal salt solutions, the hydration of dissolved ions reduces the number of free water molecules thereby increasing the amount of "un-freezable" water. As fewer bulk water molecules are available to join the growing ice crystal, the freezing point is lowered – a colligative effect.

The colligative explanation for the majority of increased freezing point depression agrees with a paper published by DeVries et al (1970) on the chemical properties of antifreeze glycoproteins. The authors described the effects of 50mM NaCl on the freezing point of various concentrations of *Trematomus borchgrevinki* AFGP solutions. Based on the parallel nature of the no-salt and salt added curves, they concluded that the increase in freezing point depression was entirely colligative (additive). However, the authors did not measure the effects of increasing salt concentration on antifreeze TH activity. As is

Figure 3-3. Thermal hysteresis curves of increasing type III AFP and AFGP concentrations measured in two different NaCl solutions. Best fit lines are drawn through individual salt curves using regression analysis performed with SigmaPlot™ software.

Type III AFP
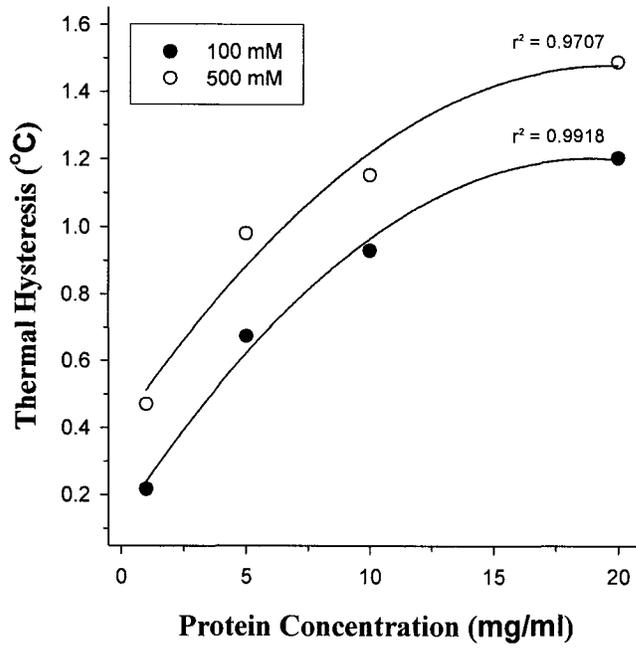
AFGP
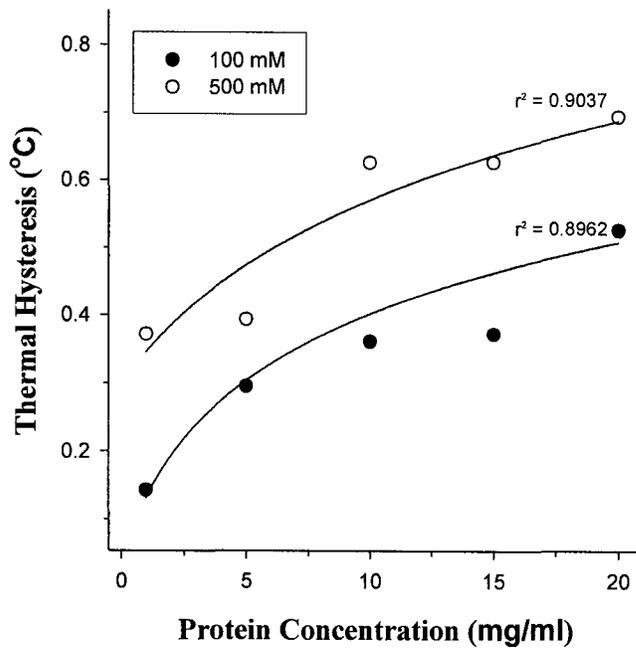
Figure 3-4. Freezing point depression of AF(G)Ps dissolved in salt solutions. The stacked bar graphs represent the proportions of FPD due to dissolved salts or AF(G)Ps as indicated. Values are averaged for the three salts used in figure 3-1. Thermal hysteresis (TH) is the combination of the no-salt portion for an AF(G)P and the extra, salt-induced, component.

Type I

Type III

AFGP

Freezing Temperature (°C)
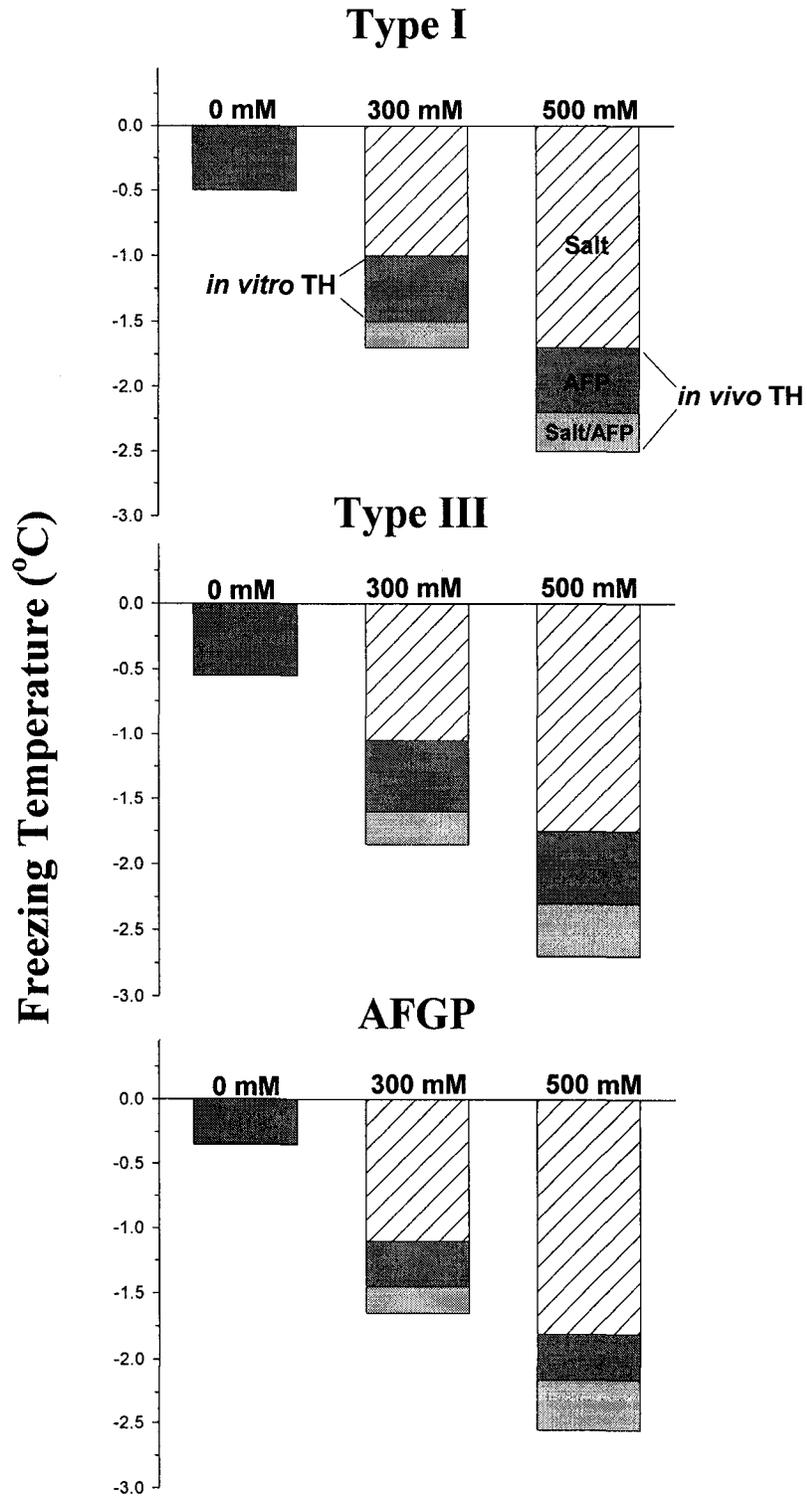
evident from these data, TH activity is compromised when measured in low ionic strength solutions, but increases as the salt concentration is elevated (see Figure 3-1). Freezing point depression can thus be divided into three components – a colligative portion produced by dissolved salts, a portion produced by AF(G)P alone (*in vitro* TH), and a portion produced by dissolved salts affecting AF(G)P (*in vivo* TH).

The effect of increasing concentrations of salt on TH activity appears to be similar for all the AF(G)Ps tested, suggesting that there is no specific interaction between the salt (dissociated ions) and the protein structure itself. The mechanism by which TH is increased in the presence of increasing concentrations of salt could be related to the effect of dissolved ions on the hydration shell surrounding the AF(G)Ps in solution. The salts used here dissociate into ions which are effective at 'salting-in' or increasing the solubility of proteins (Somero et al 1992). As the protein molecules become completely hydrated in solution (reducing the likelihood of aggregation), the protein surface area available to adsorb at the ice/water interface should ice crystals start to form is maximized. This would result in an apparent increase in thermal hysteresis activity per unit of dry protein dissolved. The increase in TH activity would presumably continue until a plateau is reached once all of the protein is fully hydrated. This outcome has been observed in beetle larvae AFP with the addition of various low molecular weight solutes (Li et al 1998). When measured in various NaCl solutions, there was an approximately 49% increase in activity at 500mM which levelled off up to 1.5M NaCl. However, it is also possible that physiologically relevant salt concentrations are required simply for correct protein functioning. Thus in a low ionic strength environment, protein structure is

compromised, TH is sub-optimal and increased salt concentration can remedy this situation. While there is evidence that even plasma TH activity is enhanced by the addition of salt (R.P. Evans and G.L. Fletcher, unpublished results) more work is needed to confirm the precise mechanism.

The current hypothesis for the *in vivo* function of antifreeze proteins in fish proposes that freeze protection is provided on two levels. Extracellular protection is supplied by AF(G)Ps which are produced in the liver, secreted into the plasma and distributed throughout the extracellular space to inhibit growth of ice within the body fluids of the fish (Fletcher et al 2001, Ewart 2002). Additional protection is provided to the external epithelial cells, in particular to gills, skin and gut, by the presence of AF(G)Ps located within the cells that are providing the first line of defense against entry of ice into the fish (Gong et al 1992, Gong et al 1996, Fletcher et al 2001, Ewart 2002, Murray et al 2002, Murray et al 2003). Murray et al (2003) also noted that in the skin of the winter flounder, AFP was present in the interstitial spaces of the skin epidermis.

In fish during winter, the concentration of plasma electrolytes can increase up to 300mM (Pearcy 1961, Fletcher 1977). As Figure 3-4 illustrates, at this salt concentration the freezing point is decreased to below approximately -1.75°C for all AF(G)Ps tested. This lowered freezing point is in the range necessary to protect fish from freeze damage during winter, should nascent ice crystals find their way into the blood stream or other extracellular fluids. The salt enhanced TH activity of AF(G)Ps appears significant physiologically since it would help fish avoid freezing without altering their electrolyte balance and causing osmotic problems.

The enhancement of low antifreeze activity by ions in solution may be particularly important in preventing ice propagation into fish from their external environment. Biological membranes have been shown to be very effective barriers to ice propagation, and experiments have demonstrated that fish skin is no exception (Valerio et al 1992). In fact, ice would not propagate across isolated winter flounder skin until a temperature considerably lower than the freezing temperature of the blood was reached, and that addition of AFPs enhanced this ability. They also suggested that for every increase in skin solute concentration, there was a doubling of the structural freezing point depression and that the solute concentration in the skin extracellular space may be higher than plasma. In tissues in immediate contact with the marine environment, elevated ion concentrations in interstitial fluids (possibly of marine origin) might be of significant importance in enhancing the freeze protection produced by AF(G)Ps at the external surface.

While those authors (Valerio et al 1992) were uncertain about the amount and location of AFPs in skin, recent studies (Murray et al 2002, Murray et al 2003) have shown that AFPs present in fish skin are localized both inside and outside cells in the epithelial layer. Results here establish that in high salt concentrations, even a low amount of AFPs would be effective in controlling ice crystal growth and therefore ice propagation into the fish. It seems that in fish skin, the effects of solutes and AF(G)Ps act in concert to provide freeze protection.

While certain studies of the AF(G)Ps require dissolution in distilled water, it is clear from the data presented here that their full thermal hysteresis activity only becomes

realized when they are dissolved in salt solutions (as in plasma). Such conditions give the most realistic picture of the way the AF(G)Ps are acting *in vivo*, and the of level of freeze protection they are capable of conferring on fish in the ocean. Thus, in order to draw conclusions between antifreeze levels, physiological levels of freeze protection, distribution within the temperature field, and survival in the wild, the antifreeze proteins and glycoproteins should be studied in solutions which better reflect the physiological environment from which they originated.

For many teleost species, the ability to survive at temperatures down to -1.8°C is unequivocal. Fish are capable of surviving in ice-laden seawater during winter and the freezing point of a fish's blood is a good measure of the freezing point of the whole animal (Fletcher et al 2001). While dissolved solutes provide ~1°C of protection in extracellular fluids, the freezing point depression is augmented with antifreeze proteins. Taken separately, these two mechanisms of freeze protection might not be sufficient to protect fish down to the lowest environmental temperatures they could face. However, the shortfall, which has long puzzled researchers in this field, can be made up *in vivo* by the addition dissolved of salts to AF(G)Ps.

CHAPTER 4:

Isolation of Antifreeze Proteins from Skin Tissues of Atlantic Snailfish,

Cunner and Sea Raven

4.1 Introduction

Previous research has shown that winter flounder and two sculpin species produce skin-type I AFPs in many epithelial tissues that are distinct from liver expressed protein (Gong et al 1996, Low et al 1998, Low et al 2001). In each case, the skin-type AFPs are synthesized as mature proteins that lack both the signal and pro-sequences typical of liver-type (plasma) proteins and thus could remain intracellular. It has been suggested that the skin-type AFPs are a widespread antifreeze class which might represent a common adaptation in many cold ocean species (Low et al 2002). Other authors further suggested that liver-type AFPs evolved from skin-type proteins (Gong et al 1996, Low et al 2002). If this hypothesis is accurate, then all fish that contain plasma AFPs should also contain evidence of skin expression or at least the remnants of an ancestral gene.

Based on the above information, it was hypothesized that skin tissue was a prime source from which to successfully clone snailfish AFP cDNAs. The initial stage in this process would require isolation of AFPs from snailfish skin followed by characterization of the purified proteins. In order to better access the distribution of skin type AFPs in general, two additional species were studied. Sea raven (*Hemitripterus americanus*), which contains type II AFP in blood plasma, was judged to be an ideal comparison species since they are closely related to sculpins and snailfish that produce type I AFPs (Scott and Scott 1988, Fletcher et al 2001). If sea raven also contains type I AFP in skin, this would help to clarify the phylogenetic distribution of skin type I AFPs.

An unrelated species, cunner (*Tautogolabrus adspersus*) is known to contain antifreeze activity in epithelial tissue but no protein has been isolated (Valerio et al

1990). The assumption has been that cunner survives in part by depending on an epidermis that is fortified by AFPs to provide a barrier to ice (Valerio et al 1990, Fletcher et al 2001). Isolation of AFPs from cunner skin would confirm the earlier results and would give credence to the argument that skin-type proteins are ubiquitous in all species producing AFPs.

4.2 Materials and Methods

4.2.1 Tissue sample collection

Twelve Atlantic snailfish (*Liparis atlanticus*) and two cunner (*Tautogolabrus adspersus*) were collected by divers near Logy Bay, Newfoundland, Canada in the winter of 2000. Two sea raven (*Hemitripterus americanus*) were collected by divers in the winter of 1998. In each case the live fish were brought into the laboratory and placed into holding tanks supplied with ambient temperature seawater. Prior to tissue collection, fish were anaesthetized using MS-222 and bled using a syringe and needle containing heparin. In the case of Atlantic snailfish and sea raven, skin epithelial tissue was peeled away from the body of the anaesthetized fish, immediately frozen in liquid nitrogen and stored at -70°C. In the case of the cunner, scales containing epithelial tissue were scraped from the body using a knife blade, frozen in liquid nitrogen and stored at -70°C.

4.2.2 Isolation and purification of skin AFPs

Frozen skin tissues were first pulverized using a mortar and pestle containing liquid nitrogen prior and homogenized in 0.1M NH_4HCO_3 using a Polytron™ homogenizer. Samples were then centrifuged at 5,000 rpm for 10 minutes; supernatants were transferred to new containers and subsequently lyophilized. The homogenates for

antifreeze activity measurements were removed both before and after lyophilization. After re-dissolving in 0.1M NH$_4$HCO$_3$, total protein was assayed using a Modified Lowry Protein Assay kit (Pierce Biotechnology, Inc.) as described by the manufacturer. Normally 1.5 ml aliquots were applied to a Sephadex G-75 gel filtration column (0.9 x 60 cm) and eluted with 0.1M NH$_4$HCO$_3$. Protein fractions that exhibited antifreeze activity were pooled and lyophilized. Following dialysis in 0.1M NH$_4$HCO$_3$, samples were further purified by reverse phase HPLC. The partially purified AFPs were separated on a Nucleosil C8 column (0.46 x 25 cm). A gradient of 35-65% acetonitrile (solvent A) and 0.1% trifluoroacetic acid (solvent B) was used with a flow rate of 1 ml/min. Individual peaks were collected, lyophilized and redissolved in 0.01M NH$_4$HCO$_3$ for activity measurements.

The purified proteins were separated on 16.5% tricine polyacrylamide gels in a Tris-Tricine buffer system (Bio-Rad Laboratories, Inc) and stained with 0.1% Coomassie® Brilliant Blue R-250 (Schagger and von Jagow 1987). A polypeptide standard (Bio-Rad Laboratories, Inc) was run in each gel to estimate the approximate molecular weight of AFPs. Amino acid analysis and mass spectrometry (MS) were performed on HPLC purified protein samples. For comparison, individual protein bands were also cut out of SDS-PAGE gels and purified using Ultrafree-MC® Centrifugal Filter Units and Zip-Tips® (Millipore Corporation) prior to amino acid and mass spectrometry analyses. Amino acid analyses and ESI-QqTOF MS were performed by the Advanced Protein Technology Centre (Hospital for Sick Children, Toronto ON).

4.2.3 Measurement of antifreeze activity

Antifreeze activity was measured as thermal hysteresis using a Clifton Nanolitre

Osmometer (Clifton Technical Physics, Hartford, NY), following the procedure of (Kao

et al 1986). Thermal hysteresis is defined as the difference between the melting and

freezing temperatures (in °C) of a test solution. Samples were dissolved in 0.1 M

NH_4HCO_3 and centrifuged before use. For each sample, measurements were made in

triplicate, and the average value taken.

4.2.4 RT-PCR and sequencing of sea raven AFP RNA

One μg of DNase-treated total RNA from sea raven skin and liver tissue was

combined with 70 pmol of an anchored poly-T primer and Superscript™ II RNase H⁻

Reverse Transcriptase (Invitrogen Canada Inc) was used to generate first strand cDNA in

a 1 hr reaction at 42°C, as described by the manufacturer. The forward and reverse

primers were designed to span an intron/exon boundary and were based on the published

sequence of sea raven type II cDNA (see Fig 4-4) (Ng et al 1986, Hayes et al 1989).

Normally, 1/10th of the RT reaction was combined with the primers and touchdown PCR

amplification was performed using ELONGase® Enzyme Mix polymerase (Invitrogen

Canada Inc) in an Eppendorf Mastercycler® thermocycler. The touchdown cycling

conditions consisted of an initial 94°C denaturing step (1 minute), followed by 10 cycles

of 94°C (15s), 72°C decreased to 60°C (15s), 72°C (60s) and 25 more cycles of 94°C

(15s), 60°C (15s), 72°C (60s). RT-PCR reaction products were separated on 1% agarose

gels and visualized using ethidium bromide.

Bands containing DNA were excised from the gel and purified using

CONCERT™ Gel Extraction System (Invitrogen Canada Inc) prior to cloning. The

pGEM®-T Easy Vector System was used to clone the purified RT-PCR products for

sequencing into a pGEM®-T Easy cloning vector, as described by the manufacturer

(Promega). Sequencing was performed on at least three independent clones using M13

Forward and M13 Reverse primers at the DNA sequencing facility in The Centre for

Applied Genomics (Hospital for Sick Children, Toronto, ON).

4.3 Results

Crude homogenates prepared from skin tissue from all three fish species

contained antifreeze activity that could be purified in order to identify the unknown

antifreeze proteins. After an initial centrifugal step to remove insoluble debris, snailfish

skin homogenate had a thermal hysteresis of 0.18 °C while cunner skin was 0.17 °C and

sea raven was 0.19 °C. The initial homogenates were then lyophilized and redissolved in

half of their original volumes. The thermal hysteresis activity of the new snailfish skin

homogenate increased to 0.26 °C, cunner to 0.22 °C and sea raven to 0.24 °C - an average

increase of 35%. The new homogenates were then used in the further purification of

AFPs.

4.3.1 Purification and analysis of snailfish skin AFP

Partially purified protein from Sephadex G-75 gel filtration chromatography

could be further resolved by HPLC into a single major peak (designated as La-sAFP) and

a few minor peaks (Fig 4-1A, B). Although all peaks were initially collected and

analyzed for the presence of antifreeze activity, only the major one contained activity.

Figure 4-1. Outline of the purification of snailfish (*Liparis atlanticus*) skin AFPs.
Panel (A) shows a typical Sephadex G-75 profile of crude skin homogenates with fractions that contained antifreeze activity indicated. (B) Sephadex G-75 purified antifreeze from skin homogenates were separated on a Nucleosil C8 column (see Methods for details). Approximately 6.4 mg of total protein was loaded onto the column with ~30% recovery from the column. The sole AFP peak found is labelled as *L. atlanticus* skin AFP (La-sAFP). Panel (C) is the SDS-PAGE separation of *L. atlanticus* skin AFP. Lane 1, pooled G-75 column fractions of skin AFPs (approx. 25 μg of total protein); Lane 2, HPLC purified protein (approx. 10 μg of protein).

A

B

La-skin

C

| | 1 | 2 | MW (kDa) |

26.6

17.0

14.3

6.5

3.5
1.4
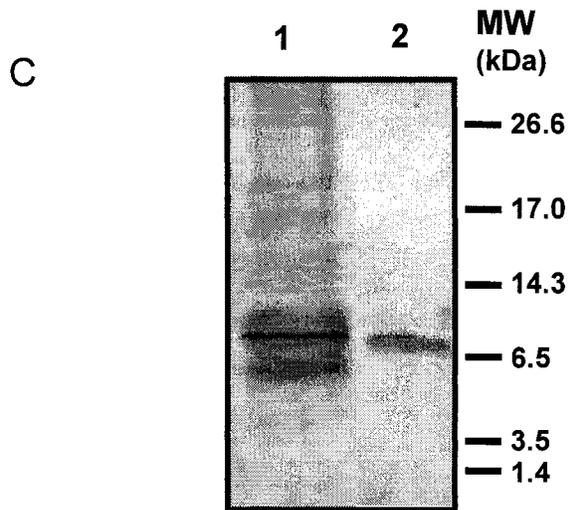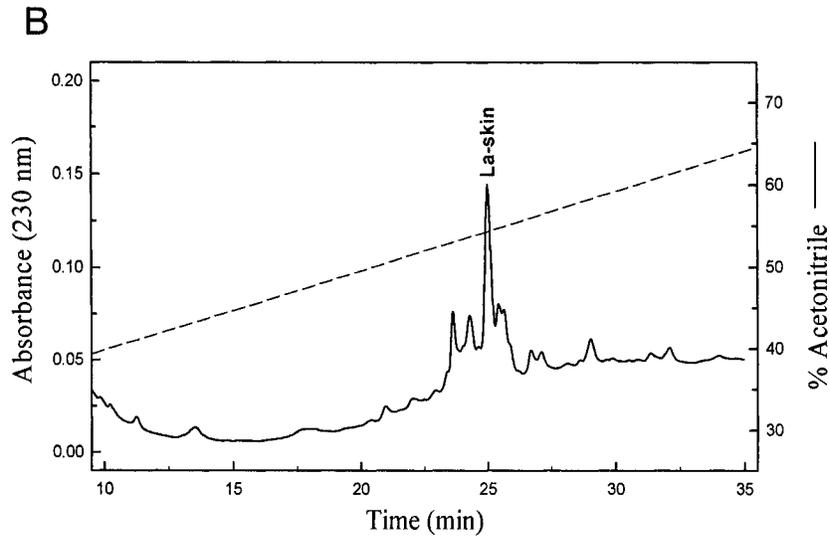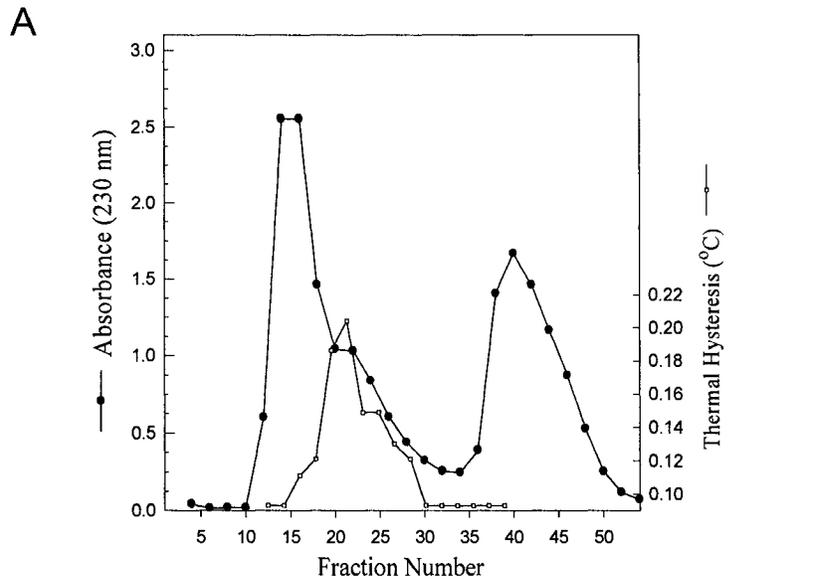
Based on the SDS-PAGE results, it appeared that the collected HPLC fraction was purified to homogeneity since there was a single band on the gel with a M_r of ~6.5 kDa (Fig 4-1C). However, analysis by mass spectrometry determined that there were actually five different proteins within the HPLC peak; two major proteins (9344 Da and 9415 Da) and three minor ones (see Table 4-1). Similar analysis of the excised SDS-PAGE band confirmed these results.

The two major skin proteins have identical molecular weights to the type I AFPs previously isolated from *L. atlanticus* plasma (Evans and Fletcher 2001; chapter 2). Previously, it was determined that these two proteins differ by a single alanine residue at their amino terminal ends and the discrepancy between electrophoretic and mass spectrometry data is likely a direct consequence of the structure of these proteins (Evans and Fletcher 2001). The amino acid content of the snailfish skin AFPs is typical of all type I AFPs in that they have very high alanine composition, around 46 mol% (Table 4-1). The abundance of alanine residues is slightly lower than the plasma AFP but the content of other amino acids such as threonine and proline were quite similar. The AFPs purified from snailfish skin give the typical hexagonal bipyramidal shape to ice crystals and these are subsequently elongated into spicule-like forms at high protein concentrations (data not shown - see chapter 2 for details).

4.3.2 Purification and analysis of cunner skin AFP

Homogenized cunner skin was initially fractionated on Sephadex G-75 gel filtration columns and the active fractions were collected and further purified using HPLC. Only a single peak collected from HPLC retained antifreeze activity, which was

Table 4-1. Amino acid composition (Mol %) and molecular mass of snailfish (*L. atlanticus*) and cunner (*T. adspersus*) skin AFPs.

Amino Acids	Snailfish (La-skin)	Cunner (Cun-skin)
ASP	5.5	5.0
GLU	4.9	1.1
SER	4.7	2.5
GLY	3.7	3.4
ARG	2.4	5.8
THR	10.8	7.3
ALA	45.9	54.1
PRO	2.9	2.8
VAL	4.9	1.8
ILE	2.1	1.2
LEU	4.1	3.1
PHE	-	1.1
LYS	4.1	8.2
Mol Mass* (Da)	9344. 9415 (major) 9457, 9387, 9501	7009, (major) 6993, 6961

*Based on ESI-MS analysis of HPLC peaks

designated as Cun-sAFP (Fig 4-2A, B). The active HPLC peak was run on SDS-PAGE

and shown to have a strong major band with a M_r of ~6.2 kDa and some very faint larger

bands indicating that the column had removed many impurities from the protein (Fig 4-

2C). Analysis by mass spectrometry determined that the HPLC peak contained a major

protein with a molecular mass of 7009 Da and two minor ones (see table 4-1). Based on

mass spectrometry determination, the molecular mass of the most prominent band

excised from the gel was identical to the major HPLC peak. The size of the cunner skin

protein is larger than all type I AFPs reported to date with the exception of snailfish

plasma/skin AFP (Evans and Fletcher 2001) and shorthorn sculpin skin AFP (Low et al

1998).

Alanine is the most prominent amino acid with just over 54 mol% of the total,

which is consistent with type I AFPs (table 4-1). Threonine and lysine levels are also

similar to type I AFPs reported before from sculpin skin and the percentage of proline

suggest that the protein contains 2 or 3 of these residues. The sequence AAAATAEAA

was determined by preliminary MS/MS sequence analysis of peptides prepared from the

excised gel band. The cunner skin AFPs produced ice crystals that had the typical

hexagonal bipyramidal shape that other fish AF(G)Ps produce.

4.3.3 Purification and analysis of sea raven skin AFP

Sea raven skin homogenates that contained antifreeze activity were initially

fractionated on Sephadex G-75 gel filtration columns and the active fractions were

collected and purified by HPLC (Fig 4-3A,B). A large HPLC peak containing antifreeze

activity, which was mixed with several smaller peaks, was collected and analyzed on an

Figure 4-2. Outline of the purification of cunner (*Tautogolabrus adspersus*) skin AFPs. Panel (A) shows a typical Sephadex G-75 profile of crude skin homogenates with fractions that contained antifreeze activity indicated. (B) Sephadex G-75 purified antifreeze from skin homogenates were separated on a Nucleosil C8 column (see Methods for details). Approximately 5.2 mg of total protein was loaded onto the column with ~30% recovery from the column. The sole AFP peak found is labelled as *T. adspersus* skin AFP (Cun-sAFP). Panel (C) is the SDS-PAGE separation of *T. adspersus* skin AFP. Lane 1, pooled G-75 column fractions of skin AFPs (approx. 25 μg of total protein); Lane 2, HPLC purified protein (approx. 10 μg of protein).

A

B

C
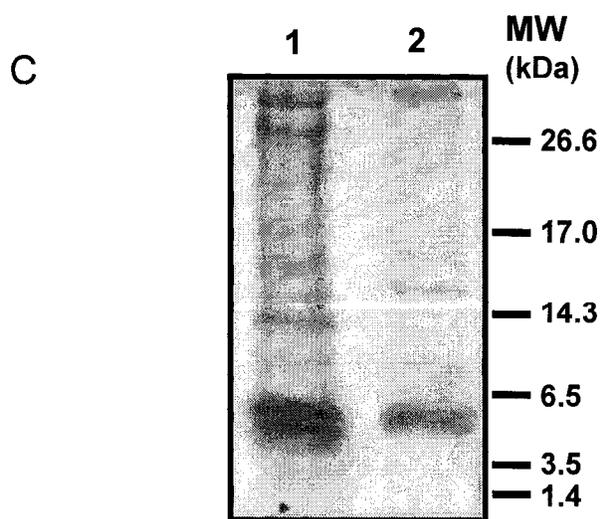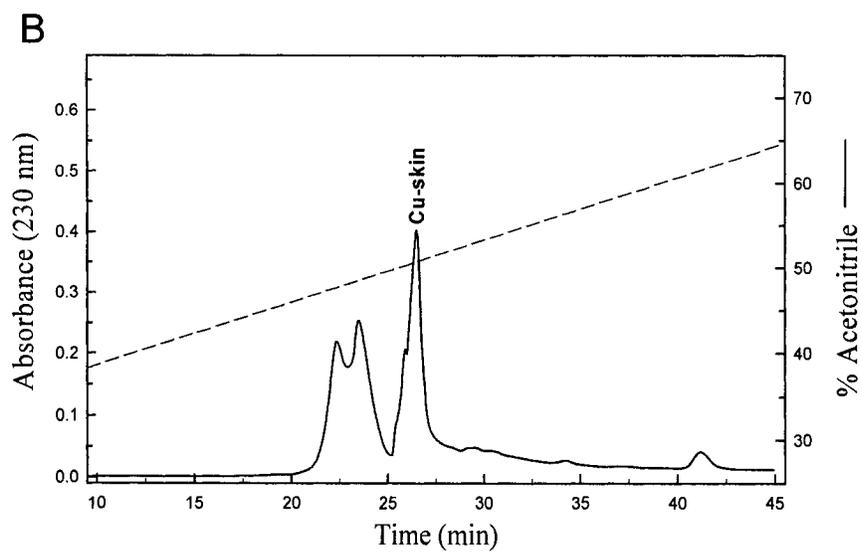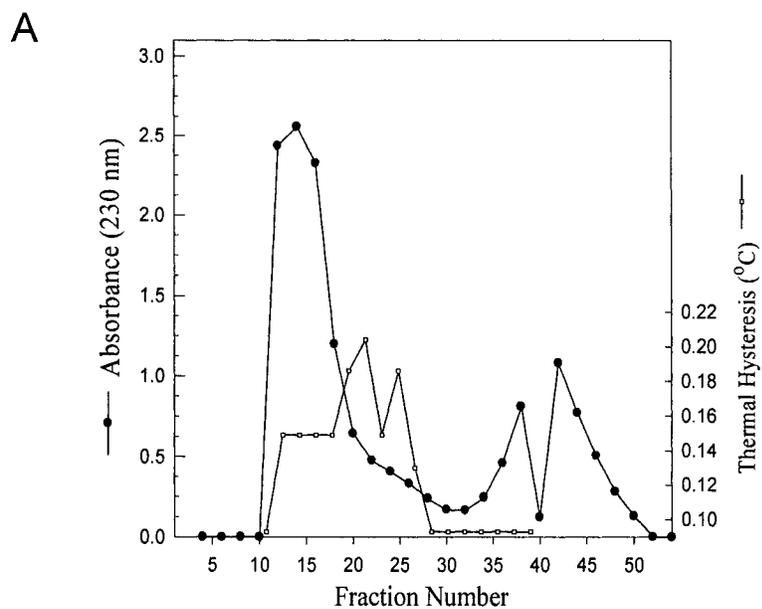
Figure 4-3. Outline of the purification of sea raven (*Hemitripterus americanus*) skin AFPs. Panel (A) shows a typical Sephadex G-75 profile of crude skin homogenates with fractions that contained antifreeze activity indicated. (B) Sephadex G-75 purified antifreeze from skin homogenates were separated on a Nucleosil C8 column (see Methods for details). Approximately 5.8 mg of total protein was loaded onto the column with ~30% recovery from the column. The sole AFP peak found is labelled as *H. americanus* skin AFP (SR-sAFP). Panel (C) is the SDS-PAGE separation of *H. americanus* skin AFP. Lane 1, pooled G-75 column fractions of skin AFPs (approx. 25 μg of total protein); Lane 2, HPLC purified protein (approx. 10 μg of protein).

A
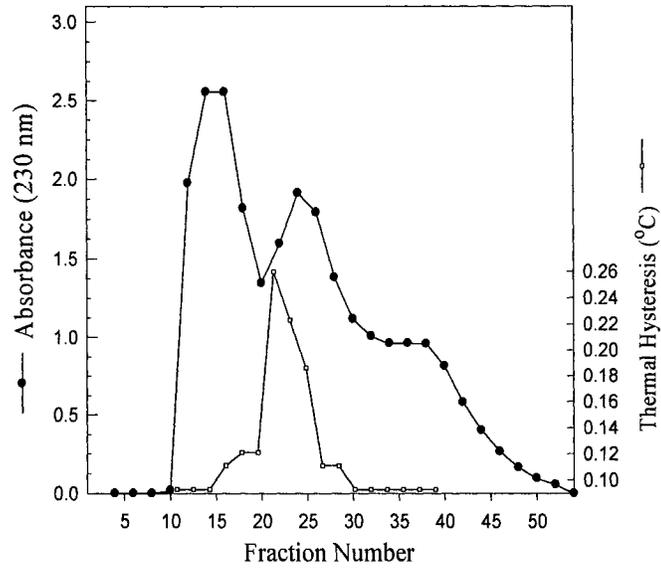
B
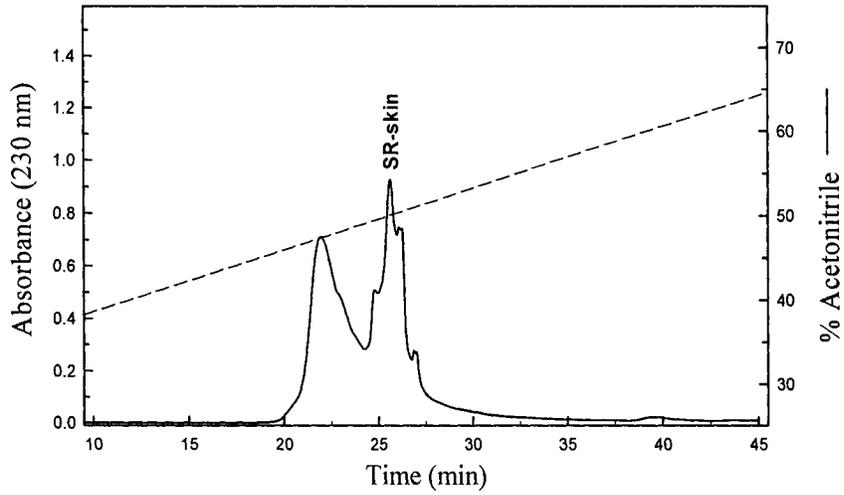
C
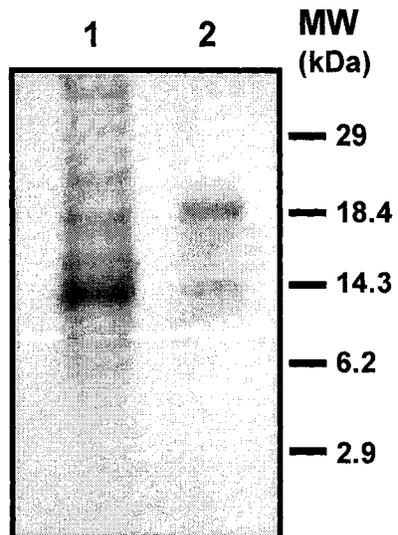
SDS-PAGE gel (Fig 4-3C). The results indicated that while impurities were removed from column fractions by HPLC there appeared to be two individual bands – a prominent one with a M_r ~18 kDa and a smaller, fainter, band of ~14 kDa. When the collected HPLC peak was analyzed by mass spectrometry, the two molecular masses were determined to be 18345 Da and 14006 Da (Table 4-2). The HPLC purified sea raven skin AFPs produced ice crystals that had modified shapes similar to previous reports for sea raven plasma AFPs. While it is likely that the gel bands correspond to true sea raven AFPs, the gel bands would need to be analyzed separately for activity before this is absolutely certain.

Results of amino acid analysis of the isolated proteins showed they had elevated cystine but only ~12% alanine. Clearly they were not type I AFPs but more resembled type II AFPs that sea raven synthesize in liver for circulation in blood plasma. When compared to the published amino acid content of the plasma type II AFP, there was considerable similarity between the skin and plasma AFPs (Table 4-2). Furthermore, it is known that the circulating AFP in sea raven is 129 amino acids long (14 kDa) and is derived from an initial 163 amino acid translation product that is 17.5 kDa (Duncker et al 1996). These two molecular masses also correspond well with the two proteins isolated from skin tissue. RT-PCR was used to determine if any AFP mRNA expressed in the skin tissue was related to the known liver sequence using primers from the published cDNA sequence (Fig 4-4A). The identity of the sea raven Type II AFP mRNA was confirmed since only two nucleotide differences were observed between the RT-PCR result and published cDNA sequence and their translation products were identical (Fig. 4-4B).

Table 4-2. Amino acid composition (Mol %) and molecular mass of sea raven (*H. americanus*) skin and liver AFPs.

Amino Acids	Sea Raven (SR-skin)	SR-liver[b] (163 AA)	SR-liver[b] (129 AA)
ASP	12.8	5.5	5.4
GLU	10.5	3.1	3.1
SER	7.3	6.7	7.0
GLY	8.7	7.4	7.8
HIS	3.3	2.5	3.1
ARG	3.4	1.8	2.3
THR	7.3	8.6	7.8
ALA	12.2	13.5	13.2
PRO	6.6	4.9	5.4
TYR	2.0	1.2	1.6
VAL	4.3	4.3	3.1
CYS	5.8	6.7	7.8
ILE	3.3	3.1	3.1
LEU	6.6	8.0	6.2
PHE	2.5	1.5	2.3
Mol Mass (Da)	18345, 14006[a]	17469[b]	13993[b]

[a]Based on ESI-MS analysis of HPLC peaks
[b]Based on published protein sequence (Ng et al 1986, Hayes et al 1989)

Figure 4-4. RT-PCR results and cDNA sequence of sea raven skin AFP mRNA.
Panel (A) lanes 1 and 2 are duplicate samples of total skin RNA; C1 and C2 are RT-PCR controls. Panel (B) sequence comparison between published sea raven type II AFP cDNA and RT-PCR results.

A
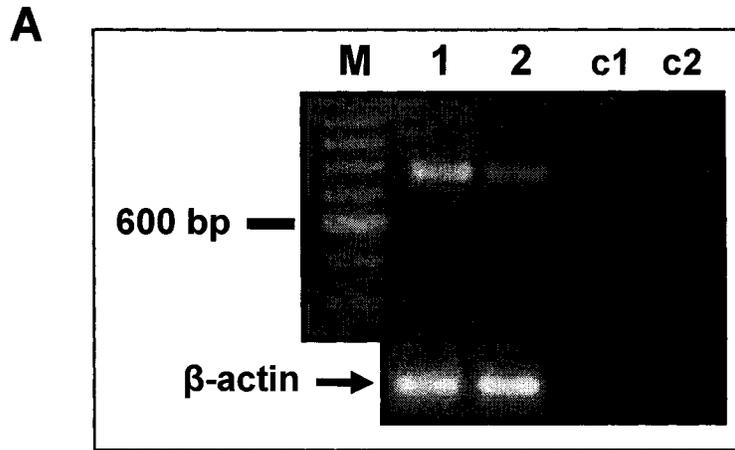
B

```
       1 (Forward Primer)CAACAGGCTGACACTGAAACAAGAG                                    70
SR-cDNA   CACACATGAATGCAGAGGCAACAGGCTGACACTGAAACAAGAGAAGATATTTCTACAGCAGGGCTATCAA
RT-PCR    ------------------..................................................

       71                                                                          140
SR-cDNA   TCATCTTCATCGTCTGCACCATCTCTACCACGAGGATGCTGACTGTGTCTCTACTGGTTTGTGCCATGAT
RT-PCR    ....................................................................

       141                                                                         210
SR-cDNA   GGCTCTGACTCAAGCTAATGATGACAAAATACTCAAAGGCACGGCTACAGAGGCTGGACCGGTCTCTCAG
RT-PCR    ....................................................................

       211                                                                         280
SR-cDNA   AGAGCCCCACCAAACTGTCCCGCTGGTTGGCAACCTCTTGGTGACCGCTGTATCTATTATGAGACAACAG
RT-PCR    ....................................................................

       281                                                                         350
SR-cDNA   CGATGACTTGGGCTCTGGCTGAGACAAACTGTATGAAATTGGGTGGACACCTTGCATCCATCCACAGCCA
RT-PCR    ....................................................................

       351                                                                         420
SR-cDNA   GGAGGAGCATAGTTTCATTCAGACCTTGAATGCTGGTGTTGTATGGATCGGAGGCTCCGCTTGCCTCCAG
RT-PCR    ....................................................................

       421                                                                         490
SR-cDNA   GCAGGTGCTTGGACCTGGTCTGATGGTACACCTATGAATTTTCGTTCCTGGTGTTCTACCAAACCTGATG
RT-PCR    ....................................................................

       491                                                                         560
SR-cDNA   ATGTACTGGCCGCGTGCTGTATGCAGATGACTGCTGCAGCTGACCAATGCTGGGATGACTTGCCTTGTCC
RT-PCR    ....................................................................

       561                                                                         630
SR-cDNA   GGCATCCCACAAATCAGTCTGCGCCATGACATTCTGAGCTAACACAGAGGCCATCCATCACACAAACACT
RT-PCR    ...G................................................................

       631                                                                         700
SR-cDNA   TTAGTGGGTGTTTGATTGTGTGTGTTCGCATACTCATCTGTGTTCGTGTCAACAGCCTCATGCTGAACCT
RT-PCR    ....................................................................

       701                                                                         770
SR-cDNA   GAAGGTTGAAAATCTCATATGACATCTTTAATTCTTTGCTATTGTTGGAGCTGCCTGAAAGGATGAGACG
RT-PCR    ....................................................................

       771                                                                         840
SR-cDNA   ACAAGAGCTGGAAAGCATCTGAGGGCTTTTAGGAAGAAATTGAATGGTTATGAAAATGATGGTCTTTTTA
RT-PCR    ...................-...............------------------------------------
                   GCATCTGAGGGCTTTTAGGAAGAAA(Reverse Primer)

       841                       874
SR-cDNA   TGTATTATGTCAAATTAAAAGGCTGACACGTTGA
RT-PCR    ----------------------------------
```

4.4 Discussion

Skin-type AFPs are thought to be a widespread class of type I AFPs, so it was decided that skin tissue would be the most appropriate source from which to isolate and characterize new snailfish AFPs. The results here confirm that snailfish skin tissue did contain antifreeze activity that could be purified by standard chromatography techniques. The purified proteins, which gave a single peak on an SDS-PAGE gel, could be characterized as type I AFPs based on their amino acid content, specifically the high levels of alanine. This is another example of a fish from the order Scorpaeniformes which expresses type I AFP in epithelial tissues. Three species were previously known to produce skin type I AFPs - winter flounder (Gong et al 1996), shorthorn and longhorn sculpins (Low et al 1998, Low et al 2001).

Unexpectedly, the two major AFPs isolated from skin had identical molecular masses to snailfish plasma AFPs (chapter 2; Evans and Fletcher 2001). Although it is practically impossible to completely avoid blood plasma contamination when isolating proteins from epithelial tissues, caution was used when removing the skin in order to minimize blood contamination. The evidence here clearly indicates that Atlantic snailfish have identical type I AFPs circulating in blood and also in skin tissues, for protection from freezing. Although it is obvious that snailfish plasma AFPs are extracellular, it is not clear whether some skin proteins remain intracellular or are exported to blood as a source of circulating AFPs (see chapter 5 for further discussion).

With the isolation and partial characterization of AFPs from cunner skin tissues, we have confirmed an earlier report from Valerio et al (1990) that cunner have antifreeze

in their skin. It is apparent that these skin AFPs have properties that would designate

them as type I. Amino acid analysis indicated that cunner skin AFPs are alanine rich and

mass spectrometry measurements show they are larger than all type I AFPs, except those

from snailfish and shorthorn sculpin skin. Moreover, their high alanine content would be

indicative of an α-helical secondary structure, which is also characteristic of type I AFP.

Cunner are from the order Perciformes, which is unrelated to the other known

orders with fish producing skin AFPs. These results provide more evidence that skin

AFPs might be ubiquitous across all teleost orders. Other data (Fletcher et al, unpublished

results) indicate that cunner also have type I AFPs circulating in their blood, although at

this time it is unclear how these are related to the skin localized protein. While the

evidence presented here is informative, it would be necessary to clone the corresponding

cDNA which codes for this protein, to determine its complete amino acid sequence. With

this sequence data, the nature of possible amino acid repeats could be used to help clarify

its relationship to other known type I AFPs from skin and the evolution of type I AFPs in

general.

Results here demonstrate that sea raven skin tissues contain antifreeze activity

that could be purified by gel chromatography and HPLC to two individual bands on an

SDS-PAGE gel. Amino acid and mass spectrometric analyses indicated that these

proteins are nearly identical to the previously identified circulating type II AFPs from sea

raven. The mature plasma sea raven AFP is 129 amino acids long (14.0 kDa) which is

derived from an initial 163 amino acid translation product (17.5 kDa) that is synthesized

in liver (Duncker et al 1996). There is a 146 amino acid (16 kDa) proAFP intermediate

stored in the liver, which is processed to mature AFP through signal peptide cleavage, during or soon after, its release into blood circulation. Given that the molecular mass of the major AFP found in skin tissue corresponds with the hepatic expressed pre-proprotein, it is unlikely to represent contaminating blood protein since no significant amount of the unprocessed AFP exists in blood (Duncker et al 1996). Furthermore, RT-PCR experiments confirmed that skin tissue does express the requisite mRNA necessary to code for the pre-proprotein. Since the primers spanned an intron/exon boundary the product generated by RT-PCR was not due to DNA contamination.

It has been assumed that sea raven type II AFPs are expressed specifically in liver tissue since a 1992 study reported that no expression of type II AFP mRNA could be detected in skin or gill tissue RNA by northern blot (Gong et al 1992). Evidence from snailfish however, indicates that there can be significant tissue variability in type I AFP mRNA expression between individual fish (chapter 5). Until expression analysis is performed on many individual sea raven using blood cells and epithelial tissues, the extent and importance of the type II expression in skin will remain unresolved. It is also not known whether the untranslated regions of the mRNA are identical or if there is any significant sequence divergence in the corresponding gene. Although the tissue contains measurable antifreeze activity, the physiological significance of the skin expressed AFP mRNA remains unknown since no northern blots were performed to determine the relative levels of expression. Data from chapter 3 shows that even a small quantity of AFPs can be effective at controlling ice growth since their activity is enhanced by co-occurring salts.

This is the first clear report of the isolation of a class – other than type I – of AFP from skin tissues and the second example of a fish having identical plasma and epithelial AFPs. These data provide more evidence that skin tissue is a common site of AFP expression. Closely related species to sea raven, sculpins and snailfish, also synthesize AFPs in skin tissue. Until now, the paradigm has been that skin AFPs are expressed as mature proteins by an independent set of genes different from the liver multigene family that express plasma AFPs. The data presented here demonstrate that AFPs can also be expressed in skin tissues that include pre-pro sequences similar to liver-type proteins. While it is clear that unprocessed protein remains in skin tissue, it is also possible that the signal sequences are cleaved such that some protein is exported into blood. The expression of antifreeze genes in sea raven is not as simple as originally believed in that these fish utilize additional means to bolster their antifreeze complement for protection from freezing during winter. Although the precise physiological function of epithelia expressed AFPs has not been unequivocally established, they likely act as an additional extracellular barrier to ice-crystal propagation into peripheral tissues of fish (see chapter 5 for further discussion).

CHAPTER 5:

Snailfish Type I AFPs Expressed in Skin Tissues are Identical to Circulating Plasma Proteins

5.1 Introduction

In 1996, a novel class of type I AFPs was isolated and characterized from the skin of winter flounder (Gong et al 1996). These AFPs, which are encoded by a separate subset of genes from liver expressed proteins, were designated as skin-type AFPs. Generally, these proteins are synthesized as mature polypeptides that lack both signal and prosequences which suggests that skin-type AFPs remain intracellular (Gong et al 1996). Recently, additional type I AFPs have been isolated from skin tissues of 2 closely related species of sculpin, shorthorn and longhorn (Low et al 1998, Low et al 2001).

The characterization of known skin-type AFPs and the presence of antifreeze activity in skin tissues of other species has led to the hypothesis that skin-type AFPs are the widespread ancestors of liver-type (plasma) AFPs. The discovery that snailfish plasma and skin tissues contain identical antifreeze proteins was entirely unexpected (see chapter 4). Further analysis of the snailfish AFPs would be helpful in determining the function and deciphering the evolutionary relationships of skin type I AFPs in general. Pursuant to this, a snailfish skin cDNA library was constructed and screened using type I AFP cDNAs from shorthorn sculpin skin. This method was used in combination with other molecular techniques to generate insight into the expression of AFPs in snailfish tissues.

5.2 Materials and Methods

5.2.1 Tissue sample collection

Twelve Atlantic snailfish (*Liparis atlanticus*) were collected by divers near Logy Bay, Newfoundland, in the winter of 2000. Two specimens of dusky snailfish (*Liparis*

gibbus) were collected from Placentia Bay, Newfoundland during the winter of 1999. Live fish were brought into the laboratory and placed into holding tanks supplied with ambient temperature seawater. Prior to tissue collection, fish were anaesthetized using MS-222 and bled using a heparin containing syringe and needle. Tissues were removed from anaesthetized fish, immediately frozen in liquid nitrogen and stored at -70°C.

5.2.2 Skin library construction and screening

Total RNA from Atlantic snailfish skin tissue was isolated using TRIzol® reagent (Invitrogen Canada Inc.) as described by the manufacturer. Poly A$^+$ mRNA was isolated from total RNA using an Oligotex mRNA Kit (QIAGEN) as described by the manufacturer. A skin cDNA library was constructed, as described by the manufacturer, using Lambda ZAP® II library and ZAP-cDNA® Synthesis Kits and Gigapack® Gold III packaging extracts (Stratagene, La Jolla, CA). The primary skin cDNA library contained around 5×10^5 clones. Normally, ~ 50,000 plaques were grown on 15 cm NZYCM plates for primary screening while 9 cm plates were used in secondary and tertiary screens.

Hybond-N nylon membranes (Amersham Biosciences) were prepared and screened according to the manufacturer. Briefly, membranes were hybridized at 42°C overnight in the following buffer: 5X SSC, 5X Denhardt's, 0.5% SDS, 50% formamide and 100 µg/ml calf thymus DNA. Probe was labelled with ^{32}P-dCTP using an All-in-One Random-Primed Labelling Mix (Sigma-Aldrich) and purified prior to use with ProbeQuant G-50 Micro Columns (Amersham Biosciences). The final wash was performed in 1.0X SSC, and 0.1 % SDS, at 52°C for 20 minutes. A 300 bp DNA

fragment corresponding to the ORF (open reading frame) of shorthorn sculpin skin (s3-2) clone (Low et at, 1998) was used as a probe to screen approximately 2.0 x 10^5 clones of the primary cDNA library. Positive plaques were first isolated and then pBluescript® phagemids, to be used for sequencing inserts, were produced using an *in vitro* excision protocol (Stratagene, La Jolla, CA).

5.2.3 Northern blot analysis

Total RNA from various tissues of Atlantic and dusky snailfish were isolated using TRIzol® reagent (Invitrogen Canada Inc.) as described by the manufacturer. Five µg aliquots of total RNA were separated on 1% formaldehyde gels and were analyzed by a non-radioactive northern blotting procedure using positively charged nylon membranes (Roche Diagnostics Corporation). RNA was transferred to membranes using a VacuGene XL Vacuum Blotting System (Amersham Biosciences) and cross-linked with UV light. The membrane was hybridized at 50°C overnight in DIG Easy Hyb buffer (Roche Diagnostics Corporation). Probe was labelled with DIG-11-dUTP using a DIG-High Prime DNA Labelling kit or in some cases with a PCR DIG Probe Synthesis Kit (Roche Diagnostics Corporation) with chemiluminescent signal detection using CDP-Star®. The final wash was performed in 0.1X SSC, and 0.1 % SDS, at 50°C for 2x 15 min. A 175 bp DNA fragment corresponding to the 3' UTR (untranslated region) of the skin clone was used as a probe.

5.2.4 Southern blot analysis

Genomic DNA was isolated from liver of Atlantic and dusky snailfish using a Wizard® Genomic DNA Purification Kit (Promega Corporation). Aliquots of RNAse A

treated genomic DNA were digested with various restriction endonucleases (Invitrogen). Five μg aliquots of the digestion products were separated in a 0.8% agarose gel, transferred to positively charged nylon membranes (Roche Diagnostics Corporation) using a VacuGene XL Vacuum Blotting System (Amersham Biosciences) and cross-linked with UV light. A chemiluminescent based non-radioactive method was used to detect sequences on the membrane (Roche Diagnostics Corporation). Briefly, the membrane was hybridized at 42°C overnight in DIG Easy Hyb buffer (Roche Diagnostics Corporation). Probe was labelled with DIG-11-dUTP using a PCR DIG Probe Synthesis Kit (Roche Diagnostics Corporation) with chemiluminescent signal detection using CDP-Star®. The final wash was performed in 0.5X SSC, and 0.1 % SDS, at 65°C for 2x 15 minutes. A 175 bp DNA fragment corresponding to the 3' UTR of the skin clone was used as a probe. Autoradiography was performed as described in the northern blot section.

5.2.5 RACE procedure

Both 5'- and 3'-RACE reactions were performed using the RNA ligase-mediated GeneRacer™ Kit, as described by the manufacturer (Invitrogen Canada Inc). One μg of DNase treated total RNA combined with Thermoscript™ Reverse Transcriptase (Invitrogen Canada Inc) was used to generate adapter-linked first strand cDNA for 1 hr in a 50°C reaction. The first strand cDNA was combined with the appropriate primers and touchdown PCR amplification was performed using DyNAzyme EXT™ DNA polymerase (Finnzymes Oy) in an Eppendorf Mastercycler® thermocycler. The touchdown cycling conditions consisted of an initial 95°C denaturing step (2 minutes),

followed by 10 cycles of 94°C (15s), 72°C decreased to 60°C (15s), 72°C (60s) and 25 more cycles of 94°C (15s), 60°C (15s), 72°C (60s). In order to obtain a product in most reactions, DMSO was added at 10% (v/v) which allows the GC base pairs to melt at a lower temperature. RACE reaction products were separated on 1% agarose gels and then purified using a spin columns provided in the kit GeneRacer™ Kit or by CONCERT™ Gel Extraction System (Invitrogen Canada Inc), as described by the manufacturer. A TOPO TA Cloning® kit was used to clone the purified RACE products for sequencing into a pCR®4-TOPO cloning vector (Invitrogen Canada Inc). At least three independent clones were isolated and the purified plasmids sequenced.

5.2.6 RT-PCR analysis

One μg of DNase treated total RNA from each of the specified tissues was combined with 70 pmol of an anchored poly-T primer. Thermoscript™ Reverse Transcriptase (Invitrogen Canada Inc) was then used to generate first strand cDNA in a 1 hr reaction at 50°C, as described by the manufacturer. Normally, 1/10th of the RT reaction was combined with the appropriate primers and touchdown PCR amplification was performed using DyNAzyme EXT™ DNA polymerase (Finnzymes Oy) in an Eppendorf Mastercycler® thermocycler. The touchdown cycling conditions consisted of an initial 95°C denaturing step (2 min), followed by 10 cycles of 94°C (15s), 72°C decreased to 60°C (15s), 72°C (60s) and 25 more cycles of 94°C (15s), 60°C (15s), 72°C (60s). RT-PCR reaction products were separated on 1% agarose gels and visualized using ethidium bromide.

5.2.7 DNA sequencing

Sequencing was performed on the pBluescript® phagemids or pCR®4-TOPO

plasmids using T3 and T7 primers at the DNA sequencing facility in The Centre for

Applied Genomics (Hospital for Sick Children, Toronto, ON).

5.2.8 Bioinformatics Programs

Homologous nucleotide and protein sequences were searched through **BLAST**

searches on the NCBI web server (www.ncbi.nlm.nih.gov/BLAST/). The NCBI **ORF**

Finder was utilized to identify putative open reading frames in the nucleotide sequences

(www.ncbi.nlm.nih.gov/gorf/gorf.html). Helical net and helical wheel diagrams were

constructed using EMBOSS package located on the Canadian Bioinformatics Resource

web page (www.cbr-rbc.nrc-cnrc.gc.ca/index_e.php). Swiss PDB software used to

generate 3D model of Las-AFP. ClustalX and TreeView (1.6.1) software were used to

create un-rooted neighbour joining trees.

5.3 Results

5.3.1 cDNA Library screening and cloning of snailfish skin AFP

After establishing the presence of AFPs in the skin of snailfish (chapter 4), a

cDNA library was constructed to investigate the presence of corresponding mRNA in

skin tissues. Approximately half of the primary library was initially screened using the

ORF of a shorthorn sculpin skin clone, s3-2, as a probe under low stringency conditions.

Following the tertiary screen, two independent clones were identified and these

phagemids were sequenced. The ~260 bp clones (las-c1 and las-c2) contained identical

sequences, apart from a small difference in the length of polyA tail and a few nucleotides

at their 5' ends. However, after closer inspection the clones appeared to be truncated versions of complete type I AFP messages. As indicated in figure 5-1, the sequence translated in one reading frame generates a mere 26 amino acid peptide, which is alanine-rich, but lacks the obligatory in-frame start codon. The sequence information was then used in 5'-RACE reactions to ascertain the remainder of the skin AFP cDNA sequence.

During the initial optimization of 5'-RACE, numerous primer sets were designed and utilized in reactions. Due to excessive GC content in this particular RNA species, the standard homopolymer tailing RACE method (Gibco-BRL) and the SMART™ system (BD Biosciences) proved ineffective since smeared products were frequently obtained. Even when single bands could be excised for analysis, the sequences were completely unrelated to AFP. Furthermore, many enzyme combinations and chemical additives failed to give acceptable results. A technique based on RNA ligase-mediated RACE (GeneRacer™) was used successfully in combination with a DNA polymerase specialized for GC rich templates (with 10% DMSO) to clone the remaining 5' portion of the snailfish AFP cDNA. The full L. atlanticus skin cDNA is 568 bp long and contains a complete 342 bp ORF (Fig 5-1). The ORF encodes an alanine-rich protein of 113 residues and was designated as Las-AFP (Liparis atlanticus skin AFP). The putative start and stop codons are underlined as well as three possible polyadenylation signal sequences (Graber et al 1999).

Figure 5-1. Nucleotide sequence and primary translation product of *Liparis*
atlanticus **skin AFP cDNA.** The ORF is in lower case letters while the 5' and 3' UTRs
are in non-bold capitalized letters. The putative start and stop codons are underlined in
bold and the three possible polyadenylation signal sequences. The sequence obtained
from the initial las-c1 and las-c2 cDNA clones are underlined. RT-PCR or RACE primer
sequences are shown above (5' → 3') or below (3' → 5') the nucleotide sequence.

Las-AFP

```
  1 CATGGACTGAAGGAGTAGAAAACTAACAGAAGGAGAAACAGAAAGAACatggccgctgca 60
  1                                                       M   A   A   A   4

 61 accccccgcccagagagccgctgccactgccactgctgccgccgccgccgccgcttccgcc 120
  5 T   P   A   Q   R   A   A   A   T   A   T   A   A   A   A   A   A   S   A   24

    (LaS-RT Forward primer) ccgccagcaccgccgccaaagtctct
121 gccgctgccgccgcagccaccaccgccagcaccgccgccaaagtctctgccggtgcagct 180
 25 A   A   A   A   A   A   T   T   A   S   T   A   A   K   V   S   A   G   A   A   44

181 gccaccgccgctgccgctgtcgtcgccgccaaaaacgctgccaccgccgttgccccaac 240
 45 A   T   A   A   A   A   V   V   A   A   K   N   A   A   T   A   V   A   P   N   64

241 accggggccatcaccgccgccaccgctgcctctgccaccgccgccgccgccgccaaagcc 300
 65 T   G   A   I   T   A   A   T   A   A   S   A   T   A   A   A   A   K   A   84

301 gcccaggccaccgccgacgccgctgccaccaaagccgccgcagccgctgtgacctccaaa 360
 85 A   Q   A   T   A   D   A   A   A   T   K   A   A   A   A   V   T   S   K   104

361 gccgccgccgctgccctcgccgccctttaaAGGGAAACCCAAAGCAGGACATTTTATCAG 420
          ccgccgctgccctcgccgcccttta (LaS-5'RACE/RT reverse primer)
105 A   A   A   A   A   L   A   A   L                                           113

421 TGGCCTCAAGTGAGCTTCGGTTTAGTTGGGATCATGTGTGTTTTCCTGTATTATGATTATTG 480

481 TAGTGGCCATTTGTCCGTCCAAAATCAACAAACAATAATCTTAATCAAACTGCCACTGAT 540

541 GCACCGGGGTCTCGGTGTTGACGCACGCA(n)570
```

The Las-AFP sequence data were utilized to design appropriate RT-PCR and 3' -

5' RACE primers for de-novo cloning of AFP sequence from dusky snailfish skin RNA.

Initial RT-PCR experiments, using primers designed from Las-AFP 3'-UTR, indicated

that there was a similar sized product in dusky snailfish skin (data not shown). The 3'-

RACE procedure (primers indicated in Fig 5-2) produced a single band that was

approximately 450 bp while 5'-RACE gave a 370 bp product. The overlapping sequences

were combined into a 587bp clone which contained a 342 bp ORF that encodes a 113

residue, alanine-rich, protein designated as Lgs-AFP (Liparis gibbus skin AFP). The

putative start and stop codons are underlined in the figure along with the standard

polyadenylation signal sequence.

The AFP cDNAs cloned from the skin tissues of Atlantic and dusky snailfish have

strikingly similar nucleotide sequences that encode nearly identical proteins (Fig 5-3). A

few minor nucleotide substitutions and insertions in the ORF translate into four amino

acids differences at the carboxy terminus of the proteins. However, there is a 19 bp

insertion in Lgs-AFP 3'-UTR just before the polyA tail region. The snailfish AFP cDNA

sequences are similar to skin-type AFPs from winter flounder and sculpins in that they do

not appear to contain signal sequence nor prosequences (Gong et al 1996, Low et al 1998,

Low et al 2001). Surprisingly, snailfish express AFPs in skin tissues that are identical to

those found in their blood plasma. The amino acid composition of proteins purified from

Atlantic snailfish skin tissue is quite similar to AFP predicted from the cDNA sequence

(Table 5-1). Any differences could be attributed to variations in analytical procedures and

the fact that mixtures of AFPs were analyzed. Most importantly, the predicted molecular

Figure 5-2. Nucleotide sequence and primary translation product of *Liparis gibbus* **skin AFP cDNA.** The ORF is in lower case letters while the 5' and 3' UTRs are in non-bold capitalized letters. The putative start and stop codons and the polyadenylation signal are underlined. RT-PCR or RACE primer sequences are shown above (5' → 3') or below (3' → 5') the nucleotide sequence.

Lgs-AFP

```
  1 CATGGACTGAAGGAGTAGAAAACTAACAGAAGGAGAAACAGAAAGAACatggccgctgct 60
  1                                                 M  A  A  A   4

 61 accccgcccagagagccgctgccactgccactgctgccgccgccgccgcttccgcc 120
  5 T  P  A  Q  R  A  A  A  T  A  T  A  A  A  A  A  A  S  A  24

    (LgS-3'RACE/RT Forward) ccgccagcaccgccgccaaagtctct
121 gccgctgccgccgcagccaccaccgccagcacagccgccaaagtctctgccggcgccgct 180
 25 A  A  A  A  A  A  T  T  A  S  T  A  A  K  V  S  A  G  A  A  44

181 gccaccgccgctgccgctgtcgtcgccgccaaaaacgctgccaccgccgttgcccccaac 240
 45 A  T  A  A  A  A  V  V  A  A  K  N  A  A  T  A  V  A  P  N  64

241 accggggccatcaccgccgccaccgctgcctctgccaccgccgccgccgccgccaaagcc 300
 65 T  G  A  I  T  A  A  T  A  A  S  A  T  A  A  A  A  A  K  A  84

301 gcccaggccaccgccgacgccgctgccaccaaagccgccgcagccgctgtgacctccaaa 360
 85 A  Q  A  T  A  D  A  A  A  T  K  A  A  A  A  V  T  S  K  104

361 gccgccgccgcgctgcctcgccgcctttaaAGGGAAACCAAAGCAGGACATTTTATCAGT 420
    ccgccgctgccctcgccgccctta (LgS-5'RACE/RT reverse)
105 A  A  A  A  L  P  R  R  L                                 113

421 GGCCTCAAGTGAGCTTCGGTTTAGTTGGGATCATGTGTTTTCCTGTATTATGATTATTGT 480

481 AGTGGCCATTTGTCCGTCCAAAATCAACAAACAATAATCTAAATCAAACTGCCACTGATG 540

541 CACCGTGGGTCTCTGTGTTGTTGCACGCAAAATAAAACAATGTCCCGA(n)588
```

Figure 5-3. Nucleotide and amino acid sequence alignments of Atlantic and dusky snailfish type I AFPs. Panel (A) is nucleotide sequence alignment and (B) amino acid alignment. Dusky snailfish (Lgs-AFP) sequence which is identical to Atlantic (Las-AFP) sequence are indicated by dots. Mismatched Lgs-AFP nucleotides or amino acids are written below Las-AFP sequences.

A

```
         1                                                                    70
LaS  CATGGACTGAAGGAGTAGAAAACTAACAGAAGGAGAAACAGAAAGAACATGGCCGCTGCAACCCCCGCCC
LgS  ....................................................................T..........

         71                                                                   140
LaS  AGAGAGCCGCTGCCACTGCCACTGCTGCCGCCGCCGCCGCCGCCGCTTCCGCCGCCGCTGCCGCCGCAGCCAC
LgS  ............................................................................

         141                                                                  210
LaS  CACCGCCAGCACCGCCGCCAAAGTCTCTGCCGGTGCAGCTGCCACCGCCGCTGCCGCTGTCGTCGCCGCC
LgS  ............A....................C..C................................

         211                                                                  280
LaS  AAAAACGCTGCCACCGCCGTTGCCCCCAACACCGGGGCCATCACCGCCGCCACCGCTGCCTCTGCCACCG
LgS  ....................................................................

         281                                                                  350
LaS  CCGCCGCCGCCGCCAAAGCCGCCCAGGCCACCGCCGACGCCGCTGCCACCAAAGCCGCCGCAGCCGCTGT
LgS  ....................................................................

         351                                                                  420
LaS  GACCTCCAAAGCCGCCGCCGC---TGCCCTCGCCGCCCTTTAAAGGGAAACCCAAAGCAGGACATTTTAT
LgS  .......................-GC....-.........-.................-.................

         421                                                                  490
LaS  CAGTGGCCTCAAGTGAGCTTCGGTTTAGTTGGGATCATGTGTTTTCCTGTATTATGATTATTGTAGTGGC
LgS  ....................................................................

         491                                                                  560
LaS  CATTTGTCCGTCCAAAATCAACAAACAATAATCTTAATCAAACTGCCACTGATGCACCG-GGGTCTCGGT
LgS  ...........................................A..........................T.......T..

         561                                       611
LaS  GTTGACGCACGC------------------AAAAAAAAAAAAAAAAAAAA
LgS  ....TT......AAAATAAAACAATGTCCCG....................
```

B

```
         1                                                                    70
LaS  MAAATPAQRAAATATAAAAAAASAAAAAAATTASTAAKVSAGAAATAAAAVVAAKNAATAVAPNTGAITA
LgS  ....................................................................

         71                                     113
LaS  ATAASATAAAAAKAAQATADAAATKAAAAAVTSKAAAAALAAL
LgS  .................................LPRR.
```

Table 5-1. Amino acid composition (Mol %) and molecular mass of *L. atlanticus*, type I AFPs.

Amino Acids	LaP-AFP (protein)	LaS-AFP (protein)	LaS-AFP (cDNA)
ASP	3.6	5.5	0.9
GLU	3.0	4.9	0.0
SER	2.8	4.7	4.5
GLY	4.6	3.7	1.8
ARG	1.6	2.4	0.9
THR	10.3	10.8	13.4
ALA	58.8	45.9	61.6
PRO	2.5	2.9	1.8
VAL	5.6	4.9	4.5
ILE	1.3	2.1	0.9
LEU	2.6	4.1	1.8
LYS	3.4	4.1	4.5
Mol Mass (Da)	9344, 9415*	9344, 9415* 9457, 9387 9501	9415**

*Based on ESI-MS analysis of HPLC peaks
**Predicted from cDNA sequence excluding Met
LaP-AFP = *Liparis atlanticus* plasma AFP; LaS-AFP = *L. atlanticus* skin AFP

Table 5-2. Amino acid composition (Mol %) and molecular mass of *L. gibbus*, type I AFPs.

Amino Acids	LgP-AFP1 (protein)	LgP-AFP2 (protein)	LgS-AFP (cDNA)
ASP	5.4	5.5	0.9
GLU	2.6	2.6	0.0
SER	2.0	2.0	4.4
GLY	3.9	3.9	1.8
ARG	1.8	0.9	2.7
THR	8.9	9.0	13.3
ALA	51.2	51.7	58.4
PRO	4.2	4.2	2.7
VAL	8.4	8.5	4.4
ILE	1.7	1.8	0.9
LEU	2.3	2.3	1.8
LYS	6.6	6.6	4.4
Mol Mass (Da)	9646* 9514, 9814	9573, 9742*	9742**

*Based on ESI-MS analysis of HPLC peaks
**Predicted from cDNA sequence including Met

mass and amino terminal sequence (see chapter 2) for Las-AFP is identical to the isolated

plasma proteins. Dusky snailfish also express the same type I AFP in skin tissue that is

circulating in their blood (Table 5-2).

5.3.2 Analysis of snailfish AFP genes

Northern blots were performed to access the tissue distribution of snailfish AFP

mRNA. A 175 bp fragment from the 3'-UTR of Las-AFP was used to probe total RNA

from Atlantic snailfish tissues (Fig. 5-4A). A very strong specific band is clearly visible

even after short exposure times in skin tissue and a fainter band from gill begins to be

detectable with longer exposures. No other tissues gave detectable signals on this

northern blot. When this experiment was repeated using another fish, the same banding

pattern was detected except that there was a definite detectable signal in liver tissue RNA

(Fig. 5-4B). The more sensitive RT-PCR technique performed using the same RNA (with

ORF primer set) illustrates that positive bands are clearly visible for skin, gill, blood

cells, kidney, spleen and liver but are not evident for any other tissues tested (Fig. 5-4C).

Additional RT-PCR reactions, using a set of primers designed within the 3' UTR of Las-

AFP, showed the same pattern of expression in tissues that the ORF primer set showed

(data not shown).

Additional northern experiments were performed to further examine possible

individual variation of AFP mRNA levels. Using the same probe DNA, skin tissues from

4 Atlantic snailfish and a dusky snailfish gave very intense autoradiographic signals (Fig

5-5A) which were confirmed by RT-PCR analysis (Fig 5-5B). The northern experiment

was repeated using liver RNA from eight individual Atlantic snailfish and a dusky

Figure 5-4. Tissue distribution of Atlantic snailfish skin AFP mRNA. Panels (A) and (B) are northern blots of samples from two individual fish with lanes labelled with RNA tissue source. Blots were probed with snailfish AFP UTR sequence. Panel (C) is a typical result of RT-PCR analysis. Numbers correspond with the tissue labels from the northern blots.
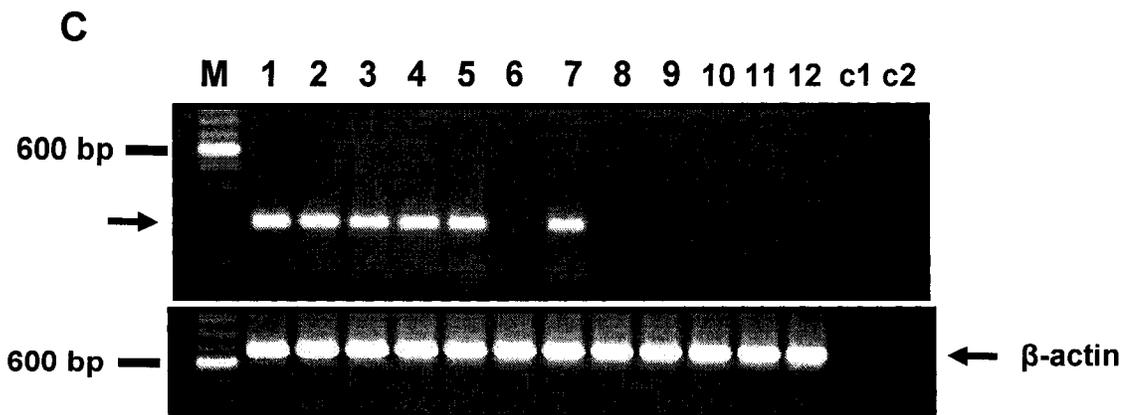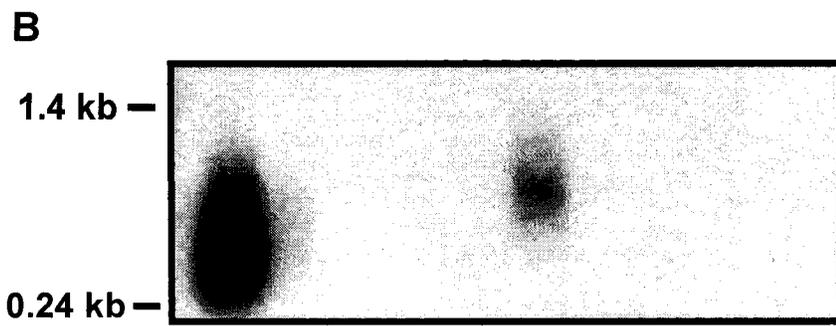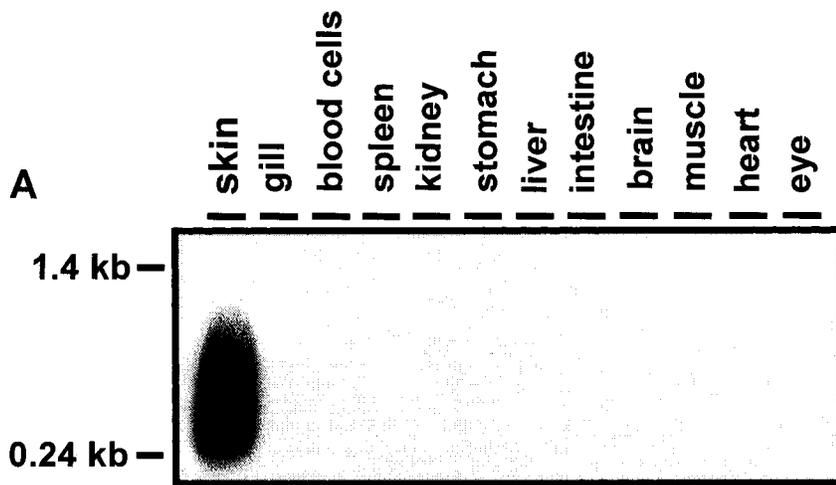
Figure 5-5. Distribution of snailfish AFP mRNA in skin tissues of 5 sample fish.
Panel (A) Northern blot analysis from four individual Atlantic snailfish and one dusky
snailfish. Blots were probed with snailfish AFP UTR sequence. Panel (B) is the
corresponding RT-PCR results from the same tissue samples.

A

L. atlanticus | Lg

Kb | 1 | 2 | 3 | 4 | 1

1.05 —

0.58 —

B

La | Lg

M | 1 | 2 | 3 | 4 | 1 | c1 | c2

600 bp —

→

600 bp — ← β-actin
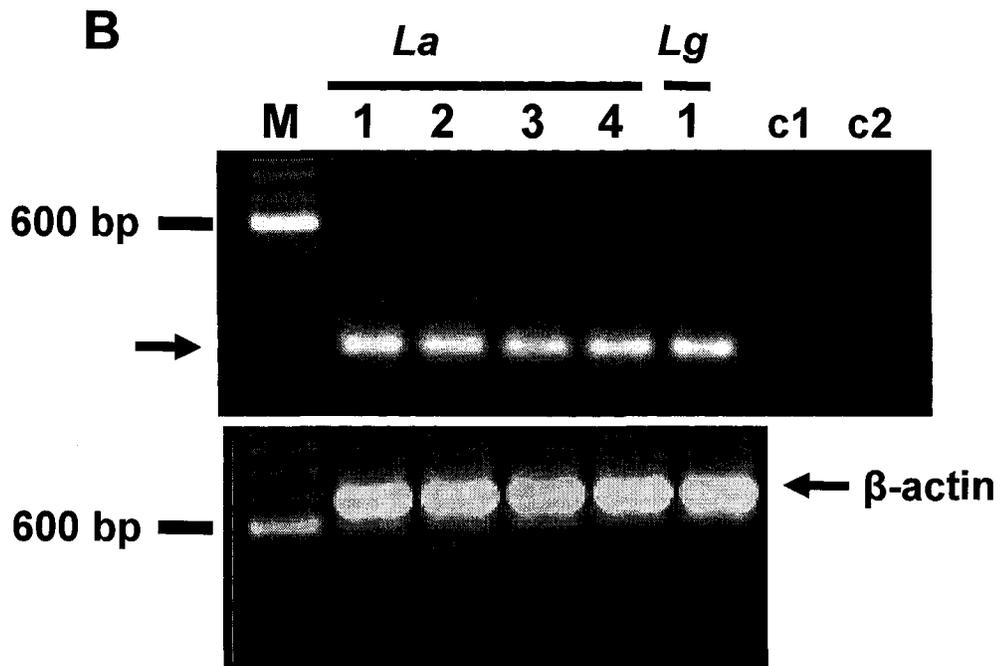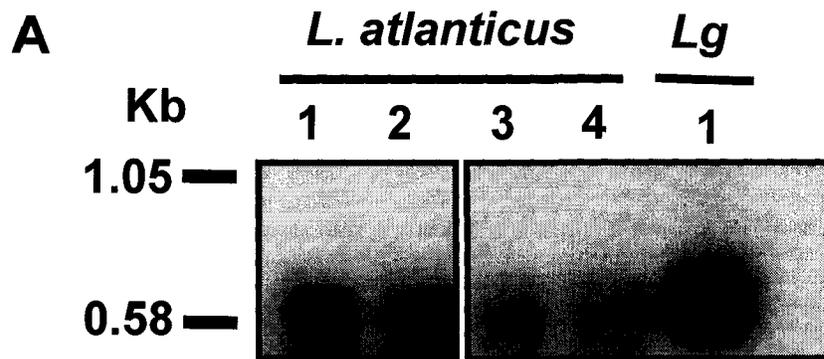
Figure 5-6. Distribution of snailfish AFP mRNA in liver tissues of 9 sample fish.
Panel (A) Northern blot analysis from eight individual Atlantic snailfish and one dusky snailfish. Blots were probed with snailfish AFP UTR sequence. Panel (B) is the corresponding RT-PCR results from the same tissue samples.

A

L. atlanticus   Lg

Kb   1   2   3   4   5   6   7   8   1

1.05

0.58
0.48

B

La   Lg

M   1   2   3   4   5   6   7   8   9

600 bp

600 bp   ← β-actin

105
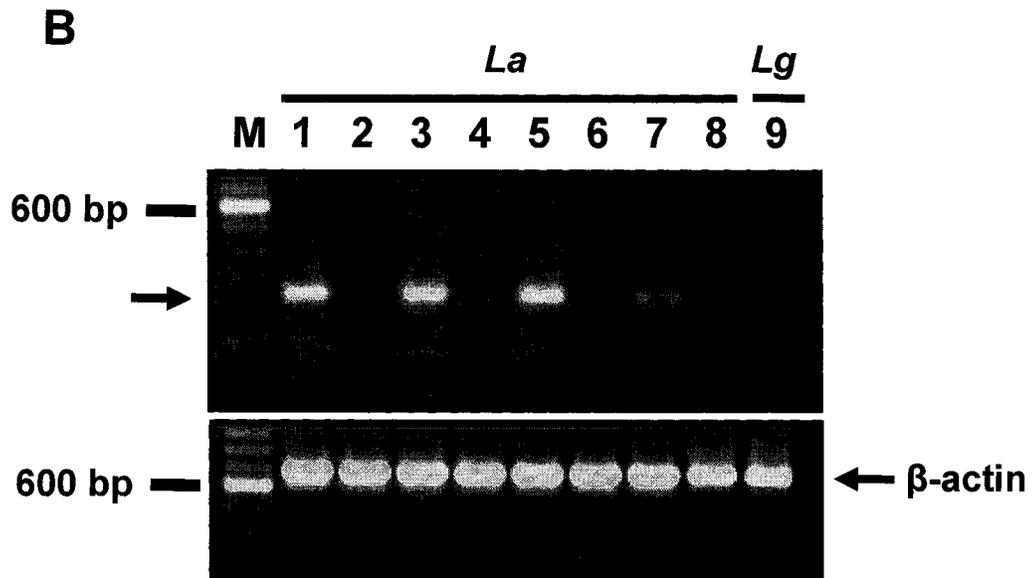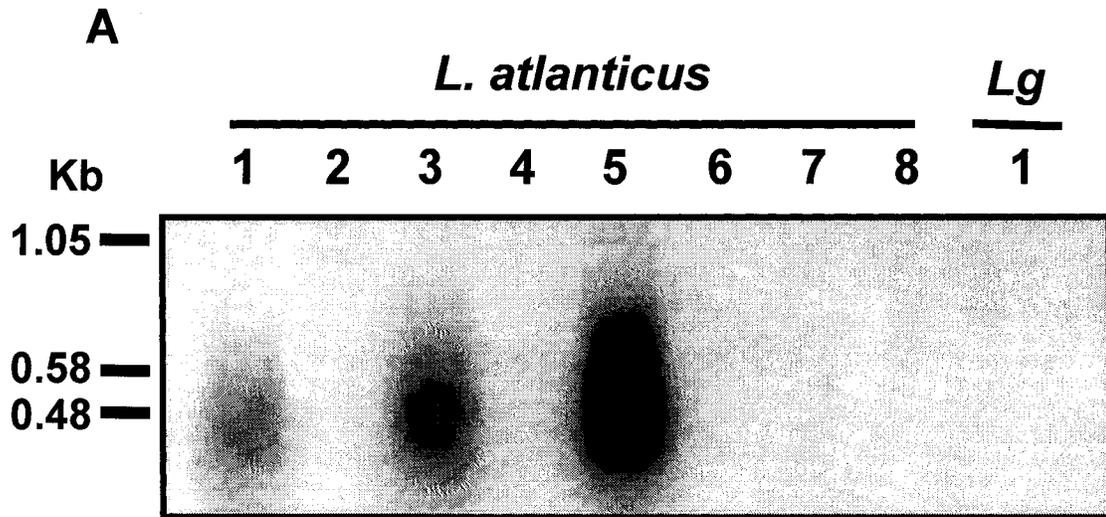
snailfish (Fig 5-6A). Results showed that three of the Atlantic snailfish samples, but not

the dusky, gave positive signals of varying intensities. RT-PCR gave the same result as

the northern blots with the exception of one of the samples (Fig 5-6B).

To analyze snailfish genes, a Southern blot was probed with the same DNA probe

applied to the previous northerns. At least nine individual bands can be distinguished

when Atlantic snailfish DNA is digested with *Hind*III (Fig 5-7; lane 3) while at least 10

are visible for dusky snailfish and the same restriction enzyme (Fig 5-7; lane 7). Results

of this experiment indicate that snailfish AFPs are expressed via a multigene family but

the exact number gene copies cannot be determined precisely here.

5.4 Discussion

Using a combination of cDNA library screening and 5'-RACE, a complete cDNA

corresponding to type I AFP was cloned from Atlantic snailfish skin tissue and

subsequently in closely related dusky snailfish. Both the nucleotide and protein sequences

are nearly identical, suggesting that these AFPs shared a common ancestral gene prior to

snailfish species divergence. This differs from taxonomically related shorthorn and

longhorn sculpin skin AFPs which produce quite contrasting proteins while untranslated

regions of mRNA are nearly identical (Low et al 2001).

Based on cDNA sequence, both snailfish species express 113 residue AFPs that

are the largest described to date. The predicted proteins lack signal or pro-sequences,

which indicates the mature polypeptides remain intracellular. This would imply that their

location and function is analogous to the presumptive intracellular skin AFPs of winter

flounder (Gong et al 1996) and sculpins (Low et al 1998, Low et al 2001). However, the

Figure 5-7. Southern blot analysis of snailfish AFP genes. 10 µg of DNA were digested with the indicated restriction enzymes. The blot was probed with snailfish AFP UTR sequence.
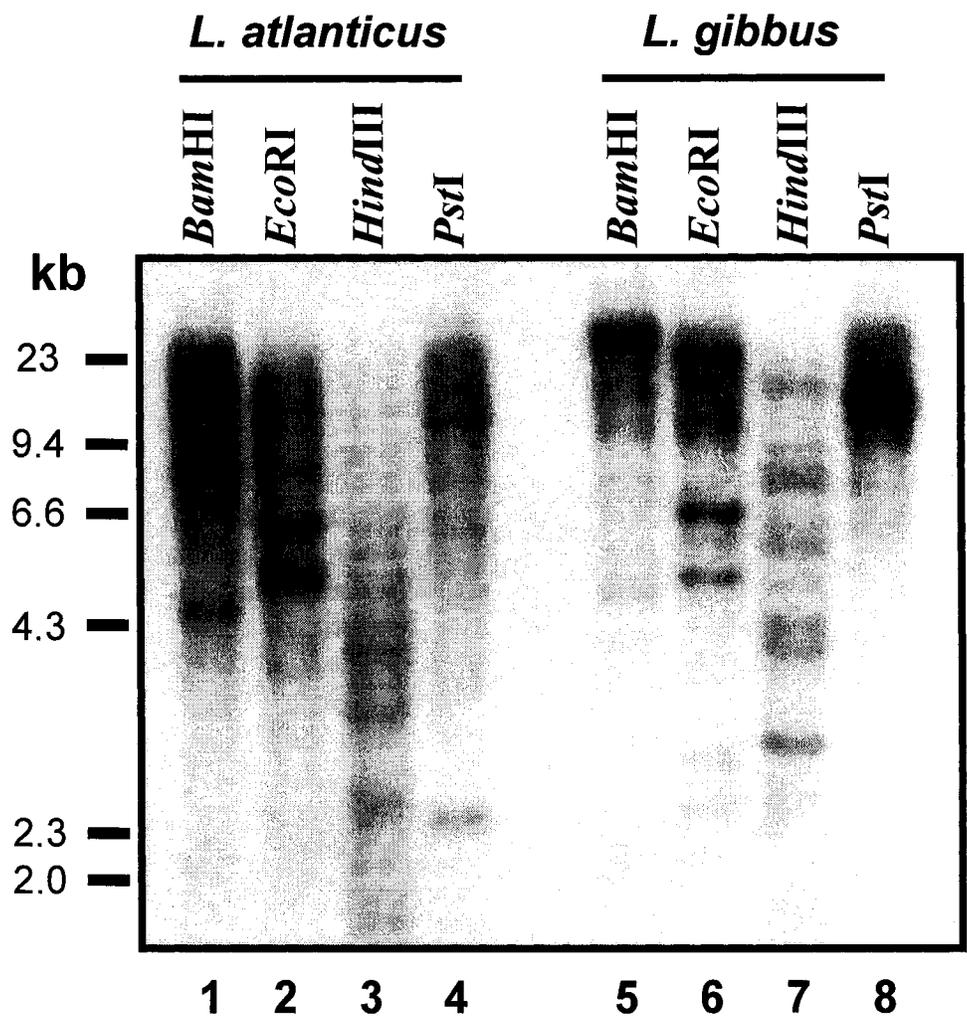
molecular mass of snailfish skin proteins predicted from cDNA and their amino terminal

sequence are identical to the results determined for their purified plasma AFPs.

Furthermore, northern blots indicate that snailfish AFP mRNA has consistently

significant expression only in skin tissue. Taken together, the evidence reported here and

in chapter 4 demonstrates that the circulating plasma AFPs and skin localized AFPs are

identical proteins that are normally expressed by the same skin specific gene.

These results represent the first definitive report of fish which synthesize identical

AFPs for protection in two different physiological locations. The assumption has been

that skin-type AFPs are expressed via a different subset of genes from the liver multigene

family (Gong et al 1996, Low et al 1998, Low et al 2001). The evidence from snailfish

contradicts the original hypothesis that separate sets of genes code for unique AFP

isoforms to provide extracellular and intracellular antifreeze protection. Although the

exact sub-cellular location has not yet been unequivocally established for skin-type AFPs,

evidence from winter flounder indicates that skin AFPs are present in gill cell cytoplasm

as well as in contact with the plasma membrane outside epithelial cells (Murray et al

2002). Clearly, snailfish AFPs produced in the cytoplasm of epithelial cells are secreted

into blood to provide extracellular protection but it is still unclear if some protein remains

inside these cells as well. It is also uncertain exactly how snailfish AFPs are secreted

from the cells that express them if they do not contain the requisite signal sequences.

Alternative pathways for protein export that circumvent the usual endoplasmic reticulum-

Golgi complex have been described previously (Mignatti et al 1992, Menon and Hughes

1999).

The northern blot experiments exhibited a startling and unexpected variation in AFP expression patterns among individual fish. While skin tissues consistently produced high levels of AFP mRNA, expression in liver ranged from undetectable to high levels. This extreme individual variation in mRNA expression has not been reported previously for any species producing antifreeze. However, studies have demonstrated geographic dependent population differences in antifreeze gene copy number (Hew et al 1988, Hayes et al 1991). In fact, individual fish from one population of Newfoundland ocean pout had demonstrable differences in antifreeze gene copies which indicates the malleability of antifreeze genes within a given fish genome. It would be informative to determine if the diverse nature of the snailfish multigene family or if regulatory control regions within snailfish AFP gene(s) are responsible for the variation in observed tissue specific gene expression.

The physiological significance of the variation in snailfish mRNA expression is not clear since all fish examined had significant levels of protein in blood and skin during the winter. It is possible that different physiological or environmental cues initiate expression in each tissue separately. Previous studies have demonstrated that type I AFP expression in liver is seasonally adjusted from low in summer to high in winter based on environmental cues (reviewed in Fletcher et al 1998, Fletcher et al 2001). Furthermore, skin AFP expression is uniformly high in winter flounder but has an annual variation in shorthorn sculpin. It seems likely that skin AFP expression provides the primary source of AFP production in snailfish and the liver is an ancillary site of expression for contributing supplementary protection. In this sense snailfish may be similar to cunner in

that they rely more on skin (and its AFP content) to provide the primary barrier to ice crystal propagation.

Overall, the evidence presented here supports the hypothesis that AFPs from skin are the primordial protein source from which the liver type (plasma AFP) later evolved (Gong et al 1996, Low et al 2002). The skin AFP acts as the first line of defense to protect from external ice damage and may also protect the integrity of cellular membranes. In order for an undercooled fish to freeze, ice must propagate into it from the external environment. Several studies have shown that biological membranes can be effective at preventing ice propagation to undercooled fluids and that epithelial tissues act as effective barriers to ice propagation (Fletcher et al 2001, Valerio et al 1992). It is possible that the dual skin/liver expression in snailfish could represent the intermediate state between primary skin specific and liver specific AFP expression prior to the divergence of skin AFPs into distinct liver isoforms – a snapshot of evolution in action. While skin appears to be the original location for ubiquitous AFP expression in marine fish, a distinct subset of genes evolved later for liver expression of different AFP isoforms for circulation in blood. Eventually, the liver specific genes further evolved into the primary production site of circulating AFPs in most species. Evidence from chapter 6 suggests that snailfish AFPs may have evolved from proteins expressed in epithelial tissues via gene cooption from a chimeric AFP intermediate. A similar gene cooption mechanism is responsible for the transformation of a trypsinogen-like serine protease gene into AFPG in notothenioids (Chen et al 1997a, Chen et al 1997b, Cheng and Chen 1999).

The primary structure of snailfish AFPs is unlike most other known type I AFPs. While they are extremely α-helical proteins – determined experimentally by circular dichroism spectrometry – they possess only moderate thermal hysteresis activity compared to other type I AFPs (chapter 2). Helical net and helical wheel representations (Fig 5-8A, B) of Las-AFP indicate that there are none of the ice binding motifs that were originally identified as important for ice binding (Sicheri and Yang 1995, Lin et al 1999, Zhang and Laursen 1998). Recently, amino acid substitution experiments have determined that it is the conserved alanine-rich hydrophobic surface which is most important for ice-binding in type I AFPs (Baardsnes et al 1999, Harding et al 1999, Baardsnes et al 2001, Fairley et al 2002). A rudimentary 3D representation of Las-AFP suggests that no full-length hydrophobic surface is free from interfering polar residue side chains (Fig 5-8C). Furthermore, snailfish AFPs do not contain the requisite hydrogen bonding amino acids necessary to create the elaborate terminal cap structures found in most type I AFPs (Sicheri and Yang 1995). The lack of complete hydrophobic face and terminal caps might be responsible for the low activity of these AFPs. It should be noted however that the predicted structure of snailfish AFP may not exactly correspond with structural data provided by experimental methods. It is possible that the protein contains kinks or bends in the backbone around the helix-breaking proline residues.

Based on protein primary structure, most type I AFPs cluster into three distinct groups, depending on the nature of their highly conserved amino terminal sequences (Figure 5-9). Two of the groups contain the classic 11 residue ($ThrX_{10}$) repeat sequence while the third group contains no such repeat structure.

Figure 5-8. Schematic representations of Atlantic snailfish AFP secondary structure.
(A) Helical net (B) Helical wheel diagrams were constructed by the EMBOSS package located on the Canadian Bioinformatics Resource web page. Hydrophilic residues DENQST are marked with diamonds. Positively charged residues HKR are marked with octagons. Aliphatic residues ILVM are marked with squares. (C) Swiss PDB software generated 3D model of Las-AFP projected from amino and carboxy terminals. Only the polar amino acid side chains are visible in the model.

A PEPNET of from 1 to 112

71 64 57 50 43 36 29 22 15 8 1

106 99 92 85 78

B PEPWHEEL of from 1 to 112

C

Amino   Carboxy
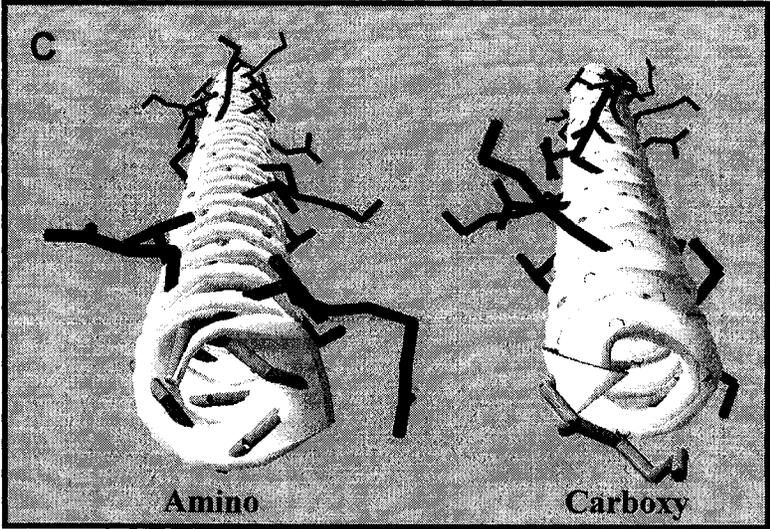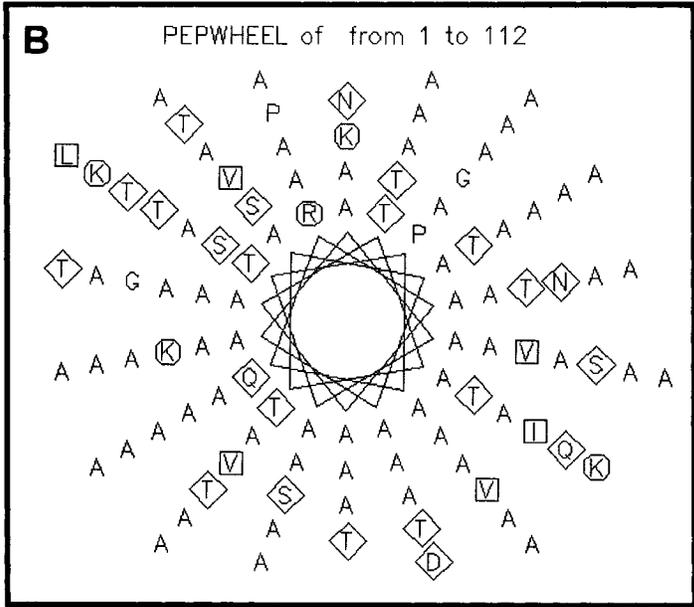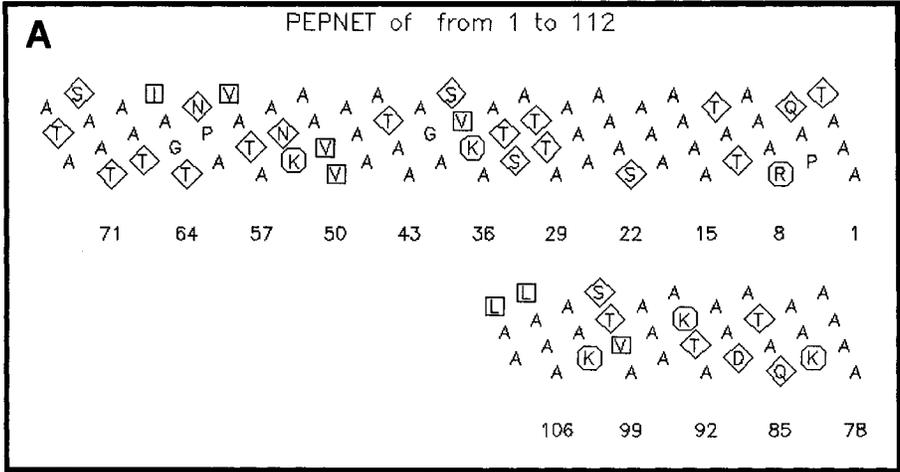
Figure 5-9. Evolutionary groupings of all known type I AFP protein/polypeptide sequences. (A) Sequence comparisons were performed using ClustalX software to create un-rooted neighbour joining trees that are visualized with TreeView (1.6.1) software. (B) Amino acid sequence alignments of the groups created by ClustalX analysis. Identical amino acids are shown with black backgrounds while similar ones are shaded.

A
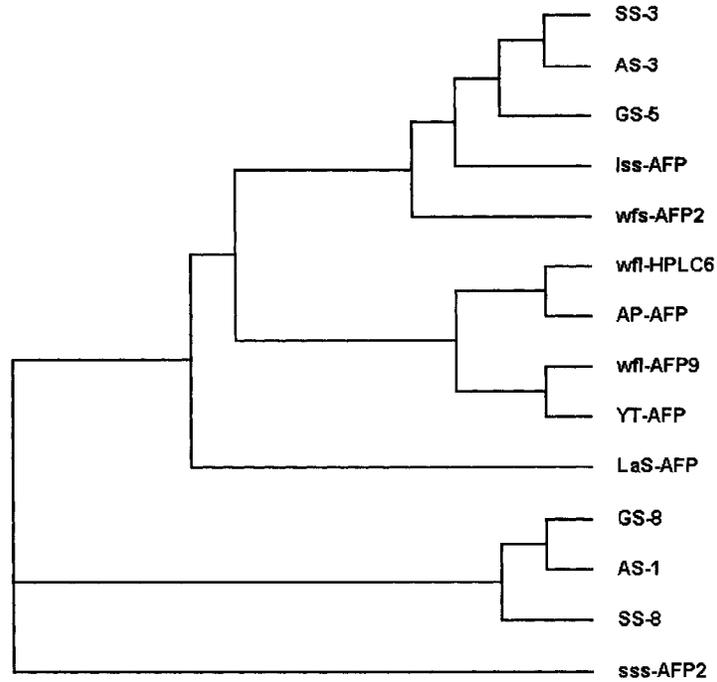
B

```
SS-3      1 NAPARAAAKTAADALAAAKKTAADAAAAAAA----------
AS-3      1 DAPARAAAKTAADALAAANKTAADAAAAAAAA---------
GS-5      1 DAPAIAAAKTAADALAAAKKTAADAAAAAAKP---------
lss-AFP   1 DAPAKAAAKTAADAKAAAKTAADALAAANKTAAAAKAAAK
wfs-AFP2  1 DAPAKAAAATAAAKAAAEATAAAAAKAAAATKAGAAR---


wfl-HPLC6 1 DTASDAAAAAALTAANAKAAAELTAANAAAAAAATAR---------------
AP-AFP    1 DTASDAAAAAAATAAAAKAAAEKTARDAAAAAAATAAAAR------------
wfl-AFP9  1 DTASDAAAAAAATAATAAAAAAATAVTAAKAAALTAANAAAAAAAATAAAARG
YT-AFP    1 DTASDAAAAAAATAAAAKAAADTAAAAAKAAADTAAAAAEAAAATAR----


GS-8      1 DGETPAQKAARLAAAAAALAAKTAADAAAKAAAIAAAA------
AS-1      1 DGETPAGKAARLAAAAA-LAAKTAADAAAKAAAIAAAA------
SS-8      1 NGETPAQKAARLAAAAA-LAAKTAADAAAKAAAKAAATAAAASA


sssAFP-2  1 AAAAKAAEAAAMAAANAAEAAATKAADAAASAAAAAIAAIAEAAEAAEAA
         51 ATKSANVAAAAAATSAAAAAKATANAAAAASAAAAAAAAVA


La-s      1 AAATPAQRAAATATAAAAAAASAAAAAAATTASTAAKVSAGAAATAAAAV
         51 VAAKNAATAVAPNTGAITAATAASATAAAAAKAAQATADAAATKAAAAAV
        101 TSKAAAAALAAL
```

While all polypeptides that fit in the three groups are small (~3.3 - 4.5 kDa), the unusually large snailfish and shorthorn sculpin skin AFPs are outliers that do not conform to either of the parsimonious categories. Interestingly, there seems to be no connection between the AFP structural groups and phylogenic classification or tissue source of the proteins. With the discovery of snailfish skin proteins, it is apparent that type I AFPs can also be separated into distinct structural subclasses based on size and the absence of traditional amino acid repeat structure. This subclass could have unique evolutionary origins and a distinctive mechanism for ice-binding separate from the three groups mentioned above. Perhaps the fundamental property of type I AFP, as represented by snailfish AFPs, is an alanine rich protein with α-helical secondary structure that is capable of ice-binding.

CHAPTER 6:

Molecular Cloning of Liver Expressed Proteins from Snailfish:

Possible Evolutionary Origins of Type I AFPs

6.1 Introduction

While type I AFPs have been characterized in many species, winter flounder (*Pseudopleuronectes americanus*) is the best studied and is considered the prototypical example for developing an understanding of AFP function and regulation. It is known that winter flounder synthesize pre-proAFP in the liver, and that it undergoes post-translational modifications before and after secretion into blood for extracellular freeze protection (Davies et al 1982, Pickett et al 1984). The expression of liver–type AFP genes in winter flounder is controlled primarily by photoperiod and follows a seasonal cycle with levels of AFP mRNA in winter increased several fold over summer levels (reviewed by Fletcher et al 1989, Chan et al 1993, Fletcher et al 1998). Currently, the genes for all AFPs from plasma are believed to be expressed primarily in liver tissue, which gave rise to the original name 'liver-type AFP' (Gong et al 1992).

Experiments described in chapter 2 revealed that both snailfish species have type I AFPs circulating in their blood plasma. To further characterize snailfish plasma AFPs, much useful information can be derived from cloning and sequencing their corresponding cDNAs from a cDNA library. Therefore, it was decided that a liver cDNA library would be constructed since it should provide high probability for successful isolation of snailfish AFP clones.

6.2 Materials and Methods

6.2.1 Tissue collection

Atlantic snailfish (*Liparis atlanticus*) were collected by divers near Logy Bay, Newfoundland in the winters of 1995 and 1996. Dusky snailfish (*Liparis gibbus*) were

collected from Placentia Bay, Newfoundland during the winter of 1995. The live fish were brought into the laboratory and placed into holding tanks supplied with ambient temperature seawater prior to tissue collection. Tissues of freshly killed fish were removed, immediately frozen in liquid nitrogen and stored at -70°C before use.

6.2.2 Northern blot analysis

Total RNA from liver and skin tissues of Atlantic and dusky snailfish were isolated using TRIzol® reagent (Invitrogen Canada Inc.) as described by the manufacturer. Ten μg aliquots of total RNA were analyzed by standard northern blotting procedures using Hybond-N nylon membrane as described by the manufacturer (Amersham Biosciences). The membrane was hybridized at 42°C overnight in the following buffer: 5X SSC, 5X Denhardt's, 0.5% SDS, 50% formamide and 100μg/ml calf thymus DNA. Probe was labelled with ^{32}P-dCTP using a Megaprime DNA Labelling System and purified prior to use with ProbeQuant G-50 Micro Columns (Amersham Biosciences). The final wash was performed in 0.2X SSC, and 0.1 % SDS, at 45°C for 15 min. A 300 bp DNA fragment corresponding to the open reading frame (ORF) of shorthorn sculpin skin (s3-2) clone (Low et at, 1998) was used as a probe. In the other blot, the ORF of a winter flounder liver AFP clone (pKenc-17) was used as a DNA probe. It was necessary to use the ORFs to ensure some signal because there is little or no sequence conservation of the untranslated regions (UTRs) from type I AFPs between the species of fish of different families. After approximately 20 hrs exposure, a hybridization signal was detected by autoradiography.

6.2.3 Library construction and screening

A liver cDNA library from a female Atlantic snailfish was obtained from Dr. Z.

Gong (the Dr. C.L. Hew lab at University of Toronto). Briefly, total liver RNA was

isolated using the guanidinium thiocyanate-phenol-chloroform extraction method

(Chomczynski and Sacchi 1987) and poly A^+ mRNA was separated using Oligo (dT)

columns. The cDNA library was constructed and amplified, as described by the

manufacturer, using Lambda ZAP® library and ZAP-cDNA® Synthesis Kits and

Gigapack® Gold packaging extracts (Stratagene, La Jolla, CA). Normally, 50,000 plaques

were grown on 15 cm NZYCM plates for primary screening while 9 cm plates were used

in secondary and tertiary screens.

Hybond-N nylon membranes (Amersham Biosciences) were prepared and

screened according to the manufacturer. Briefly, membranes were hybridized at 42°C

overnight in the following buffer: 5X SSC, 5X Denhardt's, 0.5% SDS, 50% formamide

and 100µg/ml calf thymus DNA. Probe was labelled with ^{32}P-dCTP using a Megaprime

DNA Labelling System and purified prior to use with ProbeQuant G-50 Micro Columns

(Amersham Biosciences). The final wash was performed in 1.0X SSC, and 0.1 % SDS, at

52°C for 20 minutes. A 300 bp DNA fragment corresponding to the ORF of shorthorn

sculpin skin (s3-2) clone (Low et at, 1998) was used as a probe to screen approximately

5.0×10^5 clones of the amplified cDNA library.

6.2.4 DNA sequencing

Positive plaques were first isolated and then pBluescript® phagemids were

produced using an in-vitro excision protocol according to the manufacturer (Stratagene,

La Jolla, CA). Sequencing was performed using T3 and T7 primers at the DNA

sequencing facility in The Centre for Applied Genomics (Hospital for Sick Children,

Toronto, ON).

6.2.5 Bioinformatics Programs

Homologous nucleotide and protein sequences were searched through **BLAST**

searches on the NCBI web server (www.ncbi.nlm.nih.gov/BLAST/). The NCBI **ORF**

Finder was utilized to identify putative open reading frames in the nucleotide sequences

(www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequence alignments were generated via

MultAlin (www.prodes.toulouse.inra.fr/multalin/multalin.html). Visualization of

sequence alignments were made with BOXSHADE 3.21 program on Swiss EMBnet

server (www.ch.embnet.org/index.html).

6.3 Results

6.3.1 Northern blot analysis

Total RNA from snailfish liver and skin were probed using the ORFs of type I

AFP cDNA clones from shorthorn sculpin. As is seen in Figure 6-1, the Atlantic snailfish

liver RNA produced a signal ~850 bp and the band was a little larger in dusky snailfish.

The skin tissues for both species have fainter bands ~850 bp but very strong signals up at

~2.3 kb. The results were similar when this northern blot was probed with the winter

flounder ORF cDNA probe (data not shown). Based on these results, it was concluded

that these probes would be satisfactory to successfully screen the liver cDNA library for

type I AFP clones.

6.3.2 cDNA library screening and clone isolation

The initial screen of the Atlantic snailfish liver cDNA library produced more than 100 positive signals. Of these, approximately 20 independent clones were analyzed by Southern blot (data not shown) and clones that gave strong positive signals were sequenced. The first clone isolated from the library was termed lal-A1 (for Liparis atlanticus liver-clone A1) and its sequence is shown in Figure 6-2. The sequence indicated that although it was longer than predicted from northern blots, a section toward the 5′ end with many GC-rich codons translated into alanine residues, similar to type I AFP. However, this reading frame was incomplete and no other ORFs translated entirely into type I AFP sequence.

As shown in Figure 6-2, the largest ORF contains 441 amino acids (47 kDa) and short 5′ and 3′ UTRs. The putative start and stop codons are underlined as well as the common alternative polyadenylation signal, ATTAAA (Graber et al 1999). When the nucleotide sequence of lal-A1 was used in a BLAST search, there was very significant sequence similarity with structural proteins from fish eggs (i.e. choriogenin H, zona pellucida protein etc - Table 6-1). A protein sequence BLAST search also showed strong similarity with the same proteins as the nucleotide homologies (table 6-2). In nucleotide and protein sequences, there was no homology with the fish eggshell proteins toward the end portion of lal-A1 which includes the 'AFP like' section.
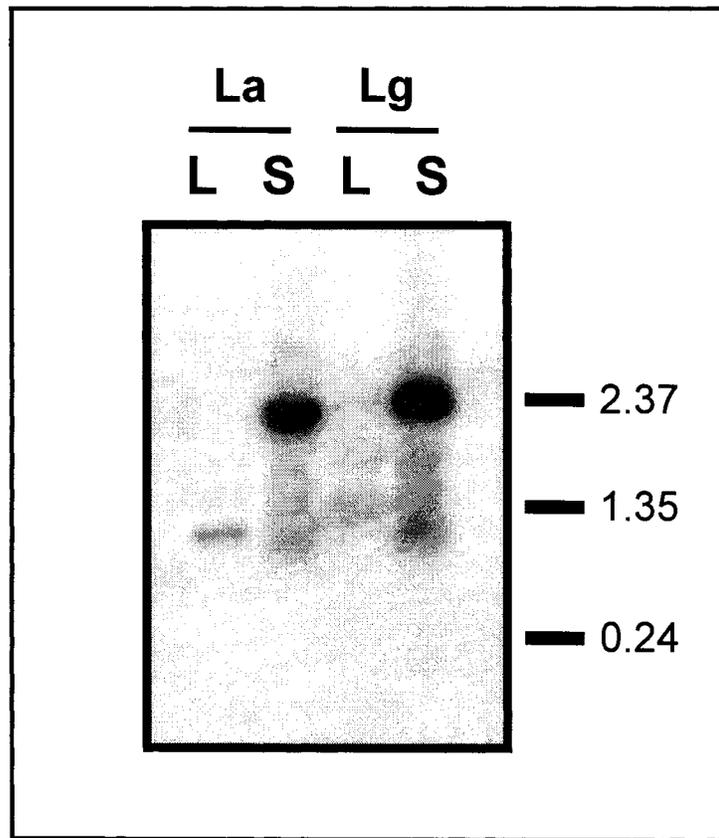
Figure 6-1. Northern blot analysis of snailfish liver and skin RNA for Type I AFPs.
Blot probed with a shorthorn sculpin ORF probe. Lanes contain 10 μg of *L. atlanticus*
(La) or 10 μg of *L. gibbus* (Lg) total RNA. L = liver RNA; S = skin RNA

Figure 6-2. Full length nucleotide sequence and primary translation product of lal-A1 cDNA clone. The ORF is in lower case letters while the 5' and 3' UTRs are in non-bold capitalized letters. The putative start and stop codons and the polyadenylation signal are underlined.

```
   1 GCACGAGTCAAACTGCTGTTGAAACATGGCGACACCTGGGGTGTCACCTCTTTTGTGGCCCTAGCACTGCTAGCATGCTT 80

  81 TGCTGGGATGGAAGTAGAAGCTCAGGACAAGACCTTTTCAAATCCTCCTCCAAAGGTCATTCAACAGGGTAGCTCTCCTC 160

 161 CATTAGCCGACGGCGGTGACGGTGGCGACGGCGGCCACGGAGGCTTCATCGGCAGCGGTGGCCatggcggcagcggcggc 240
   1                                                                  M  A  A  A  A  A 6

 241 gacggcagctggaccggcagcggcggcagcggcggccacggcggccatggcggcttcatcggcagcggcggcgacggtgg 320
   7  T  A  A  G  P  A  A  A  A  A  A  A  T  A  A  M  A  A  S  S  A  A  A  A  T  V  E 33

 321 agaaggcggagacgccctccccggctacatcccctgctggggtagccattgtccccatgtttggtttgatcagaagcagc 400
  34     K  A  E  T  P  S  P  A  T  S  P  A  G  V  A  I  V  P  M  F  G  L  I  R  S  S 59

 401 gaaaccccaaaaactcacagaatcaaatcagacacattttgccaaataattctcctgcccagagttgtgacgtgccaaaa 480
  60 E  T  P  K  T  H  R  I  K  S  D  T  F  C  Q  I  I  L  L  P  R  V  V  T  C  Q  K 86

 481 aaacacgagagtcacatgtggacttcctgatatctctactaaacactgcgaagagataaactgctgctctgatggacaac 560
  87 N  T  R  V  T  C  G  L  P  D  I  S  T  K  H  C  E  E  I  N  C  C  S  D  G  Q  H 113

 561 actgcttttatggaaaggcagtgaccgttcagtgcaccaaggatggccagttcattgtcgtagtggcgagagatgccact 640
 114    C  F  Y  G  K  A  V  T  V  Q  C  T  K  D  G  Q  F  I  V  V  V  A  R  D  A  T 139

 641 ctgcccaacattgacctcgagtcaatctcacttttgagaagcggtccaggctgtacacatgttgactctaattcaggctt 720
 140 L  P  N  I  D  L  E  S  I  S  L  L  R  S  G  P  G  C  T  H  V  D  S  N  S  G  F 166

 721 tgccatctataactttggtgttaccgcatgtggcaccgttgtttcggaggagcctggtgttataatctatgagaacagaa 800
 167 A  I  Y  N  F  G  V  T  A  C  G  T  V  V  S  E  E  P  G  V  I  I  Y  E  N  R  M 193

 801 tgatctcctcatatgaagtggacactgggcctgttggagtcattagcagagacagtcaatatgaattgctcttccaatgt 880
 194    I  S  S  Y  E  V  D  T  G  P  V  G  V  I  S  R  D  S  Q  Y  E  L  L  F  Q  C 219

 881 cggtacatcggcaccactgttcaaactgtggttgtagaagtatcaccgttactagatcctcctatatcagttgctgctgt 960
 220 R  Y  I  G  T  T  V  Q  T  V  V  V  E  V  S  P  L  L  D  P  P  I  S  V  A  A  V 246

 961 gggacccatcagagtagaactgaggttgggtaatggacagtgcatttcaaagggttgtgttgaagaggatgtggcctatg 1040
 247 G  P  I  R  V  E  L  R  L  G  N  G  Q  C  I  S  K  G  C  V  E  E  D  V  A  Y  A 273

1041 cttcgtactacaccggtgctgactatcctgtcagtaaagtactgagggaccctgtgtacgtggaggttcgactccttgag 1120
 274    S  Y  Y  T  G  A  D  Y  P  V  S  K  V  L  R  D  P  V  Y  V  E  V  R  L  L  E 299

1121 aagacagataccaaccttgtcctgactcttggtcgctgttgggcaaccacaagccccaaccctcacactctgccccagtg 1200
 300 K  T  D  T  N  L  V  L  T  L  G  R  C  W  A  T  T  S  P  N  P  H  T  L  P  Q  W 326

1201 ggacattctgacagacgggtgtccaaacaggaacgataaatacttgtcttcactgattccaatcggtccctcctctggtc 1280
 327 D  I  L  T  D  G  C  P  N  R  N  D  K  Y  L  S  S  L  I  P  I  G  P  S  S  G  L 353

1281 tgttctaccccagtcactacagacgtttccttttcaaaatgtttacctttgttggccaaacgtcatcaaagtcgatcaaa 1360
 354    F  Y  P  S  H  Y  R  R  F  L  F  K  M  F  T  F  V  G  Q  T  S  S  K  S  I  K 379

1361 tctcaacccttgagggaacaggtgtacattcactgcagtacagcagtgtgcactcctgtgaagggatactcctgtgaacc 1440
 380 S  Q  P  L  R  E  Q  V  Y  I  H  C  S  T  A  V  C  T  P  V  K  G  Y  S  C  E  P 406

1441 agtgtgctacagaaaaaagagagatgtcatggatgtggaccagacaagttctcagccaaaggtcgtggcttctgtaggac 1520
 407 V  C  Y  R  K  K  R  D  V  M  D  V  D  Q  T  S  S  Q  P  K  V  V  A  S  V  G  P 433

1521 cagtggacatgggtgcgtcttgggagtaaaCAGCAGTGTCAAACTCAAGAATTTGGCCGAGATCATGAAGTGAAAGATTT 1600
 434    V  D  M  G  A  S  W  E                                                          1600
                                                                                       441
1601 GCATATTACAGACTGTATATTGTAATGATTGCAAACGTATTACAAAGTGTGCTGCAGGAAGAATGAAATTGATCCCCGAA 1680

1681 AGTATTGTAGACACAATTGTATTAAAATATATGGCTTTGA(n)                                      1720
```

Table 6-1. Nucleotide Sequences with Significant Sequence Similarity to lal-A1.

Organism	Sequence Type/Name	E value*
Winter flounder *(Pseudopleuronectus americanus)*	zona pellucida protein gene	3e-24
Japanese medaka *(Oryzias latipes)*	choriogenin H mRNA	3e-15
Japanese medaka *(Oryzias latipes)*	choriogenin H gene	3e-12
Rainbow trout *(Oncorhynchus mykiss)*	vitelline envelope protein beta mRNA	3e-10
Rainbow trout *(Oncorhynchus mykiss)*	zona radiata structural protein mRNA	3e-08

*The lower the E-value, or the closer it is to "0" the more significant the match is.
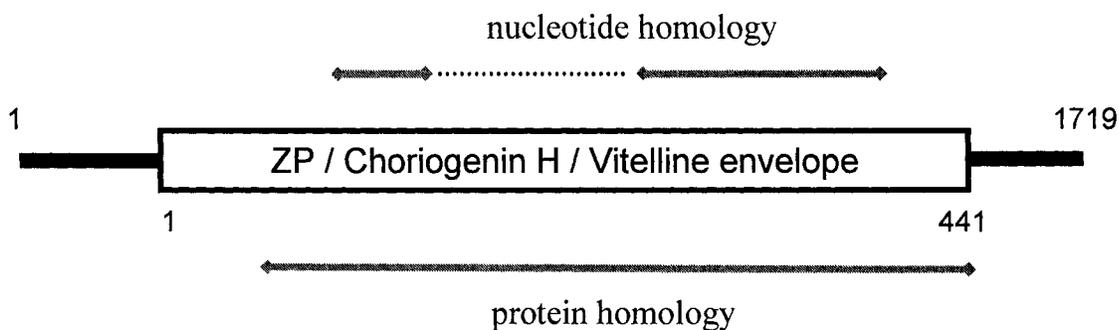
Table 6-2. Protein Sequences with Significant Sequence Similarity to lal-A1.

Organism	Sequence Type/Name	E value
Rainbow trout *(Oncorhynchus mykiss)*	vitelline envelope protein beta	1e-118
Japanese medaka *(Oryzias latipes)*	choriogenin H	1e-118
Winter flounder *(Pseudopleuronectus americanus)*	zona pellucida / egg envelope protein	1e-117
Zebrafish *(Danio rerio)*	egg envelope protein ZP2 variant C	1e-104
Zebrafish *(Danio rerio)*	zona pellucida glycoprotein 2	1e-102

Amongst the group of twenty clones picked initially, the most common clone isolated from the library was termed **lal-B2** (for _L_iparis _a_tlanticus liver-clone B2) and its sequence is shown in Figure 6-3. Overall, of the clones isolated for sequencing, at least 90% were lal-B2 while the remainder was either lal-A1 or lal-C14. In one of the reading frames, toward the 5' end, many alanine residues were translated which suggested that it was type I AFP sequence. However, this reading frame was not complete in that it did not have the required start and stop codons in frame. The largest lal-B2 ORF contains 494 amino acids (52 kDa) with numerous glycine residues toward the amino terminus. The putative start and stop codons are underlined as well as the standard polyadenylation signal (Fig 6-3). This clone is of similar length to lal-A1 and also has very short 5' and 3' UTRs.

When the nucleotide sequence of lal-B2 was used in a BLAST search, there was also very significant sequence homology with mRNA of structural proteins from fish eggs (Table 6-3). The sequence of lal-B2 is very similar to chorion protein mRNA from sea bream and L-SF from medaka. A protein sequence BLAST search also showed strong similarity with the same egg structural proteins as the nucleotide homologies (Table 6-4). As was the case for lal-A1, there was no homology for the nucleotide and protein sequences with the fish eggshell proteins in the section of the clone that initially appeared to contain type I AFP sequence.

Figure 6-3. Full length nucleotide sequence and primary translation product of lal-B2 cDNA clone. The ORF is in lower case letters while the 5' and 3' UTRs are in non-bold capitalized letters. The putative start and stop codons and the polyadenylation signal are underlined.

```
   1 ACGAGAGCACTTGTTGTTGTGAAGCatggtgatgaagtatactgccgtgtgcctttttggtactagccttgtttggcactt 80
   1                         M  V  M  K  Y  T  A  V  C  L  L  V  L  A  L  F  G  T  F 19

  81 tttgtgaggctcaaagggggggtttccagaaaccataccagaagccagcatcacccaaacaggtgccttatgaacctcaa 160
  20  C  E  A  Q  R  G  G  F  Q  K  P  Y  Q  K  P  A  S  P  K  Q  V  P  Y  E  P  Q 45

 161 cagcaggcaaagcagaactttgagaaacctctcacctggattttccctgaagatcctcagcccgaagcagcagttgaagt 240
  46 Q  Q  A  K  Q  N  F  E  K  P  L  T  W  I  F  P  E  D  P  Q  P  E  A  A  V  E  V 72

 241 gcctttcgagctgagatatcctgttgctgctgcatctgtctctgttgagtgtagagagagtgctgtccatgtggaggtca 320
  73 P  F  E  L  R  Y  P  V  A  A  A  S  V  S  V  E  C  R  E  S  A  V  H  V  E  V  K 99

 321 agaaagacatgtttggaattggccagttcatcaatgcagctgaccttactctgggaaactgtggcgctgtcgcagaggac 400
 100 K  D  M  F  G  I  G  Q  F  I  N  A  A  D  L  T  L  G  N  C  G  A  V  A  E  D 125

 401 tctgctggtcaagtgttggtctttgaagctgagctccagaattgtctcagctcattggggatgaccgaagattccctcat 480
 126 S  A  G  Q  V  L  V  F  E  A  E  L  Q  N  C  L  S  S  L  G  M  T  E  D  S  L  I 152

 481 ctacaccttcaccatgaactacattcccaacctctggggggatctcctgttgtgaggaccagccaggctgctgtaattg 560
 153 Y  T  F  T  M  N  Y  I  P  Q  P  L  G  G  S  P  V  V  R  T  S  Q  A  A  V  I  V 179

 561 tcgaatgccactacccaaggaagcacaatgtgagcagccttgctcttgatcccctgtggatcccattttctgcggttaag 640
 180 E  C  H  Y  P  R  K  H  N  V  S  S  L  A  L  D  P  L  W  I  P  F  S  A  V  K 205

 641 atggcagaggaattcctgtacttcaccctgaaactcatgactgacgactggcagtatgagaggccaagctaccaatattt 720
 206 M  A  E  E  F  L  Y  F  T  L  K  L  M  T  D  D  W  Q  Y  E  R  P  S  Y  Q  Y  F 232

 721 cctgggagacaccatcaatattgaagctgtcgtcaagcagtacttccacgtgcccctccgtatatatgtggaaagctgcg 800
 233 L  G  D  T  I  N  I  E  A  V  V  K  Q  Y  F  H  V  P  L  R  I  Y  V  E  S  C  V 259

 801 tagctactctggaacctgacacatccgccaacccccagatatgccttcattgataacaatgggtgtttgctcgatgcaagg 880
 260  A  T  L  E  P  D  T  S  A  N  P  R  Y  A  F  I  D  N  N  G  C  L  L  D  A  R 285

 881 ctcacaggatccaactctaagttctatcttcgctctgcagacaacaagcttcagttccagttggaggccttcaggttcca 960
 286 L  T  G  S  N  S  K  F  Y  L  R  S  A  D  N  K  L  Q  F  Q  L  E  A  F  R  F  Q 312

 961 gaatgctgaaagtggtctgctctacatcacttgccacttgaaagcaacatctgcttcctcttctatcgataatgatcata 1040
 313 N  A  E  S  G  L  L  Y  I  T  C  H  L  K  A  T  S  A  S  S  I  D  N  D  H  R 339

1041 gagcttgttcttggaacaacgggtggcatgaggccagcaaaatcgactcagtttgtggcagctgtgaatctgccggtttg 1120
 340  A  C  S  W  N  N  G  W  H  E  A  S  K  I  D  S  V  C  G  S  C  E  S  A  G  L 365

1121 ccaccagctgccaacccaaggggagctaactttgttgccagcggcccctacaccatcagcggcggcggcggcggcggcgg 1200
 366 P  P  A  A  N  P  R  G  A  N  F  V  A  S  G  P  Y  T  I  S  G  G  G  G  G  G 392

1201 cagcggcggcagcggtaactaccccagcggcagcggtaactaccccagcggtagcggcggcctcggcggtgccggcggcg 1280
 393 S  G  G  S  G  N  Y  P  S  G  S  G  N  Y  P  S  G  S  G  G  L  G  G  A  G  G  G 419

1281 gaagcggcggctacaacggcgcccatggcggccatggcgcccacggtatcaacggcggcggcggcggcggcggcggcggc 1360
 420  S  G  G  Y  N  G  A  H  G  G  H  G  A  H  G  I  N  G  G  G  G  G  G  G  G 445

1361 ggcggcggcggtggcggcggctacaacggtaacagcggcggcggcatctacaacccttcaagaaagatacgtgatgtgtc 1440
 446 G  G  G  G  G  G  Y  N  G  N  S  G  G  G  I  Y  N  P  S  R  K  I  R  D  V  S 472

1441 tgaagtattcgaatgggaaggtgatgtcaccctgggacccatccccattgcagacaagatggttgcctaaCTCAATTCCC 1520
 473 E  V  F  E  W  E  G  D  V  T  L  G  P  I  P  I  A  D  K  M  V  A          494

1521 TGCCATGCCTAGGGCCATTAAAATAAACAGTCGTATTGGTTCATA(n)                                1565
```

130

Table 6-3. Nucleotide Sequences with Significant Sequence Similarity to lal-B2.

Organism	Sequence Type/Name	E value*
Gilthead sea bream (*Sparus aurata*)	chorion protein mRNA	1e-103
Japanese medaka *(Oryzias latipes)*	L-SF mRNA	6e-38
Japanese medaka *(Oryzias latipes)*	choriogenin L gene	1e-08
Rainbow trout (*Oncorhynchus mykiss*)	vitelline envelope protein gamma mRNA	1e-08

*The lower the E-value, or the closer it is to "0" the more significant the match is.

Table 6-4. Protein Sequences with Significant Sequence Similarity to lal-B2.

Organism	Sequence Type/Name	E value
Gilthead sea bream (*Sparus aurata*)	chorion protein	1e-148
Japanese medaka *(Oryzias latipes)*	L-SF precursor	1e-145
Japanese medaka *(Oryzias latipes)*	choriogenin L	1e-142
Rainbow trout (*Oncorhynchus mykiss*)	vitelline envelope protein gamma	1e-114
Common Carp (*Cyprinus carpio*)	egg membrane protein ZP3	7e-94
Carp (*Carassius auratus gibelio*)	egg envelope glycoprotein ZP3	4e-90

One other independent clone was isolated from the cDNA library during the first screen. The clone was ~600 bp, included a poly A tail and GC rich sequence which translated into many alanine codons. However, the sequence did not contain a proper start codon in the expected reading frame and a preliminary BLAST nucleotide search showed there was some homology with keratin mRNA. The library was re-screened and a single clone with identical sequence at the 5' end was found. The complete sequence of this clone, named **lal-C14** (for _Liparis atlanticus_ liver-clone C14) is shown in Figure 6-4. The largest, complete, ORF translated in lal-C14 contains 569 amino acids (62 kDa) and is rich in glycine residues at the carboxy and amino termini. The putative start and stop codons are underlined as well as the standard polyadenylation signal (Fig 6-4). Clearly the 600 bp clone originally sequenced was merely a truncated version of lal-C14.

When the complete nucleotide sequence of lal-C14 was used in a BLAST search, extremely significant sequence homology was found with rainbow trout and Zebrafish type II keratin mRNA (Table 6-5). The same homologies were revealed when the protein sequence was used in a BLAST search (Table 6-6). Similar to the clones described previously, a portion of lal-C14 that had no significant hits in homology searches. This portion contains sequence that appears similar to type I AFP.

Figure 6-4. Full length nucleotide sequence and primary translation product of lal-C14 cDNA clone. The ORF is in lower case letters while the 5' and 3' UTRs are in non-bold capitalized letters. The putative start and stop codons and the polyadenylation signal are underlined.

```
   1 GAAAACTCAGAGGGACTCGGCTCTCCTTTTGCAGACGGCTCATCTTCACTTTTCTTCTTGTCAGATAGTTGACCACCACT 80

  81 GCAAACACGatgaggaagtcatactcagtctcaggcggcagcagcagcatcaggaggtccaatgcacctagcagctactc 160
   1           M  R  K  S  Y  S  V  S  G  G  S  S  S  I  R  R  S  N  A  P  S  S  Y  S  24

 161 cgtccaaagaaccagctatggttccggtggcggtggaggtggtggctttgcttccggtggtttcagtagctccagtggtt 240
  25  V  Q  R  T  S  Y  G  S  G  G  G  G  G  G  G  F  A  S  G  G  F  S  S  S  G  F 51

 241 tcggggggctccggtagttatggtggtggatccgctggttttggttctggtatgtcttcaatgggtggtagttatggctttttct 320
  52  G  G  S  G  S  Y  G  G  S  A  G  F  G  S  G  M  S  S  M  G  G  S  Y  G  F  S 77

 321 tcgagccaaggaggcggctgcatgcccccaccgatcaccgctgtccaagtcaaccagagcctgctggcccccctgaacct 400
  78 S  S  Q  G  G  G  C  M  P  P  P  I  T  A  V  Q  V  N  Q  S  L  L  A  P  L  N  L 104

 401 ggcgatcgacccatccatccaggtggtccgtacccaggagaaagagcagatcaagaccctcaacaaccgtttcgcctcct 480
 105  A  I  D  P  S  I  Q  V  V  R  T  Q  E  K  E  Q  I  K  T  L  N  N  R  F  A  S  F 131

 481 tcatcgacaaggtccgtttcctggagcagcagaacaagatgctggagaccaaatggagcctcctgcaggaccagaccaca 560
 132  I  D  K  V  R  F  L  E  Q  Q  N  K  M  L  E  T  K  W  S  L  L  Q  D  Q  T  T 157

 561 acccgctccaacatcgatgccatgttcgaagcctacatttccaacctgcgcagacagctggacggggctcggcaacgaga 640
 158 T  R  S  N  I  D  A  M  F  E  A  Y  I  S  N  L  R  R  Q  L  D  G  A  R  Q  R  E 184

 641 agtcaagatggagggagagctgaggaacatgcagggccatgttgaggacttcaagaggaagtatgaagatgaaatcaaca 720
 185  V  K  M  E  G  E  L  R  N  M  Q  G  H  V  E  D  F  K  R  K  Y  E  D  E  I  N  K 211

 721 aacgtgcgagtgcagagaatgaatttgtgctcctgaagaaggatgttgatgctgcttacataaacaaggtggagctggag 800
 212  R  A  S  A  E  N  E  F  V  L  L  K  K  D  V  D  A  A  Y  I  N  K  V  E  L  E 237

 801 gccagagctgacgctcttcaggatgagatcaacttcctcagggccgtctatgaggctgagctccgtgagctgcagggcca 880
 238 A  R  A  D  A  L  Q  D  E  I  N  F  L  R  A  V  Y  E  A  E  L  R  E  L  Q  G  Q 264

 881 gatcaaggacacctccgtcgtcgtggagatggacaacagccgtaacctggacatggactccgttgtggctgaagtgcgtg 960
 265  I  K  D  T  S  V  V  V  E  M  D  N  S  R  N  L  D  M  D  S  V  V  A  E  V  R  A 291

 961 ctcagtatgaggacatcgccaaccgcagcaaggcggaagcagagacctggtacaaacagaagtatgaggagatacagagc 1040
 292  Q  Y  E  D  I  A  N  R  S  K  A  E  A  E  T  W  Y  K  Q  K  Y  E  E  I  Q  S 317

1041 tctgccggacagtatgggggaggacatccgatctaccaagactgagatctctgagctgaaccgcatgatctcccgtcttca 1120
 318 S  A  G  Q  Y  G  E  D  I  R  S  T  K  T  E  I  S  E  L  N  R  M  I  S  R  L  Q 344

1121 gaacgagattgagtccgtcaagggacagaggagcagccttgaggcccagatcgcagaggctgaggagcgtggtgagctgg 1200
 345 N  E  I  E  S  V  K  G  Q  R  S  S  L  E  A  Q  I  A  E  A  E  E  R  G  E  L  A 371

1201 cagtgaaggacgccaagctccgcatccgggacctggaggacgctctgcagagagagccaagcaggacatggcccggcagatc 1280
 372  V  K  D  A  K  L  R  I  R  D  L  E  D  A  L  Q  R  A  K  Q  D  M  A  R  Q  I 397

1281 agagaaatcacgagagaagccctgcagagagccaagcaggacatggcccggcagatcagagagtaccaggacctgatgaa 1360
 398 R  E  I  T  R  E  A  L  Q  R  A  K  Q  D  M  A  R  Q  I  R  E  Y  Q  D  L  M  N 424

1361 cgtcaagctggctctggacattgagatcgccacctacaggaagctgctggagggagaggaggacaggctggcgaccggca 1440
 425  V  K  L  A  L  D  I  E  I  A  T  Y  R  K  L  L  E  G  E  E  D  R  L  A  T  G  I 451

1441 tcaagtccatcaacatttcccaacagagcacgagctacggcggcttccccatggagaagagcagctactcaagcggctac 1520
 452  K  S  I  N  I  S  Q  Q  S  T  S  Y  G  G  F  P  M  E  K  S  S  Y  S  S  G  Y 477

1521 tccagcggtttcagcggcggggtacggcggcgggtacggcggcagcatgagcggcggcagcatgagcggcggctacagcag 1600
 478 S  S  G  F  S  G  G  Y  G  G  G  Y  G  G  S  M  S  G  G  S  M  S  G  G  Y  S  S 504

1601 cggcggcggcggctacagcagcggcggcggccacagcagcggcggcggcggcggcggcgccggattcagcagcggcagca 1680
 505  G  G  G  Y  S  S  G  G  G  H  S  S  G  G  G  G  G  G  A  G  F  S  S  G  S  T 531

1681 ccggcggcggcggcagcagctacagcaccacccagagcaagaagaacgttgtgatcaaggtgatcgagacccgggacggc 1760
 532  G  G  G  G  S  S  Y  S  T  T  Q  S  K  K  N  V  V  I  K  V  I  E  T  R  D  G 557

1761 agggtggtgtccgagtcctccgaggtcatggaggagtgaGCCGTCAGGTGTAAAGGTGTAGCTACCTGTCTGGTATTGTC 1840
 558 R  V  V  S  E  S  S  E  V  M  E  E                                              569

1841 GCCTCTCCTGTCTGGTAGTTTTATACTTACAGTTCAACCCCCCCCACCTGTTCCCCCCCCCCCCCCACCCCCACCCCCCAAC 1920

1921 TATGAAGTAAAACGCTTGCCAGCCACTCTCCGACGGAGTCTAAACATTTCCATGAAGCAATAAATGATCCTAACACTACC 2000

2001 AAAGATCTGA(n)                                                                   2010
```

Table 6-5. Nucleotide Sequences with Significant Sequence Similarity to lal-C14.

Organism	Sequence Type/Name	E value*
Rainbow trout (*Oncorhynchus mykiss*)	type II keratin E3 mRNA	0.0
Zebrafish *(Danio rerio)*	keratin 8 mRNA	1e-160
Rainbow trout (*Oncorhynchus mykiss*)	simple type II keratin mRNA	5e-82
African clawed frog (*Xenopus laevis*)	cytokeratin type II mRNA	1e-45

*The lower the E-value, or the closer it is to "0" the more significant the match is.

nucleotide homology

Type II keratin

1   2009

1   569

protein homology
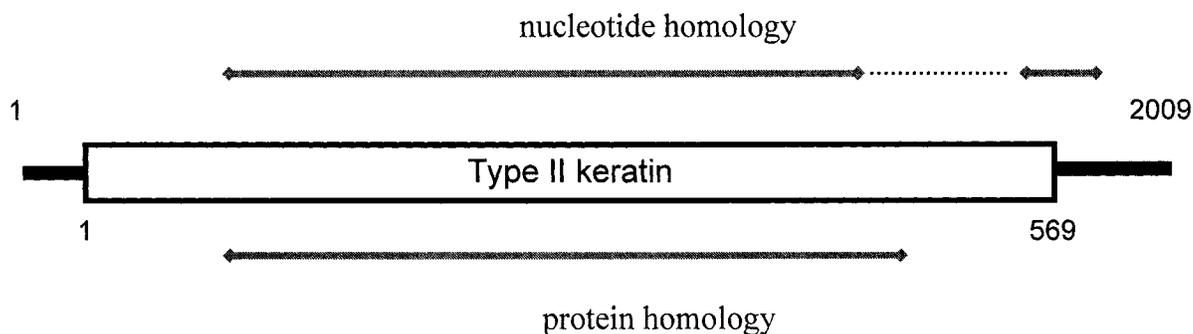
Table 6-6. Protein Sequences with Significant Sequence Similarity to lal-C14.

Organism	Sequence Type/Name	E value
Rainbow trout (*Oncorhynchus mykiss*)	type II keratin E3	1e-141
Rainbow trout (*Oncorhynchus mykiss*)	simple type II keratin	1e-138
Goldfish *(Carassius auratus)*	Intermediate filament protein ON3	1e-136
Zebrafish *(Danio rerio)*	type II basic cytokeratin	1e-136
Rat (*Rattus norvegicus*)	cytokeratin 8 polypeptide	1e-110

135

6.4 Discussion

The initial northern blot experiment indicated there was expression of type I mRNA in snailfish liver tissue. This result was expected given that, in other species that produce type I AFPs, circulating AFPs are initially synthesized in liver and then secreted into blood. Additional northern blots, using the same probes, showed that skin tissue also expressed putative type I AFP transcripts, but of a much larger size. These positive results provided enough evidence that construction and screening of a snailfish liver cDNA library would have a reasonable chance for successful isolation of the corresponding cDNA clones.

6.4.1 Possible evolutionary relationship between chorion proteins and snailfish type I AFPs

The snailfish liver cDNA library was screened several times using the same probes used for the northern blots producing many positive signals. However, although numerous positive plaques were picked and subsequently sequenced, none of the clones coded for complete type I AFPs corresponding to known amino terminal sequence of snailfish plasma AFP (chapter 2). Two of the clones coded for important fish egg structural proteins while the other coded for a ubiquitous epithelial protein, type II keratin. Because there is not any significant sequence conservation amongst type I AFP untranslated regions, the ORF of winter flounder or shorthorn sculpin skin type I AFP cDNAs had to be used as probes. Unfortunately, this poses the problem of using GC rich DNA that can generate possible false positive signals. Nevertheless, all three independent

clones were identified because there was sufficient sequence homology with the type I AFP probes.

The clone identified as lal-A1 and the highly abundant clone lal-B2 each code for proteins containing domains nearly identical to important structural proteins in the chorion of fish eggs. Unfertilized fish eggs are surrounded by the chorion, which is a thick extracellular envelope, composed of a few large glycoproteins. This structure is called zona pellucida in mammals, and perivitelline membrane or vitelline envelope in amphibians and birds (Bausek et al 2000). Normally chorion proteins are maternally synthesized in liver tissues under the influence of estrogen for deposition around the oocyte during oogenesis but are also produced in granulosa cells, oocyte and ovary (Bausek et al 2000, Murata et al 1997, Hyllner et al 2001). Shortly after fertilization, the chorion transforms into a rigid structure via hydrolysis and polymerization of proteins which protects the egg during embryonic development (Shibata et al 2000).

The translation product of lal-A1 is a chimeric protein with an 'AFP like' domain at the amino terminus of the chorion protein. A comparison of the 'AFP like' region with the ORF of snailfish type I AFP (chapter 5) gives >60% alignment in the amino acid sequence and over 50% in a nucleotide section (Fig 6-5A, B). The majority of the conserved amino acids are alanine, which would impart an α-helical secondary structure on the hypothetical protein. For lal-B2, a shift in the largest open reading frame produces an 85 amino acid 'AFP like' translation product that has high alanine content.

Figure 6-5. Homology of chorion liver clone lal-A1 and snailfish AFP. (A) Schematic representation of the nucleotide and amino acid 'AFP like' region of clone lal-A1 (B) Sequence alignment of lal-A1 'AFP like' protein and *L. atlanticus* type I AFP. Sequence alignments were generated via MultAlin located on the French INRA web server (www.prodes.toulouse.inra.fr/multalin/multalin.html). Visualization of sequence alignments were made with BOXSHADE 3.21 program on Swiss EMBnet server (www.ch.embnet.org/index.html).

A

55% identity in 165 nt region

'AFP like' region

ZP / Choriogenin H / Vitelline envelope

'AFP like' region

61% identity in 36 aa region
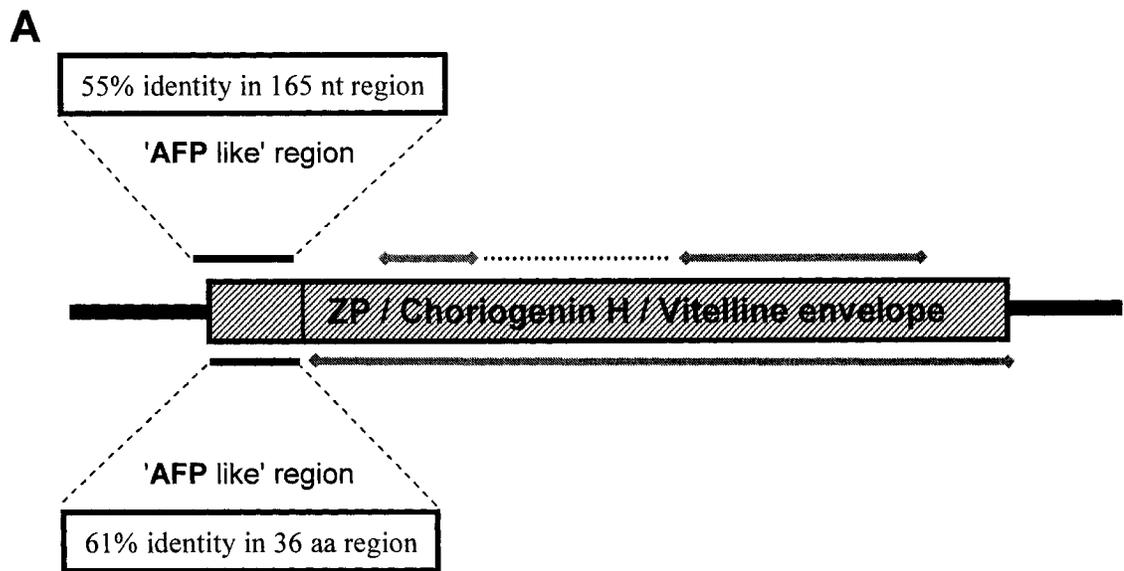
B

```
A1      1  --------------MAAAAATAAGPAAAAAAATAAMAASSAAAATVEKAE-----------
La-S    1  MAAATPAQRAAATATAAAAAAAASAAAAAAATTASTAAKVSAGAAATAAAAVVAAKNAATA

A1         -----------------------------------------------------------
La-S   61  VAPNTGAITAATAASATAAAAAAKAAQATADAAATKAAAAAVTSKAAAAALAAL
```

When this 'AFP like' protein is compared to the sequence of snailfish type I AFP, there is a 60% identity in the amino acid sequence (Fig 6-6A, B). While the majority of the identical residues are alanine, there are conserved threonines which may give the homology increased significance.

Through a process termed co-option, genes can acquire new functions against selective pressure to maintain the original function, via alterations in the amino acid coding sequence and/or by regulatory changes in gene expression patterns (True and Carroll 2002). The most common mode of gene co-option is through gene duplication events which are followed by sequence divergence to create novel functions. However, genes may also evolve new functions via a non-duplicative mode if changes occur in nonessential regions of the amino acid sequence to create novel protein domains. Either or both of these mechanisms are responsible for the independent evolution of teleost antifreeze proteins (Fletcher et al 2001). Evidence has been reported linking the evolution of different classes of AFPs and an AFGP through the co-option of unrelated genes. The gene for notothenoid AFGP evolved from a trypsinogen-like serine protease via gene duplication and amplification of a portion of nine nucleotide sequence that straddles an intron/exon junction (Chen et al 1997a, Chen et al 1997b, Cheng and Chen 1999). Type II AFPs evolved from C-type lectins at separate times (Ewart and Fletcher 1993, Ewart et al 1998, Gronwald et al 1998) and there is evidence that type IV AFP from longhorn sculpin plasma is structurally related to apolipoproteins (Deng et al 1997, Zhao et al 1998).

Figure 6-6. Homology of chorion liver clone lal-B2 and snailfish AFP. (A) Schematic

representation of the nucleotide and amino acid 'AFP like' region of clone lal-B2 (B)

Sequence alignment of lal-B2 'AFP like' protein and *L. atlanticus* type I AFP. Sequence

alignments were generated via MultAlin located on the French INRA web server

(www.prodes.toulouse.inra.fr/multalin/multalin.html). Visualization of sequence

alignments were made with BOXSHADE 3.21 program on Swiss EMBnet server

(www.ch.embnet.org/index.html).

A

54% identity in 260 nt overlap

'AFP like' region

Chorion protein / L-SF precursor

'AFP like' region

60% identity in 85 aa overlap
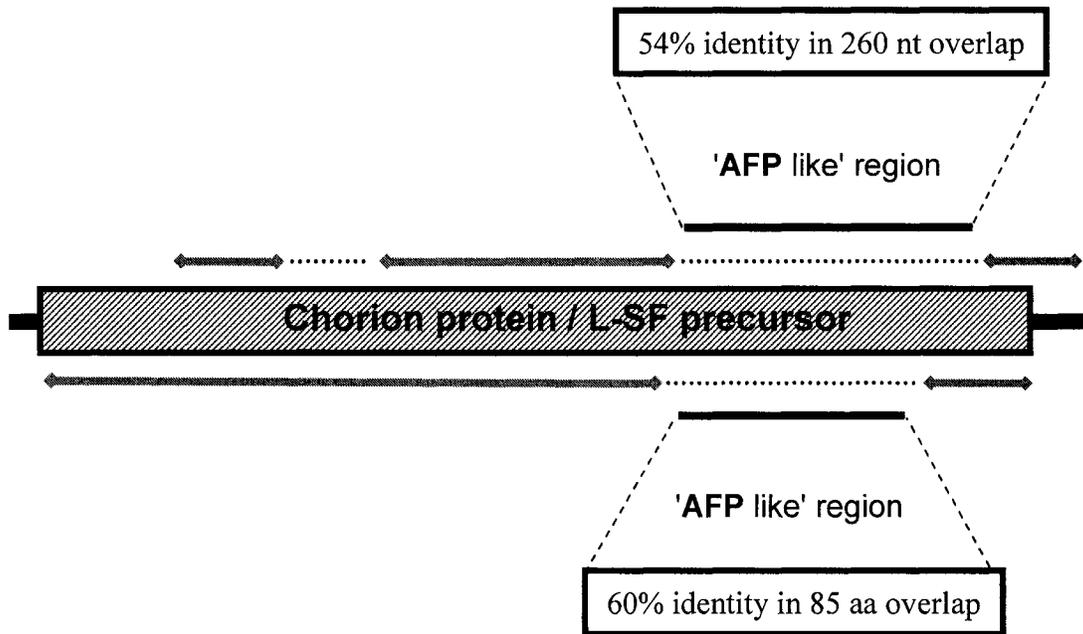
B

```
B2      1 ----------PAAPTPSAAAAAAAAAAAAVTTPAAAVTTPAVAAASAVPAAEAAATTAPM
La-S    1 MAAATPAQRAAATATAAAAAAASAAAAAAATTASTAAKVSAGAAATAAAAVVAAKNAATA

B2     51 AAMAPTVSTAAAAAAAAAAAAVAAATTVTAAAAST-----------------
La-S   61 VAPNTGAITAATAASATAAAAAKAAQATADAAATKAAAAAVTSKAAAAALAAL
```

There is reasonable evidence here to support the hypothesis that egg chorion proteins might be progenitor proteins linked to the evolution of snailfish type I AFPs. The chorion proteins, which are localized on the outer membrane of snailfish eggs to provide structural integrity, are important for protection from environmental insult. Spawning snailfish lay demersal eggs in shallow, ice-laden, inshore water during the winter months January to March (Scott and Scott 1988). Clearly the eggs would be susceptible to freezing or ice damage prior to water hardening or while in the ovary. It would therefore be important to protect outer membranes from ice damage to ensure the egg contents do not freeze. Eggshell or chorion proteins are extremely important to ensure this protection. A study in 1992 concluded that the ability of cod (*Gadus morhua*) eggs to survive in icy seawater depended upon the integrity of their chorion (Valerio et al 1992) while others have reported the cold hardiness and even freeze resistance of eggs from capelin (*Mallotus villosus*) and plaice (*Pleuronectes platessa*) (Aarset and Jorgensen 1988, Davenport and Stene 1986).

The internal contents of unfertilized snailfish eggs contain identical type I AFPs as the maternally expressed circulating proteins (R.P. Evans and G.L. Fletcher, unpublished results). Presumably, the eggs contain these AFPs because they are required for protection from freezing and could help protect the internal membrane structure of the egg. It is possible that the genes for abundantly expressed chorion proteins were originally co-opted to help protect the egg from external freeze damage. A shift in the reading frame of the GC rich portion could produce the codons for alanine residues which could produce a candidate AFP if it had ice binding capability. In the case of snailfish

type I AFPs, the presence of abundant alanine residues conferring a highly helical secondary structure is required for antifreeze activity (see chapter 5). Furthermore, the gene could be generally expressed to protect all peripheral tissues from freeze/ice damage through changes in gene regulatory elements. It would be very instructive to express recombinant lal-A1 to determine if the protein is a proper chimeric protein with an AFP moiety that is capable of ice crystal modification or antifreeze activity. Additionally, characterization of snailfish chorion genes, to determine if any significant homology exists between the non-coding or regulatory regions and snailfish AFP genes could help decipher any evolutionary links.

6.4.2 Possible evolutionary relationship between type II keratin and snailfish type I AFPs

The clone lal-C14 has significant nucleotide and amino acid sequence similarity with type II keratins from fish. Keratin proteins constitute the most diverse class of intermediate filament proteins, which form an important structural component of eukaryotic cells, primarily in epithelia. The proteins can be subdivided into two types based on their amino acid sequence, the type I (acidic) keratins and the type II (neutral/basic) keratins (Lodish et al 2000). Simple epithelial tissues contain only a single type I and a single type II keratin while more complex epithelia contain six or more keratins. Immunohistochemical and *in situ* hybridization experiments have shown that fish, like mammals, contain multiple keratin polypeptides that are expressed in different tissues and cell types (Gong et al 2002). However, unlike mammals, the distribution of fish keratins appears to be more complex since expression is not limited to epithelial

tissues. The type II keratin protein coded for lal-C14 has a glycine rich carboxy terminal amino acid sequence. However, when this region of the nucleotide sequence is analyzed in another reading frame, a 79 residue 'AFP like' amino acid sequence is translated which contains 56% identity with the primary structure of snailfish AFP and 55% identity with its nucleotide open reading frame (Fig 6-7 A,B). While the majority of the conserved residues are alanine, there is some conservation of other important amino acids which would give the primary structure homology increased significance.

Simple slippage in the reading frame of lal-C14 could convert codons for keratin glycine residues into alanine residues. Since type II keratin is ubiquitous in epithelial tissues, the co-option of this gene to create a candidate skin AFP seems quite plausible. Based on what is known about snailfish AFP expression in skin tissues (chapter 5), the strong signal observed at ~2.3kb in the northern blot (Fig 6-1) is likely from the 'AFP like' type II keratin transcripts. It has been hypothesized that skin AFP is the primordial protein which gave rise to the circulating liver type AFP (Gong et al 1996). If keratin is such a protein, it would indicate that this hypothesis might valid, at least in snailfish.

The evolution of all teleost AF(G)Ps in species of the Northern hemisphere is believed to have taken place during the last Arctic ocean cooling period when ice-laden seawater provided strong selective pressure (reviewed in Cheng 1998, Fletcher et al 2001). The long term survival of species in that harsh environment would depend on the co-option of proteins with ice binding ability to provide freezing protection. Presumably,

Figure 6-7. Homology of type II keratin liver clone lal-C14 and snailfish AFP. (A) Schematic representation of the nucleotide and amino acid 'AFP like' region of clone lal-C14 (B) Sequence alignment of lal-C14 'AFP like' protein and *L. atlanticus* type I AFP. Sequence alignments were generated via MultAlin located on the French INRA web server (www.prodes.toulouse.inra.fr/multalin/multalin.html). Visualization of sequence alignments were made with BOXSHADE 3.21 program on Swiss EMBnet server (www.ch.embnet.org/index.html).

A

55% identity in 251 nt overlap

'AFP' like' region

Type II keratin

'AFP' like' region

56% identity in 79 aa overlap
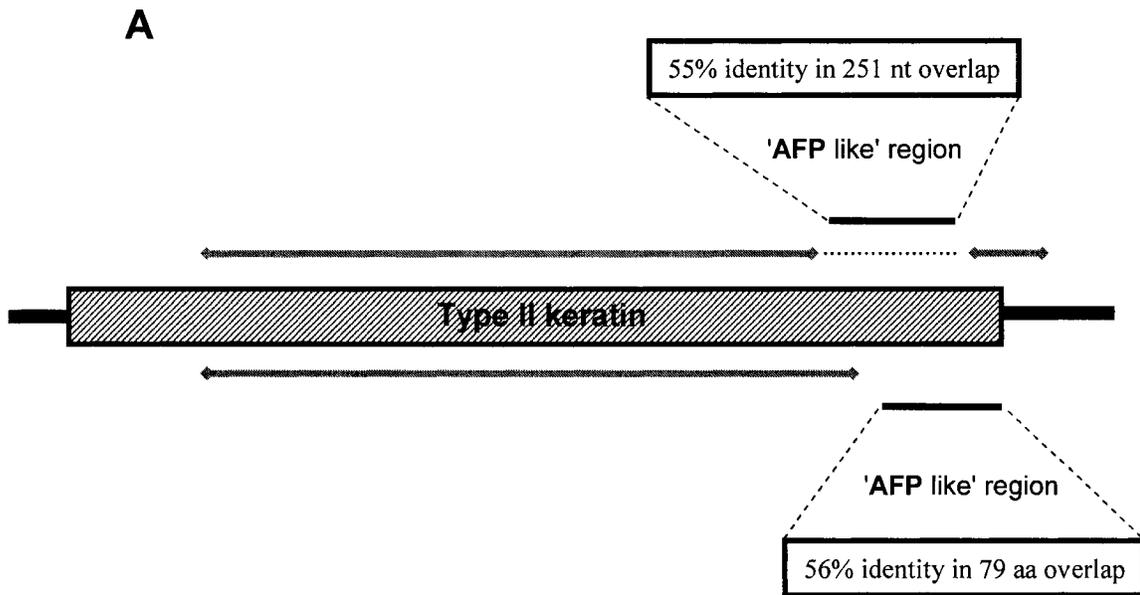
B

```
C14    1  --------RARATAASPWRRAATQAATPAASAAGTAA--GTAAAAAAAAAVTAAAAAATA
La-S   1  MAAATPAQRAAATATAAAAAAASAAAAAAATTASTAAKVSAGAAATAAAAVVAAKNAATA

C14   51  AAAATAAAAAVAAARDSAAAAPAAAAAAT----------------------
La-S  61  VAPNTGAITAATAASATAAAAAKAAQATADAAATKAAAAAVTSKAAAAALAAL
```

proteins that offered the best protection improved the overall fitness of species and enabled them to foray further into areas where others could not. It would be necessary to determine the sequence of the entire keratin gene to identify any similarity between it and snailfish AFP genes if an evolutionary relationship is to be substantiated. Snailfish AFPs do not appear to contain amino acid repeats and seem to be related to type I AFPs in their alanine content and helical secondary structure alone (see chapter 5). The alanine residue conservation found here implies an evolutionary relationship between snailfish type II keratins and their type I AFPs. Both of the scenarios proposed in this discussion for the evolution of type I AFPs support the current hypothesis that epithelial tissues are the original source of AFPs that gave rise to separate liver expressed proteins over time.

CHAPTER 7:

General Discussion

7.1 Review of primary research objectives

The general purpose of the research presented in this thesis was to determine the type and characteristics of AFPs produced by two snailfish species. This information would be used in conjunction with current information to create a clearer understanding of snailfish AFP function and their relationship to teleost AFP evolution in general. The specific goals were to use standard biochemical techniques to first isolate and characterize the AFPs from snailfish plasma and skin tissues and then molecular cloning methods to determine their nucleotide and protein sequences. While the main objectives were realized, the process from protein isolation to gene identification did not always follow the expected route.

I was successful in characterizing type I AFPs from both Atlantic snailfish (*Liparis atlanticus*) and dusky snailfish (*Liparis gibbus*) plasma and skin tissues. Furthermore, the clones for the corresponding AFP transcripts were sequenced and some aspects of their gene expression were also established. Fortuitously during the screening cDNA library for AFP clones, possible progenitor proteins were identified which might explain the origin and evolution of Type I AFPs. While several questions were answered by this study, unanticipated results have complicated the specific conclusions. These surprising results have opened exciting new possibilities and generated interesting questions that will need to be addressed in the future.

7.2 Expression of Type I antifreeze proteins in snailfish

7.2.1 Plasma AFPs

Significant thermal hysteresis activity was detected in all Atlantic and dusky snailfish blood samples that were taken during the winter months. Based on some preliminary analysis, there was a seasonal cycle in that measured thermal hysteresis declined during spring and summer. In order to identify the type and the properties of the presumptive AFPs in snailfish plasma, they had to be first isolated and purified. The strategy to purify AFPs from plasma of both snailfish species using column chromatographic and HPLC methods was successful. It was determined that the HPLC pure snailfish AFPs contained micro-heterogeneity that is typical of AFPs from many different species. In some cases, the mixtures of individual proteins differed by a single residue at their amino terminus.

Amino acid analysis and CD measurements demonstrated that snailfish AFPs are type I due to their elevated alanine content and α-helical secondary structure. These are the largest, circulating, type I AFPs identified to date with molecular masses greater than 9.3 kDa. Compared to other type I AFPs, the snailfish proteins have moderate activity on a molar basis but low on a weight basis. The initial portion of this study has shown that, like other related teleost fish, snailfish produce AFPs that circulate in their blood to protect them from extracellular freezing during the winter months.

7.2.2 Skin proteins

After successfully purifying AFPs from snailfish plasma, I decided to pursue the other known major source of the proteins – skin tissue. It was already known that winter

flounder, and closely related longhorn and shorthorn sculpins, express type I AFPs in their epithelia (Gong et al 1996, Low et al 1998, Low et al 2001). Antifreeze activity was measurable in *L. atlanticus* skin extracts, indicating that antifreeze protein was present. AFPs that were isolated and purified using gel filtration chromatography and HPLC methods had a M_r very similar to the plasma AFPs. Subsequent amino acid and MS analysis confirmed that these skin proteins were identical to the ones isolated from plasma.

The discovery that the type I AFPs expressed in snailfish skin tissue are identical to the major plasma protein was completely unexpected. The current hypothesis contends that skin AFPs are isoforms of, but different from liver type proteins. Furthermore, they are typically expressed from a separate subset of genes and are possibly the ancestral protein of the liver class (Gong et al 1996, Fletcher et al 1998, Low et al 2002). This surprising result from snailfish has generated numerous questions concerning the differential expression of liver and skin type AFPs in general. Some of the questions are addressed in other parts of the study.

7.2.3 Tissue distribution and gene expression patterns

After an initial strategy to clone the cDNA corresponding to Atlantic snailfish AFPs from a liver cDNA library proved unsuccessful, a skin library was subsequently constructed and screened. A partial skin AFP clone was isolated and the full sequence was later obtained using RACE and RT-PCR methods. Using the same methods, a nearly identical full length clone was obtained from dusky snailfish skin total RNA. The nucleotide and amino acid sequences were nearly identical from both species and the

proteins lacked the pre- or prosequences, suggesting that they remain intracellular following translation. However, northern blot and RT-PCR experiments demonstrated that snailfish AFP genes are expressed almost exclusively in skin tissues. Taken together, the evidence clearly indicates that snailfish AFPs are expressed in skin tissue and exported into blood for extracellular protection. As of now, it is unknown if some protein also remains inside the cells for intracellular freeze protection as is the case in winter flounder skin tissues (Murray et al 2002).

Southern blot analysis revealed that snailfish AFPs are the products of a multigene family with several copies per genome. The diverse nature of this multigene family is probably responsible for some of the micro-heterogeneity of snailfish AFPs. A confounding result was obtained in northern experiments to determine tissue distribution of AFP mRNA expression. While all fish analyzed had consistently high expression levels in skin tissue (the primary site), signals in liver ranged from undetectable to intense. This is the first known report of such extreme individual variability of AFP expression levels – but the significance is unknown. It is also not known whether the skin and liver AFP mRNAs are expressed from the same gene or a distinct subset of genes, as in winter flounder and sculpins. This evidence clearly indicates that the liver is a secondary source of AFP production in some snailfish that could provide enhanced freeze protection in extreme winter conditions. This suggests that while skin is the original location for ubiquitous AFP expression in some marine fish, a distinct subset of genes evolved later for liver expression of different AFP isoforms for circulation in blood. Eventually, the liver specific genes further evolved into the primary production site of

circulating AFPs in most species. The dual skin/liver expression in snailfish could represent the intermediate state between primary skin specific and liver specific AFP expression.

7.3 Evolution of Snailfish AFPs – Homology to Liver Expressed Proteins

While screening the liver cDNA library for possible AFP clones, numerous positive clones were isolated that had significant sequence homology with snailfish type I AFPs. The positives were attributed to three different liver expressed proteins – two of these are maternally expressed egg-shell proteins while the third is a type II keratin that is constitutively expressed in epithelial tissues. When the sequences of the isolated egg protein cDNAs were examined more closely, small GC rich sections had substantial homology with type I AFPs. A shift in reading frame translated these sections into alanine rich domains that resembled snailfish AFP sequences which could represent the ancestral link between these liver expressed proteins and the snailfish type I AFPs. It is conceivable that a simple shift in reading frame could produce alanine rich candidate AFPs with possible antifreeze activity or ice crystal modification properties. More experiments will be required to confirm this possibility. This fortuitous discovery could be quite important for finally identifying the progenitor proteins of all type I AFPs.

7.4 Effects of Salt Concentration on AF(G)P Thermal Hysteresis Activity

A confusing result of snailfish AFP isolation was that measured thermal hysteresis activity of the final HPLC pure protein did not equal levels predicted by activity measurements of blood plasma. This phenomenon has been observed by other

researchers when AFP was purified, some of their measurable activity was lost. Furthermore, the intrinsic *in vitro* thermal hysteresis activity of snailfish AFPs seemed to be inadequate to protect the fish in their over-wintering environment in normal physiological concentrations. In an attempt to resolve this dilemma, several experiments were conducted to mimic the known salt increase in fish blood as the winter season progresses.

Marine fish are known to increase the salt in their plasma during winter months which would have the effect of lowering the freezing point depression by colligative effects (Fletcher 1977, Fletcher 1981). Experiments were designed to determine if increased salt levels would increase the effectiveness AF(G)Ps. Results indicated that the increased salt concentration did have significant ancillary effects on the freezing point depression (FPD) of AF(G)P solutions. While the majority of the lowered FPD was strictly derived from colligative effects of salt, there was an increase in thermal hysteresis activity of AF(G)Ps. The colligative effects of plasma solutes and the hysteresis of AFPs combine to lower extracellular freezing levels enough to protect fish from freezing. These experiments contribute evidence that offers a reasonable explanation for observed diminished activity during AF(G)P purification.

7.5 Skin AFPs Isolated from Other Species – Cunner and Sea Raven

As a comparison to snailfish and to determine if skin AFPs are as widespread as has been proposed, skin AFPs were isolated from unrelated cunner and a closely related sea raven. Both fish species had measurable thermal hysteresis activity in extracts made from skin tissue. AFPs were initially isolated and purified from cunner skin tissue using

the standard techniques employed for snailfish AFP isolation. Cunner skin AFPs were a mixture of individual proteins that were determined to be ~7 kDa type I by amino acid and MS/MS micro-sequencing methods. This result confirms an earlier observation by Valerio et al that cunner skin contains antifreeze (Valerio et al 1990).

Sea raven had an AFP in skin tissue with a similar amino acid profile and molecular mass to type II AFP previously reported in this species (Slaughter et al 1981, Duncker et al 1996). The corresponding cDNA was later cloned from skin total RNA using RT-PCR and the results verified that the skin protein message was nearly identical to published sequence. However, skin tissues contain the larger (163 aa and 146 aa) proteins which correspond to the unprocessed gene product expressed in liver prior to secretion into blood. This interesting discovery represents the first type II AFP isolated from skin tissues and the second example of the same transcript used to express a protein in liver and skin. Since the protein contains a secretion signal sequence, it is possible that is also cleaved and exported into extracellular space and finally blood circulation.

The level of AFP expression in sea raven skin appears to be secondary to liver expression and therefore not as physiologically important (Gong et al 1992). If the skin protein is the ancestral source of AFPs then why would expression be lost from skin tissues? These results suggest that superfluous expression of AFPs in skin has been reduced over time in some species. This site is redundant as long as extracellular freeze protection is provided by plasma AFPs that are expressed in liver and exported by the standard Golgi route. More rigorous investigation is required before this puzzle can be unraveled fully. However, the cunner and sea raven results do provide more evidence that

expression of AFPs in skin is a very widespread phenomenon that is not restricted to type I proteins alone.

7.6 Future Research Possibilities

7.6.1 Evolution and origin of snailfish type I AFPs

To confirm the possible evolutionary relationship between the 'AFP like' proteins expressed in liver and snailfish AFPs, a two pronged approach would be required. The first step would be to compare the sequences of the entire snailfish AFP gene and the liver expressed "AFP like" chorion and type II keratin genes to determine if the regulatory regions or introns have significant similarity. It is quite possible that the evolution of snailfish AFPs is analogous to the process by which notothenioid AFGPs evolved from part of a trypsinogen-like serine protease gene. The gene sequence comparison could help prove this hypothesis if one of the liver "AFP like" genes was co-opted to form a product with antifreeze capability. This information could be applied more generally to describe the evolution of all type I AFPs.

The second approach would involve examination of the chimeric proteins that are coded for by the liver clones. Using standard molecular techniques, recombinant proteins that contain chimeric "antifreeze like" domains would be tested for possible antifreeze activity or ice crystal modification properties. The recently invented cold finger apparatus for purifying AFPs by adsorption to ice could be very useful in identifying candidate proteins (Kuiper et al 2003). Positive results would provide definitive evidence for an ancestral link to snailfish type I AFPs.

7.6.2 Structure and function of snailfish type I AFPs

Currently there are a few different ice-binding models for type I AFPs that have at least some experimental support. Generally, the models have used smaller type I AFPs from winter flounder or sculpin to generate the data. Snailfish AFPs offer a unique opportunity for a broader test of these theories because their large size and structural characteristics make them easier to express in bacteria without the need for expression tags. The initial step would be to determine the solution structure of snailfish type I AFP via NMR or x-ray crystallographic methods. This would provide information regarding any kinks or bends in the protein backbone around the helix-breaking proline residues. Site directed mutagenesis methods would be used to determine if the structure of snailfish AFP is responsible for its low thermal hysteresis activity, as predicted by the current structure-function models for type I AFP mechanism of action. Mutants could be designed to specifically test what effect an incomplete hydrophobic face has on antifreeze activity. The mechanism of action might be specific to snailfish AFPs or a general feature of all type I AFPs.

7.6.3 Expression patterns of snailfish AFPs

The results of this research have generated numerous questions regarding the complex nature of snailfish AFP gene expression. To develop a more complete understanding of the relationship between skin expression and plasma protein requires three fundamental questions to be answered. First, it would be very informative to examine if there is significant antifreeze protein translation in liver tissue. This would help to determine the physiological significance of the individual variability in liver

expression of antifreeze mRNA. Next, experiments to establish if there is a separate subset of genes which express AFP mRNA in liver and skin should be conducted. Even though the identical protein is located in plasma and skin there might be differences in the regulatory elements of either gene. Finally, the sub-cellular localization of snailfish AFPs in skin tissue cells should be identified to determine if significant protein remains intracellular.

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